UNIVERSITY OF BRASILIA

FACULTY OF AGRONOMY AND VETERINARY MEDICINE

Applications of Essential Oils in Poultry: Natural Strategies for Sanitizing Hatching Eggs

Gabriel da Silva Oliveira

Doctoral Thesis in Animal Sciences

Brasilia/DF

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Doctoral thesis submitted to the Postgraduate Program in Animal Sciences at the University of Brasilia as part of the requirements for obtaining the degree Doctorate in Animal Science.

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Dedication

I dedicate this work to my mother, Divonê, my father, Demerval, my sisters, Brenda and Silvana, and my brother, Romário. They are the greatest supporters of my goals and dreams. I love you all deeply!

Acknowledgments

To God, for always being by my side and giving me the health to achieve all of this.

To my supervisor, Dr. Concepta McManus, for giving me the opportunity and trusting me from the very beginning and for everything you have done for me thus far. Words cannot express how important you have been on this journey.

To my co-supervisor, Dr. Vinícius Machado dos Santos, who has guided me this far. Everything I am today is largely due to your support and help. It wasn't easy, but you showed me that everything is possible. Eternal gratitude for the person you are and for your life. Words cannot express how important you have been on this journey.

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To the University of Brasília for the opportunity to pursue my doctoral studies.

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To everyone who has contributed, in one way or another, to this journey.

Thesis Structure

The thesis was organized in a sequential and logical manner, beginning with broad reviews on the sanitization of hatching eggs and gradually progressing to more specific approaches, concluding with practical experiments and multivariate analyses. The following presents the step-by-step development of this thesis:

Paper one: Initially, a broad review was conducted on the different sanitizers used for hatching eggs, with a focus on their microbiological effects on the eggshell and the implications for poultry health during embryogenesis and after hatching. This chapter established the foundational understanding of the importance of microbiological control prior to pre-incubation.

Paper two: A specific review was then conducted on essential oils, addressing their antimicrobial efficacy, potential toxic effects on embryos and chicks, and their implications for productive parameters in poultry. This chapter highlighted the promising potential of essential oils as natural alternatives to conventional sanitizers.

Paper three: This chapter gathered evidence on the use of essential oils from *Citrus aurantifolia* (CAEO), *Ocimum basilicum* (OBEO), and *Allium sativum* (ASEO) as antimicrobials in poultry. The main microorganisms inhibited were identified along with the main antimicrobial applications of these essential oils in various contexts of poultry production.

Paper four: Based on the previous data, this chapter provides a critical synthesis on the feasibility of using essential oils as a sanitizing strategy, focusing on the production of microbiologically safe hatching eggs. It emphasizes the role of essential oils in addressing microbiological challenges in poultry farms.

Paper five: Following structure presented in the previous chapters, this experimental study investigated the effects of CAEO, OBEO, and ASEO on the eggshell microbiota and newly hatched chicks. The objective was to demonstrate the potential benefits of essential oils in the sanitization of hatching eggs in response to the microbiological challenges faced by poultry farms.

Paper six: A multivariate analysis was performed on the microbiological and incubation data, aiming to identify the most influential variables on hatchability rates, particularly those related to contamination of the eggshell and yolk sac of eggs that were sanitized or not with essential oils.

Concluding Remarks: After a comprehensive investigation covering both theoretical and experimental aspects, this thesis concludes by reaffirming the importance of sanitizing hatching eggs for poultry production. The use of essential oils, such as CAEO, OBEO, and ASEO, represents a promising natural alternative to conventional chemical sanitizers. Although the results are encouraging, further studies are needed to assess their economic viability and large-scale application in poultry production.

Table of contents

Paper one

Effects of Sanitizers on Microbiological Control of Hatching Eggshells and Poultry Health during Embryogenesis and Early Stages after Hatching in the Last Decade

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animals

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Review

Effects of Sanitizers on Microbiological Control of Hatching Eggshells and Poultry Health during Embryogenesis and Early Stages after Hatching in the Last Decade

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Simple Summary: Poultry systems, especially conventional comprehensive production systems to meet the global demand for eggs and meat, are constantly challenged by pathogens, requiring intense sanitary practices. Operations, including the sanitization of hatching eggs, can employ synthetic chemical sanitizers as well as natural plant extracts to minimize the microbial challenge. As the application of formaldehyde sanitizer in hatching eggs cannot be justified in terms of safety for embryonic and human health, studies are underway to assist the industry in adopting new alternative sanitizers. This review aims to evaluate the effects of different sanitizers on the microbiological quality of hatching eggshells and poultry health during embryogenesis and early stages after hatching.

Abstract: The sanitization of hatching eggs is the backbone of the hygienic–sanitary management of eggs on farms and extends to the hatchery. Poultry production gains depend on the benefits of sanitizers. Obtaining the maximum yield from incubation free of toxic sanitizers is a trend in poultry farming, closely following the concerns imposed through scientific research. The toxic characteristics of formaldehyde, the primary sanitizer for hatching eggs, are disappointing, but it is a cheap, practical and widely used antimicrobial. To overcome this shortcoming, multiple synthetic and natural chemical sanitizers have been, and continue to be, tested on hatching eggs. This review aims to evaluate the effects of different sanitizers on the microbiological quality of hatching eggshells and poultry health during embryogenesis and early stages after hatching.

Keywords: eggshells; embryonic health; hatchery; hatching eggs; microbiological safety; natural materials; poultry farm; poultry production; sanitizers; synthetic chemical materials

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1. Introduction

In poultry, embryonic mortality from pathogenic microbial infection is preventable through simple, cheap and efficient preventive guidelines. In most countries, the sanitization of hatching eggs is the primary countermeasure to the attacks of pathogenic microorganisms on the embryo. Studies have shown that sanitizing hatching eggs with synthetic products such as hydrogen peroxide [1] and natural products such as clove essential oil [2] reduced the pathogenic microbiota in eggshells and increased the percentage of hatched chicks. These active materials are non-toxic, non-corrosive and non-damaging to the eggshell. However, unsatisfactory effects such as possible severe toxicity in embryos that led to their death were reported in eggs sanitized with formaldehyde [3]. Microfragments were found in the cuticle and the vertical crystalline layer in eggs sanitized with peracetic acid [4], and reduced hatchability was found in eggs sanitized with propolis [5].

The beneficial and non-beneficial effects of sanitizers in hatching eggs result from synchrony (favorable) or non-synchrony (unfavorable) factors, such as concentration and application time [4,6]. As mentioned earlier, it is clear that sanitizers, when applied to hatching eggs under certain conditions, can generate a repertoire of adverse effects that affect embryonic development. Embryonic health is undoubtedly an important aspect that influences the entire poultry sector. It is through a healthy embryo that a healthy chick will be born. In turn, if handled properly, this chick will become a healthy broiler that will reach the consumer's table without undue influence on human health. At the same time, the poultry chain experiences significant economic gains for maintenance and growth. However, no sanitizers should be definitively rejected before being fully and continuously evaluated unless the compound is known to be lethally toxic to the point that humans cannot manipulate it with personal protective equipment. Human health must be a priority over all matters considered when choosing a sanitizer for hatching eggs.

Formaldehyde is the primary sanitizer in the routine sanitization of hatching eggs on European poultry farms (for example, Germany and Poland), as well as in Brazil and Egypt, among other countries [2,7–10]. However, it has genotoxic and cytotoxic properties [11] that subject poultry farmers and chicken embryos to a high risk of hazardous chemical exposure and possible irreversible bodily harm. Indoors, a short exposure not exceeding 0.1 mg/m^3 (0.08 ppm) of formaldehyde is recommended to avoid damage to human health [12]. Cadirci [13] reported that the concentration required to reduce practically 100% of the microbial load of hatching eggshells is at least 600 mg/m^3 (489 ppm) of formaldehyde, which is an excessively high concentration when compared to those recommended for human exposure. Therefore, formaldehyde needs to be removed from the routine sanitizing of hatching eggs.

There is a versatile repertoire of synthetic and natural sanitizing formulations for hatching eggs that have contributions from researchers dedicated to studying this line of research in various parts of the world. However, are these formulations able to meet the safety tripod (eggshell microbiological, embryonic and human health) at the oviposition–hatch interface? The compilation of this information is vital for helping the industry by showing it the potential products that can replace formaldehyde once and for all because the trend is for formaldehyde to be banned entirely from poultry farming. This review aims to evaluate the effects of different sanitizers on the microbiological quality of hatching eggshells and poultry health during embryogenesis and early stages after hatching.

2. Eggshell and Its Contamination

The eggshell is a physical, physiological and immunological protective surface that morphologically and functionally regulates the health of the embryo and supports its development through structural impermeability to pathogens and the expression of proteins that mobilize an antimicrobial response to pathogens [14,15]. Disturbances in antimicrobial functions of the shell by effects on its structure, as well as the resistance and motile capacity of some microorganisms [15–18] and exposure time of the shell to the microorganism [19,20], are possible causes of horizontal transmission of pathogens (shell–embryo) (Figure 1B), inducing infectious and inflammatory processes. Pathogens such as *Escherichia coli*, *Klebsiella*, *Micrococcus*, *Proteus*, *Pseudomonas*, *Staphylococcus* spp. and *Salmonella* Enteritidis may be associated with egg penetration and embryonic mortality [17,21–23]. The sanitation process on the farm is continuously controlled to avoid this burden on embryonic health, chick fatality and eggshell contamination by fungal and bacterial organisms. The latter can be favored by the microclimate on farms and hatcheries [24] and persists from pre-lay to pre-hatch (Figure 1A) [25].

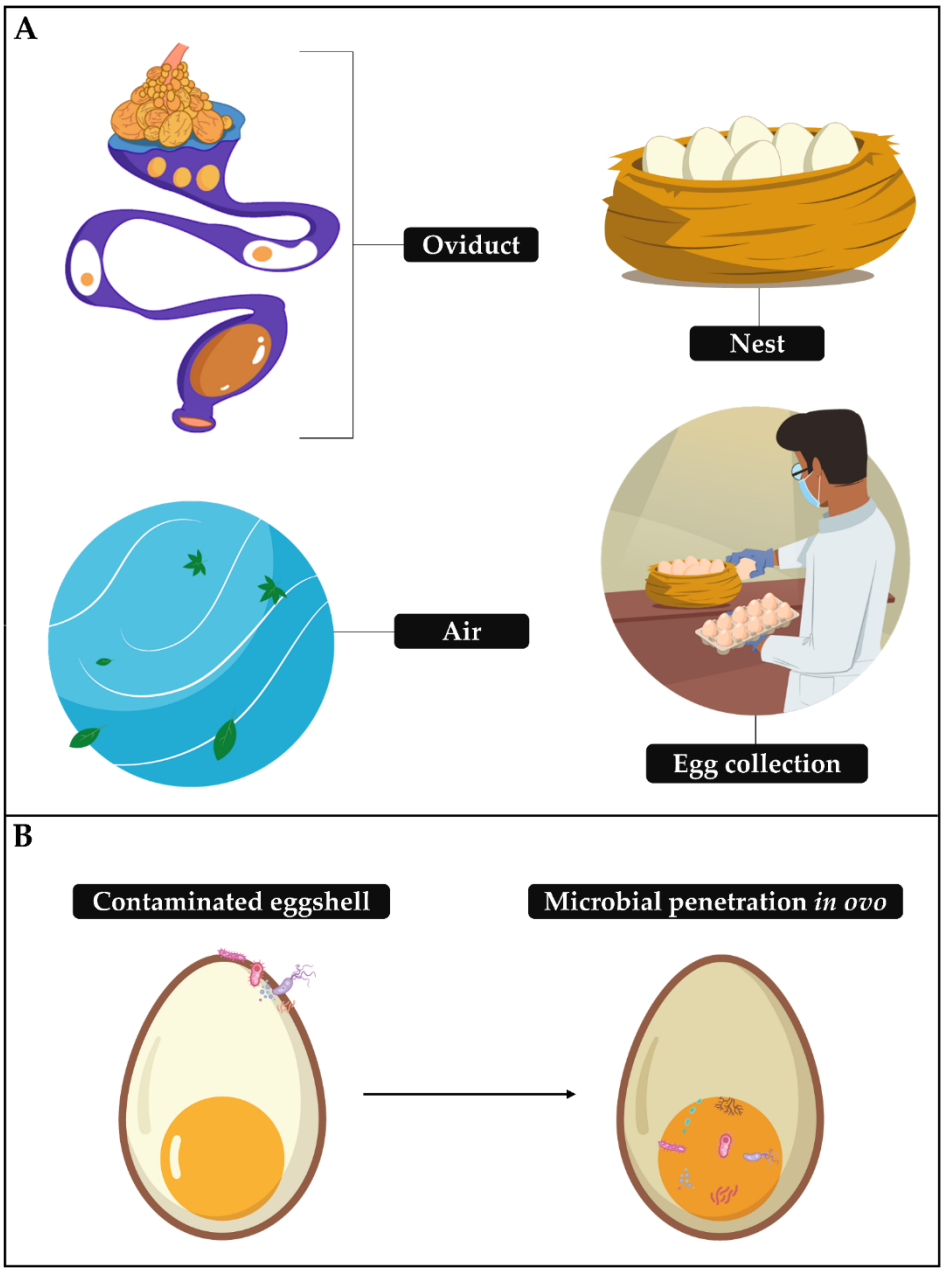

Figure 1. (**A**) Some sources of egg contamination in pre-incubation; (**B**) horizontal transmission of microbes in eggs.

3. Sanitizers and the Sanitization of Hatching Eggs

3.1. Articles and Search Criteria

Google Scholar was searched using the following keywords: hatching egg sanitization, hatching egg sanitizers and hatching egg disinfectants, in that order. The search process included papers published (between January 2012 and May 2022) in peer-reviewed journals published in English. The first 20 papers were considered for each keyword search, totaling 600 papers. For each year, 10 papers were selected (the first 10 papers found in the search order). A total of 120 papers could have been included. However, after analyzing the title and abstract of the 600 papers, only 69 papers were reviewed, as they studied and evaluated sanitizers for hatching eggs. Review articles on the topic published in this period or other research papers that did not meet all the search criteria were considered to reinforce the discussion.

The research studies of papers from the search process were carried out mainly in Egypt, Brazil and Turkey (Figure 2), and the papers were published primarily in *Poultry Science*, *Egyptian Poultry Science Journal* and the *Journal of Applied Poultry Research*.

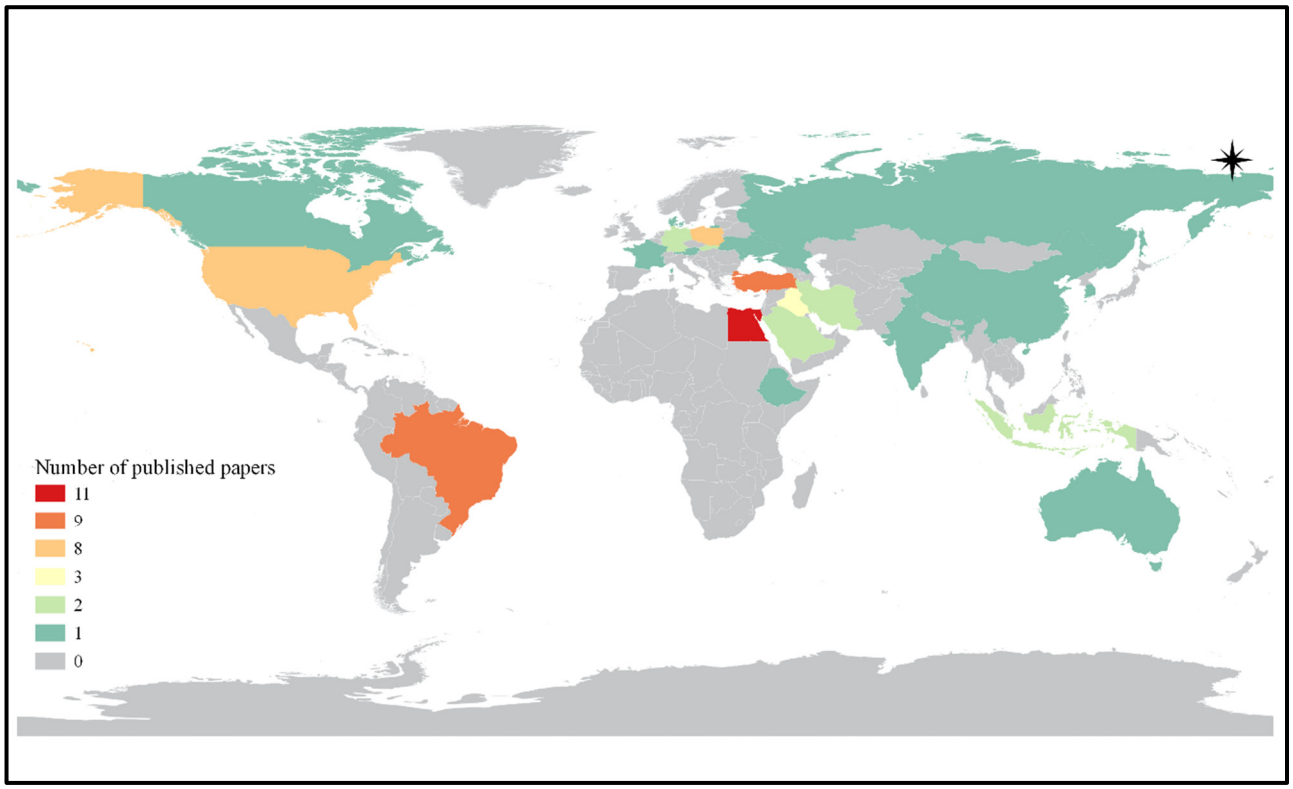

Figure 2. Number of published papers in each country. Countries in the same color have the same number of published papers.

3.2. Objective, Optimal Timing and Methods for Sanitizing Hatching Eggs

Egg contamination triggers an embryonic health crisis and threatens the world's poultry economy. This state of affairs can be alleviated by sanitizing hatching eggs, a relatively simple protocol in which the eggs must be submitted, soon after collection, to intervention in the high proliferation of pathogens in the eggshell and their possible mobility to the microenvironment of embryonic development, making the egg suitable for generating a chick. The ideal time to sanitize hatching eggs is up to 30 min after oviposition or collection (if it is immediate) [26–28]; otherwise, the probability of having no effect or worsening production results is very high. This is corroborated in [29], which reported improved hatchability of eggs sanitized immediately compared with those sanitized six hours after laying, probably due to microbial penetration. In this protocol, the contact of the sanitizer with the eggs occurs through gaseous or indirect means and by liquid or direct means (Figure 3):

- Fumigation: the release of sanitizing vapors on the surface of hatching eggshells in an enclosed space.
- Spraying: the dispersion of a sanitizing mist on the surface of hatching eggs.
- Immersion: the act of immersing hatching eggs in sanitizer until there is an interaction between them.

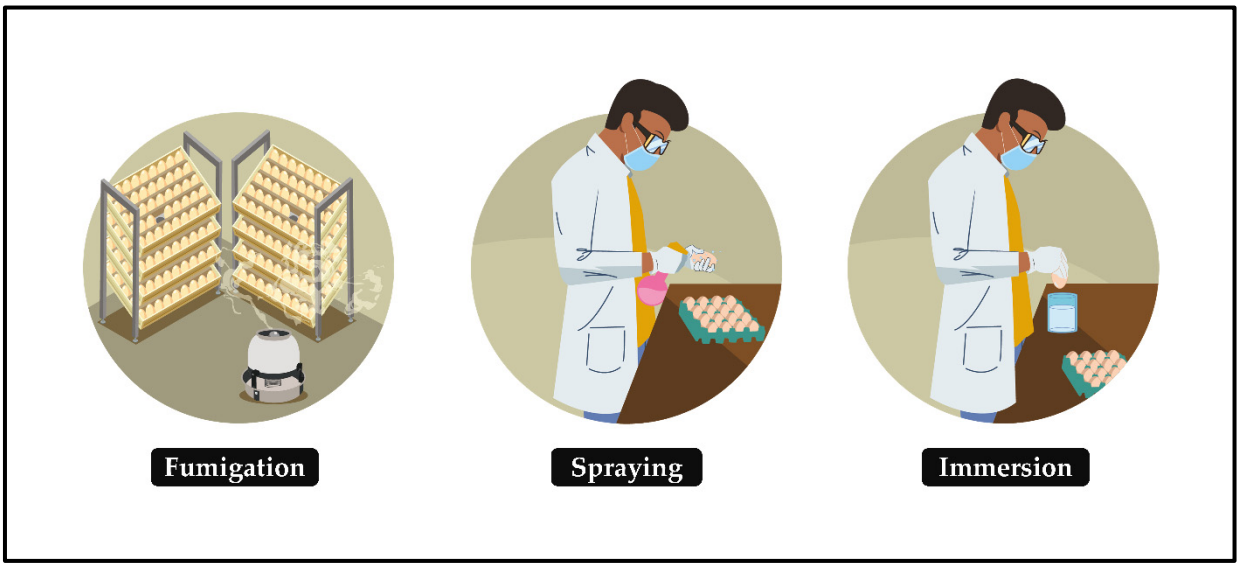

Figure 3. Main methods of sanitizing hatching eggs.

The use of each method is based on the size of the production system, number of eggs produced daily, costs and availability of equipment and facilities, type of sanitizer, number of professionals involved in the process and the specific limitations of each method.

3.3. Formaldehyde

Formaldehyde (liquid or gaseous; also called paraformaldehyde-polymerized phase) has been linked to reduced eggshell microbiota and increased hatchability percentage. There are also reports that it did not affect any of these variables (Table 1). Nevertheless, it is also associated with reports of toxicity and permanent harmful damage to embryos and chicks when applied to hatching eggs (Table 1). Although these effects depend on the concentration, length of time and method of application of formaldehyde and the period in which the egg is exposed [13], formaldehyde itself is carcinogenic because it impairs and inhibits DNA repair [30]. Therefore, its use is unjustifiable regarding embryonic life safety, health and protection. Poultry production should value lower risks to bird life (whether during development or after hatching), which will benefit the highest priority condition of preserving human health. Given the possible future restrictions on using formaldehyde in the poultry industry, other sanitizers must be readily available and approved by competent bodies to meet the global poultry demand.

Table 1. Some reports of the effects of formaldehyde on hatching eggs.

Study (Reference)	Effect on Eggshell Microbial Count *	Effect on Hatchability *
[31]	Non-evaluated	No effects
[32]	No effects	No effects
[33]	-	No effects
[34]	Reduced	Increased
[35]	Reduced	Non-evaluated
[36]	-	Increased
[37]	Reduced	Non-evaluated
[38]	Reduced	No effects
[2]	Reduced	Increased
[39]	Non-evaluated	Increased
Study (Reference)	**Some Reports of Adverse Effects on Embryos and Chicks**	
[40]	Underweight, underdeveloped and malformed embryos.	
[41]	Increased embryonic mortality in the early stage.	
[42–44]	Reduced chick survival rate in the first post-hatch week.	
[45]	Increased embryonic mortality in early, mid and late stages.	
[46]	Reduced chick quality score as a result of slow activities and high number of unclosed navels.	

* Effect compared to a negative control (non-sanitized eggs) and in the absence of negative control compared to the other sanitizers tested.

4. Sanitizers and Their Effects on the Microbiological Quality of Hatching Eggshells and the Health and Survival of Poultry in the Developmental Phase and Early Period after Hatching

The health and survival of embryos and chicks remain the most vital issues for the constant advancement of industrial poultry. This is due to the presence of dangerous microbial agents and the use of risky sanitizers for hatching eggs, representing unquestionable concern for the safety of these animals. In the last 10 years, much research has been conducted on the manufacturing and evaluation of sanitizers to minimize risks during embryogenesis and post-hatch (Table 2). The aim is for these sanitizers to provide vitality and supplements to support the poultry's quality of life during their development and further growth in the production system. Thus, the effects of sanitizers on eggshell microbiology and hatchability were reviewed (Table 2). Based on the studies reviewed, synthetics stand out over natural sanitizers in the number of published studies. However, when it comes to reducing the microbial load of the shells and increasing hatchability, the positions are reversed, as natural sanitizers present better results than synthetic ones (Table 2). Hatchability and eggshell microbial level are partially capable of predicting health and fully predicting embryo survival and level of risk of damage by pathogens, respectively. They are also associated with production profitability [47]. Studies must be complemented with other analyses, including quality, microbial counts, blood constituents and organ development during embryogenesis and post-hatch, to ensure health and survival.

Table 2. Reports of the effects of sanitizers on eggshell microbial counts and hatchability.

Study (Reference)	Sanitizer	Effect on Eggshell Microbial Count *	Effect on Hatchability *
[31]	Ozone	-	No effects
[48,49]	Propolis	Reduced	No effects
[50]	PotoClean	Reduced	-
[32]	Orthophenylphenol Stabilized hydrogen peroxide + peracetic acid + acetic acid Sodium hypochlorite + chlorine dioxide + sodium chlorite + ozone + water	Reduced	No effects
[51]	Ethanol	Reduced	No effects
[52]	Propolis	Reduced	No effects
[18]	Ultrasonic waves	-	Increased
[53]	Bac-D	Reduced	No effects
[54]	Quaternary ammoniums + bronopol + biguanide Quaternary ammoniums + polyhexamethylenebiguanide hydrochloride moiety Hydrogen peroxide Ammonium chlorides + hydrogen peroxide Quaternary ammoniums	Reduced	-
[34]	Propolis Thyme essential oil	Reduced	Increased
[55]	Quaternary ammoniums + a polyhexamethylenebiguanide hydrochloride moiety	Reduced	Reduced (commercial facility testing) and no effects (lab testing)

Table 2. *Cont.*

Study (Reference)	Sanitizer	Effect on Eggshell Microbial Count *	Effect on Hatchability *
[56]	Biosentry 904 Egg-Washer-Pro Virkon S	-	No effects
[57]	Trans-cinnamaldehyde Eugenol	Reduced	-
[7]	Ultraviolet light	-	No effects
[58]	Ultrasonic waves	Reduced	No effects
[40]	Hydrogen peroxide Sodium chloride Betadine Virkon S Cumin essential oil Oregano essential oil Cumin + oregano essential oils	Reduced	-
[59]	Hydrogen peroxide + ultraviolet irradiation	Reduced	-
[35]	Sodium dichlorocyanurate Hydrogen peroxide Electrolyzed oxidizing water	Reduced Reduced No effects	-
[3]	Virkon S	-	No effects
[29]	Propolis TH4 Virkon S	Reduced	-
[60]	Hydrogen peroxide + ultraviolet irradiation	Reduced	-
[61]	Hydrogen peroxide TH4 Virkon S	Reduced	Increased
[42]	Colloidal silver	Reduced	No effects
[62]	Live yeast	Reduced	Increased
[63]	Virocid	Reduced	-
[64]	Amino-Steril Oxydion Viron FF Hydro-Clean	-	No effects
[65]	Volatile pyrazines	Reduced	-
[1]	Hydrogen peroxide	Reduced	Increased
[66]	Nanosecond electron beam	Reduced	No effects
[67]	Garlic oil	Reduced	Increased
[8]	Hydrogen peroxide TH4	Reduced	Reduced
[68]	Lysozyme	Reduced	No effects
[69]	Chlorine dioxide gas	Reduced	No effects
[41]	Garlic extract	-	No effects
[70]	Grapefruit juice	Reduced	No effects
[37]	Ozone Ultraviolet light Hydrogen peroxide Peracetic acid	No effects Reduced No effects No effects	-
[43]	Propolis	No effects	No effects
[71]	Olive oil Albumin	-	Reduced
[72]	Hydrogen peroxide + ultraviolet light	Reduced	Increased
[73]	Noni leaf extract	-	No effects

Table 2. *Cont.*

Study (Reference)	Sanitizer	Effect on Eggshell Microbial Count *	Effect on Hatchability *
[6]	Lemongrass essential oil Pedestrian tea essential oil Lemongrass + pedestrian tea essential oils	Reduced	Reduced
[74]	Vinegar	Reduced	Increased
[75]	Ozone Ultraviolet light Hydrogen peroxide	No effects Reduced No effects	No effects
[2]	Peracetic acid Clove essential oil	Reduced Reduced	Increased
[5]	Propolis Clove essential oil	-	Reduced No effects
[76]	Fenugreek seed extract Oat seed extract Basil seed extract	-	No effects
[77]	Eucalyptus alcoholic extract	Reduced	Increased
[78]	Polydez Sterylii AB	Reduced No effects	-
[79]	Virosan Dergall	Reduced Reduced	No effects
[9]	Hydrogen peroxide Low-energy electron irradiation Peracetic acid	-	No effects
[38]	Essential oil (not specified) Low-energy electron irradiation	Reduced	No effects
[44]	Hydrogen peroxide Ozone	Reduced	No effects Reduced
[80]	Hydrogen peroxide	-	No effects
[81]	Pulsed ultraviolet light	Reduced	No effects
[82]	Oregano essential oil	-	Increased
[83]	Ultraviolet light	Reduced	-
[39]	Ozone Aldekol Virkon S	-	Increased
[84]	Ozone	Reduced	No effects
[85]	Garlic oil	-	Increased
[46]	Oregano juice	Reduced	No effects
[86]	Slightly acidic electrolysis	Reduced	No effects

* Effect compared to non-sanitized eggs (or water or alcohol control) and formaldehyde (or other positive control). The negative control (non-sanitized eggs) had priority in the comparison. An increase was also considered when the sanitizer was tested at different concentrations or methods and at least one of those concentrations or methods showed improvement. (-) When the tested sanitizer did not have the variable evaluated or when it did, the study did not apply or clarify a statistical analysis and was not compared to a positive or negative control group. Studies that evaluated only formaldehyde were not included in this table, as the focus was on alternatives.

Among the synthetic chemical sanitizers, hydrogen peroxide, ozone and Virkon S were most commonly tested. Hydrogen peroxide is a reactive oxygen type that exerts antimicrobial activity by inducing oxidative damage to cell DNA [87]. Ozone is a strong oxidant that exhibits antimicrobial characteristics by degrading cellular constituents, impairing their metabolic activity [88]. Virkon S is a combined formulation that includes peroxygen compounds, with antimicrobial action associated with cell wall damage and inhibition of enzymatic systems [89]. These three sanitizers appear to have a safety profile for humans [89–91]. Most studies that evaluated hydrogen peroxide reported the ability to reduce eggshell microbial load with almost no damage to hatchability (Table 2). The effectiveness of ozone in reducing eggshell microbial load is still dubious based on the studies reviewed, as half reported no reduction. On the other hand, only 20% reported a significant adverse effect on hatchability, the same ones that reported reduced microbial

load. Therefore, the lower hatchability was possibly a response to the toxic action of ozone on the embryo, not seeming to be a good option to sanitize hatching eggs. Regarding the control of the eggshell microbial load, Virkon S performed very well, as demonstrated in all studies, and had no record of significant loss in hatchability. Other examples that may be viable for hatching eggs are ammonium compounds [64], peracetic acid [75], nanosecond electron beam [66], low-energy electron irradiation [38] and ultraviolet radiation [83].

Among the natural sanitizers for hatching eggs, essential oils, volatile liquids produced in flowers, leaves, fruits, seeds, stems and roots [92]; propolis, a resinous product produced by bees using resins and other plant substances [93,94]; and garlic, which is a herb with bulbous flowering [95], are the most tested materials. These have more beneficial than harmful characteristics in terms of antibacterial and antifungal effects and production rates (Table 2), supported by three recently published reviews [25,27,28]. Propolis can improve hatchability by up to 11% [28], and essential oils by up to 12.59% [27]. There are no negative records of garlic in hatchability [25]. The effects of these compounds on eggshell microbial reduction ultimately influence an increase in hatchability. They kill bacterial and fungal pathogens by fully compromising the cell membrane/wall, leading to cell dysfunction and loss [96–99]. These results, added to the recognized safety of most natural compounds, are essential for preparations of natural origin intended for hatching eggs to acquire a consensual reputation that will be useful for their insertion and permanence in commercial practice to sustain the sanitation management of hatching eggs. They will also be well accepted in free-range, organic and agroecological poultry farming. Other examples include live yeast, vinegar and alcoholic extract of eucalyptus, which showed potential as alternatives as sanitizers for hatching eggs [62,74,77].

The degree of pathogenicity and the concentration of eggshell microorganisms are key considerations in embryo infection, particularly in yolk sac infection [21,25]. If the yolk sac becomes infected, the embryo dies or survives after hatching and remains infected (the microorganism causing the infection (e.g., *Escherichia coli*) can remain for months). Clinical signs include swelling, edema and redness, in addition to limited movement due to abdominal distention, which negatively affects weight distribution, causing balance disturbance. This infectious framework will result in the deprivation of nutrients and maternal antibodies and the absorption of toxins [100]. Therefore, prevention through the application of sanitizers to eggs is the way forward. Upadhyaya et al. [57] reported that essential oil substances, trans cinnamaldehyde and eugenol, reduced *Salmonella enterica* serovar Enteritidis (inoculated on the surface of eggs) to undetectable levels in embryos after being applied to eggshells. The rate of embryonic *Escherichia coli* infection can be minimized in eggs sanitized with Virkon S [3]. Mousa-Balabel et al. [29] reinforced that contaminated hatched chicks are reduced when eggs are efficiently sanitized with propolis. In eggshells experimentally contaminated with *Salmonella* (primary poultry isolate of *Salmonella* Typhimurium), the sanitizer combining hydrogen peroxide and ultraviolet irradiation ensured that this microorganism was undetectable in chicks up to two weeks post-hatch [60]. This is due to the potential of many sanitizers to provide ongoing antimicrobial protection that restricts microbial penetration. Li et al. [68] experimentally inoculated nalidixic acid-resistant *Escherichia coli* (isolated from broiler digestive tract) into hatching eggshells. They indicated that lysozyme prevented this microorganism's penetration into the egg's internal environment. This reduces the risk of bacterial infection for embryos and chicks during the early stage of their life, supported by the significant reduction in *Escherichia coli* in the yolk sac. The hatching egg must also be of quality to minimize cracks or shell breaks, reduce incubation residues and infection of birds and increase immunological resistance [78]. In addition, litter eggs should be avoided, as the dirtier the shell, the greater the possibility of containing more pathogenic microorganisms [63].

Sanitizing hatching eggs can optimize embryonic and chick development (based on body weight, organs and length) as well as chick blood hematology and immunity, in addition to microbiological protection of the embryo and chick. These effects have been reported with sanitizers based on garlic oil [67], live yeast [62] and vinegar [74].

Other reports were also described. Sanitizers based on hydrogen peroxide (Hydro-Clean), ammonium compounds (Amino-Steril), peracetic acid (Oxydion) and aldehydes (Viron FF) promote a low frequency of embryonic defects and death, discarding toxic and teratogenic effects [64]. Mousa-Balabel et al. [61] reported that eggs sanitized with Virkon S did not generate weak chicks (inability to hatch) or chicks with incomplete feathers and distorted and wet beaks. Cantu et al. [72] reported the best percentage of hatching ducklings without defects after sanitizing was with hydrogen peroxide plus ultraviolet light. Sanitizers such as Polydez (which contains hydrogen peroxide, benzalkonium chloride, cocamidopropyl betaine, neonol and other components) and Virosan (which contains benzalkonium chloride, glutaraldehyde and excipients) did not harm the development of poultry in the embryonic and post-hatch period [78]. In vitro and in vivo tests performed by Patrzałek et al. [79] confirmed that sanitizing eggs with Dergall (organomodified trisiloxanes) is not toxic to chicken embryos. Oliveira et al. [2] demonstrated that eggs sanitized with clove essential oil improved the physical quality of chicks. This same result was found when the eggs were sanitized with oregano juice [46]. No harmful effects on organ development during embryogenesis and post-hatch were reported in eggs sanitized with clove essential oil [10]. Bekhet and Sayed [82] observed that treating eggs with essential oregano oil did not cause malformations in embryos, benefiting them by restoring their antioxidant balance. Gholami-Ahangaran et al. [3], Batkowska et al. [43] and Oliveira et al. [10] reported improvement in the survival percentage of chicks from eggs sanitized with Virkon S, propolis and clove essential oil, respectively, in the first days of post-hatch life.

Sanitizers capable of inducing damage that prematurely interrupts the development and growth of poultry or that reduces their quality of life have been reported. In the study of Shafey et al. [18], low hatchability was associated with the sanitization of eggs with ultrasonic waves. According to this report, embryos exposed to these waves can develop abnormally. Low hatchability has also been described in hatching eggs sanitized with lemongrass and pedestrian tea essential oils [6]. Mousa-Balabel et al. [61] noted that eggs sanitized with hydrogen peroxide recorded weak chicks and a high percentage of omphalitis, and eggs sanitized with TH4 recorded weak chicks with distorted beaks. Oliveira et al. [5] observed that the few chicks that managed to hatch from eggs sanitized with propolis were super-hydrated. Wlazlo et al. [44] showed that ozone has a toxic profile for interrupting embryonic development, justified by the high mortality rate recorded. These studies say much about the sensitivity of embryos to the stressful effects of sanitizers in hatching eggs. Hasyim et al. [101] found a numerical increase and decrease in hatchability when eggs were sanitized with cherry leaf extract at low and high concentrations, respectively, justifying the reduction in hatchability due to the occlusion of the shell pores. Chung et al. [69] reported that the use of chlorine dioxide at low concentrations has no adverse effect on hatchability as seen at high concentrations. The side effects of chlorine dioxide on the embryo were associated with low temperature, high concentration of sanitizer and contact time with the egg [102]. Reducing incidents of sanitizer toxicity can be achieved by adequately balancing the intrinsic factors linked to efficiency that influence toxicity, such as efficiency, safety, minimum concentration and shorter contact time.

Progress in sanitizer evaluation offers some possibilities and future avenues of application at the commercial level. Hydrogen peroxide and Virkon S are among the synthetic chemicals, and essential oils, propolis and garlic are among the natural products due to their antimicrobial efficiency and little or no adverse effect recorded on embryos and chicks, in addition to meeting safety requirements for humans. However, we believe that it is necessary to continuously deepen the evaluations carried out (mainly in vivo toxicity analyses at different concentrations) during embryogenesis and post-hatch after sanitizing the eggs with these antimicrobials to find the most suitable, affordable, efficient and safe protocol possible. We need to reinforce the benefits of existing protocols or discard those that, in part, may persistently cause some disadvantages to the process. While hydrogen peroxide, Virkon S, essential oils, propolis, and garlic may meet safety criteria, proper protective clothing and other safety precautions are necessary during exposure.

Despite being a challenge, a problem observed among the studies reviewed is the non-standardization of the time for sanitizing eggs after collection. Some studies performed this outside the ideal timeframe, for example, very close to or during the incubation process. This can negatively affect the process. Oliveira et al. [25] recommended that eggs should be sanitized in the shortest possible time after collection, which also requires speed, to achieve the objective of minimizing in ovo penetration and ensuring the chances of increasing the hatchability rate healthily. Laboratory studies should be complemented with egg sanitization repetitions on commercial farms. If carried out efficiently and adequately after collection, a single treatment should be sufficient until hatching, keeping all other surfaces where the eggs pass clean and sanitized.

5. Conclusions

Knowing that the abusive and poisonous use of formaldehyde fumigation for hatching eggs cannot be underestimated, this review demonstrates that research advances in the last decade have defended, at different levels, powerful safe alternatives based on synthetic and natural products. In addition to their antimicrobial capacity, these substances can mitigate the toxic effects that decrease bird health and survival by respecting the protocols recommended by researchers. This is a big step for the poultry industry, helping to understand and limit the use and availability of formaldehyde towards its total exclusion, making future handling of hatching eggs increasingly free of toxicity.

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References

1. Al-Shemery, N.J.; Kamaluddin, Z.N. Effect of Using Different Concentrations of Hydrogen Peroxide and Formalin Compared to Formaldehyde Evaporation in Sterilization of Hatching Eggs of Broiler. *Euphrates J. Agric. Sci.* **2018**, *10*, 36–41.
2. Oliveira, G.S.; Nascimento, S.T.; dos Santos, V.M.; Silva, M.G. Clove Essential Oil in the Sanitation of Fertile Eggs. *Poult. Sci.* **2020**, *99*, 5509–5516. [CrossRef] [PubMed]
3. Gholami-Ahangaran, M.; Shahzamani, S.; Yazdkhasti, M. Comparison of Virkon S and Formaldehyde on Hatchabil-ity and Survival Rate of Chicks in Disinfection of Fertile Eggs. *Rev. Med. Vet.* **2016**, *167*, 45–49.
4. Soares, C.E.S.; Cartabiano-Leite, C.E.; Ferreira, W.X.; Maiorka, A.; Dahlke, F.; Scussel, V.M.; de Dea Lindner, J. Peracetic Acid: Effect on the Chicken Eggshell Cuticle and Decontaminating Action on Filamentous Fungi. *Jokull J.* **2021**, *71*, 82–96.
5. Oliveira, G.S.; dos Santos, V.M.; Nascimento, S.T.; Rodrigues, J.C. Alternative Sanitizers to Paraformaldehyde for Incubation of Fertile Eggs. *Poult. Sci.* **2020**, *99*, 2001–2006. [CrossRef]
6. Nogueira, W.C.L.; Pena, A.C.S.; de Souza, C.N.; Azevedo, I.L.; Fariafilho, D.E.; Almeida, A.C. Disinfection of Fertile Eggs of Free-Range Poultry with Essential Oils. *Rev. Bras. Saude Prod. Anim.* **2019**, *20*, e0822019. [CrossRef]
7. Al-Shammari, K.I.; Batkowska, J.; Gryzi, M.M. Assessment of Ultraviolet Light Effect in Hatching Eggs Disinfection on Hatchability Traits of Two Breeds of Quails and Chickens. *Acta Sci. Pol. Zootec.* **2015**, *14*, 33–44.
8. Badran, A.M.M.; Osman, A.M.R.; Yassein, D.M.M. Comparative Study of the Effect of Some Disinfectants on Em-bryonic Mortality, Hatchability, and Some Blood Components. *Egypt. Poult. Sci. J.* **2018**, *38*, 1069–1081. [CrossRef]
9. Tebrün, W.; Motola, G.; Hafez, M.H.; Bachmeier, J.; Schmidt, V.; Renfert, K.; Reichelt, C.; Brüggemann-Schwarze, S.; Pees, M. Preliminary Study: Health and Performance Assessment in Broiler Chicks Following Application of Six Different Hatching Egg Disinfection Protocols. *PLoS ONE* **2020**, *15*, e0232825. [CrossRef]

10. Oliveira, G.S.; Nascimento, S.T.; dos Santos, V.M.; Dallago, B.S.L. Spraying Hatching Eggs with Clove Essential Oil Does Not Compromise the Quality of Embryos and One-Day-Old Chicks or Broiler Performance. *Animals* **2021**, *11*, 2045. [CrossRef]
11. Zhang, L.; Steinmaus, C.; Eastmond, D.A.; Xin, X.K.; Smith, M.T. Formaldehyde Exposure and Leukemia: A New Meta-Analysis and Potential Mechanisms. *Mut. Res./Rev. Mut. Res.* **2009**, *681*, 150–168. [CrossRef] [PubMed]
12. World Health Organization. *WHO Guidelines for Indoor Air Quality: Selected Pollutants*; World Health Organization: Geneva, Switzerland, 2010; pp. 103–156.
13. Cadirci, S. Disinfection of Hatching Eggs by Formaldehyde Fumigation-a Review. *Eur. Poult. Sci.* **2009**, *73*, 116–123.
14. Hincke, M.T.; Da Silva, M.; Guyot, N.; Gautron, J.; McKee, M.D.; Guabiraba-Brito, R.; Réhault-Godbert, S. Dynamics of Structural Barriers and Innate Immune Components during Incubation of the Avian Egg: Critical Interplay be-tween Autonomous Embryonic Development and Maternal Anticipation. *J. Innate Immun.* **2019**, *11*, 111–124. [CrossRef] [PubMed]
15. Kulshreshtha, G.; D'Alba, L.; Dunn, I.C.; Rehault-Godbert, S.; Rodriguez-Navarro, A.B.; Hincke, M.T. Properties, Genetics and Innate Immune Function of the Cuticle in Egg-Laying Species. *Front. Immunol.* **2022**, *13*, 838525. [CrossRef]
16. de Reu, K.; Grijspeerdt, K.; Messens, W.; Heyndrickx, M.; Uyttendaele, M.; Debevere, J.; Herman, L. Eggshell Factors Influencing Eggshell Penetration and Whole Egg Contamination by Different Bacteria, Including *Salmonella* Enteritidis. *Int. J. Food Microbiol.* **2006**, *112*, 253–260. [CrossRef]
17. Gantois, I.; Ducatelle, R.; Pasmans, F.; Haesebrouck, F.; Gast, R.; Humphrey, T.J.; van Immerseel, F. Mechanisms of Egg Contamination by *Salmonella* Enteritidis. *FEMS Microbiol. Rev.* **2009**, *33*, 718–738. [CrossRef] [PubMed]
18. Shafey, T.M.; Hussein, E.O.S.; Al-Batshan, H.A. Effects of Ultrasonic Waves on Eggshell Strength and Hatchability of Layer-Type Breeder Eggs. *S. Afr. J. Anim. Sci.* **2013**, *43*, 56–63. [CrossRef]
19. Williams, J.E.; Dillard, L.H.; Hall, G.O. The Penetration Patterns of *Salmonella* Typhimurium through the Outer Structures of Chicken Eggs. *Avian Dis.* **1968**, *12*, 445–466. [CrossRef] [PubMed]
20. Berrang, M.E.; Frank, J.F.; Buhr, R.J.; Bailey, J.S.; Cox, N.A.; Mauldin, J. Eggshell Characteristics and Penetration by *Salmonella* Through the Productive Life of a Broiler Breeder Flock. *Poult. Sci.* **1998**, *77*, 1446–1450. [CrossRef]
21. Rezaee, M.S.; Liebhart, D.; Hess, C.; Hess, M.; Paudel, S. Bacterial Infection in Chicken Embryos and Consequences of Yolk Sac Constitution for Embryo Survival. *Vet. Pathol.* **2021**, *58*, 71–79. [CrossRef]
22. Orajaka, L.J.E.; Mohan, K. Aerobic Bacterial Flora from Dead-in-Shell Chicken Embryos from Nigeria. *Avian Dis.* **1985**, *29*, 583–589. [CrossRef] [PubMed]
23. Higenyi, J. Microbial Contamination Load of Hatching Eggs in Butaleja, Eastern Uganda. *Anim. Vet. Sci.* **2014**, *2*, 22. [CrossRef]
24. Zhelev, G.; Lyutzkanov, M.; Urumova, V.; Mihaylov, G.; Petrov, V.; Marutsov, P. Microbial Contamination in a Duck Hatchery. *Rev. Med. Vet.* **2012**, *163*, 319–322.
25. Oliveira, G.D.S.; McManus, C.; dos Santos, V.M. Garlic as Active Principle of Sanitiser for Hatching Eggs. *Worlds Poult. Sci. J.* **2022**, *78*, 1–16. [CrossRef]
26. Araújo, W.A.G.; Albino, L.F.T. *Incubação Comercial [Commercial Incubation]*; Transworld Research Network.: Trivandrum, India, 2011.
27. Oliveira, G.D.S.; dos Santos, V.M.; Nascimento, S.T. Essential Oils as Sanitisers for Hatching Eggs. *Worlds Poult. Sci. J.* **2021**, *77*, 605–617. [CrossRef]
28. Oliveira, G.D.S.; dos Santos, V.M.; McManus, C. Propolis: Effects on the Sanitisation of Hatching Eggs. *Worlds Poult. Sci. J.* **2022**, *78*, 261–272. [CrossRef]
29. Mousa-Balabel, T.M.; Mohamed, R.A.; Al-Midani, S.A.; El-Samad, M.S.A. Impact of Boiler Breeders Hatching Eggs Disinfection Time on Some Hatchability Parameters. *Int. J. Sci. Basic Appl. Res.* **2016**, *30*, 230–240.
30. Ge, J.; Yang, H.; Lu, X.; Wang, S.; Zhao, Y.; Huang, J.; Xi, Z.; Zhang, L.; Li, R. Combined Exposure to Formaldehyde and PM2.5: Hematopoietic Toxicity and Molecular Mechanism in Mice. *Environ. Int.* **2020**, *144*, 106050. [CrossRef]
31. Hrnčár, C.; Prachárová, S.; Bujko, J. The Effect of Disinfection of Hatching Eggs on Hatchability of Oravka Chickens. *Sci. Pap. Anim. Sci. Biotechnol.* **2012**, *45*, 411–414.
32. Durmus, I. Determining Effects of Use of Various Disinfecting Materials on Hatching Results and Total Bacterial Count. *Asian J. Anim. Vet. Adv.* **2012**, *7*, 739–744. [CrossRef]
33. Fidan, E.D.; Turkyilmaz, M.K.; Ahmet Nazligul, A. The Effects of Different Storage and Fumigation Lengths on Hatchability and Hatching Weight in Japanese Quails (*Coturnix coturnix* Japonica). *J. Anim. Vet. Adv.* **2012**, *11*, 1400–1404. [CrossRef]
34. Shahein, E.H.A.; Sedeek, E.K. Role of Spraying Hatching Eggs with Natural Disinfectants on Hatching Characteris-tics and Eggshell Bacterial Counts. *Egypt. Poult. Sci. J.* **2014**, *34*, 213–230. [CrossRef]
35. Keïta, A.; Huneau-Salaün, A.; Guillot, A.; Galliot, P.; Tavares, M.; Puterflam, J. A Multi-Pronged Approach to the Search for an Alternative to Formaldehyde as an Egg Disinfectant without Affecting Worker Health, Hatching, or Broiler Production Parameters. *Poult. Sci.* **2016**, *95*, 1609–1616. [CrossRef] [PubMed]
36. Bekele, M.W.; Leta, M.U. Effect of Egg Storage Temperature and Fumigation on Hatchability of Cobb 500 and Hub-bard Broiler Strains. *Afr. J. Agric. Res.* **2016**, *11*, 3418–3424. [CrossRef]
37. Clímaco, W.L.D.S.; Melo, É.D.F.; Vaz, D.P.; Saldanha, M.M.; Pinto, M.F.V.D.S.; Fernandes, L.C.C.; Baião, N.C.; Oliveira, L.G.D.; Sant'Anna, F.M.D.; Souza, M.R.D.; et al. Eggshell Microbiology and Quality of Hatching Eggs Subjected to Different Sanitizing Procedures. *Pesq. Agrop. Bras.* **2018**, *53*, 1177–1183. [CrossRef]

38. Pees, M.; Motola, G.; Hafez, M.H.; Bachmeier, J.; Brüggemann-Schwarze, S.; Tebrün, W. Use of Electron Irradiation versus Formaldehyde Fumigation as Hatching Egg Disinfectants-Efficacy and Impact on Hatchability and Broiler Performance. *Tierärztliche Prax. Ausg. G Großtiere/Nutztiere* **2020**, *48*, 406–413. [CrossRef]
39. Hrnčár, C.; Hanusová, E.; Hanus, A.; Arpášová, H.; Kokoszyński, D.; Bujko, J. The Effect of Various Disinfectants on Hatching Results in Chickens. *Sci. Pap. Anim. Sci. Biotechnol.* **2021**, *54*, 193–196.
40. Zeweil, H.S.; Rizk, R.E.; Bekhet, G.M.; Ahmed, M.R. Comparing the Effectiveness of Egg Disinfectants against Bacte-ria and Mitotic Indices of Developing Chick Embryos. *J. Basic Appl. Zool.* **2015**, *70*, 1–15. [CrossRef]
41. Baylan, M.; Akpınar, G.C.; Canogullari, S.D.; Ayasan, T. The Effects of Using Garlic Extract for Quail Hatching Egg Disinfection on Hatching Results and Performance. *Rev. Bras. Cienc. Avic.* **2018**, *20*, 343–350. [CrossRef]
42. Batkowska, J.; Al-Shammari, K.I.A.; Gryzinska, M.M.; Brodacki, A.; Wlazlo, L.; Nowakowicz-Debek, B. Effect of Us-ing Colloidal Silver in the Disinfection of Hatching Eggs on Some Microbial, Hatchability and Performance Traits in Japanese Quail (Coturnix Cot. Japonica). *Eur. Poult. Sci.* **2017**, *81*, 211. [CrossRef]
43. Batkowska, J.; Al-Shammari, K.I.A.; Lukasz, W.; Nowakowicz-Debek, B.; Gryzinska, M. Evaluation of Propolis Ex-tract as a Disinfectant of Japanese Quail (*Coturnix coturnix* Japonica) Hatching Eggs. *Poult. Sci.* **2018**, *97*, 2372–2377. [CrossRef] [PubMed]
44. Wlazlo, L.; Drabik, K.; Al-Shammari, K.I.A.; Batkowska, J.; Nowakowicz-Debek, B.; Gryzińska, M. Use of Reactive Oxygen Species (Ozone, Hydrogen Peroxide) for Disinfection of Hatching Eggs. *Poult. Sci.* **2020**, *99*, 2478–2484. [CrossRef] [PubMed]
45. Bekhet, G.M. Impact of Hatching Egg Disinfection on Hatching Characteristics and Chick Embryos. *Indian J. Anim. Res.* **2021**, *55*, 353–358. [CrossRef]
46. Taşdemir, A.N.; Onbaşılar, E.E.; Yalçın, S.; Boyalı, B.; Aygören, H.; Tülek, E.; Sarıçam, S.; Akan, M. Effects of Oregano Juice on Eggshell Microbial Load, Layer Embryo Development, Hatching Results, and Growth at the First 2 Weeks after Hatch. *Trop. Anim. Health Prod.* **2021**, *53*, 404. [CrossRef]
47. Harikrishnan, S.; Narayanankutty, K.; Chacko, B.; Anitha, P. The Effect of Various Sanitizing Agents on the Economics of Hatching Kuttanad Duck Eggs. *Indian J. Vet. Sci. Biotechnol.* **2014**, *9*, 67–68.
48. Vilela, C.O.; Vargas, G.D.; Fischer, G.; Ladeira, S.; de Faria, R.O.; Nunes, C.F.; de Lima, M.; Hübner, S.O.; Luz, P.; Osório, L.G.; et al. Propolis: A Natural Product as an Alternative for Disinfection of Embryonated Eggs for Incubation. *Arq. Inst. Biol.* **2012**, *79*, 161–167. [CrossRef]
49. Aygun, A.; Sert, D.; Copur, G. Effects of Propolis on Eggshell Microbial Activity, Hatchability, and Chick Performance in Japanese Quail (*Coturnix coturnix* Japonica) Eggs. *Poult. Sci.* **2012**, *91*, 1018–1025. [CrossRef]
50. Uçan, U.S.; Gök, A. Efficacy of a Water-Based Disinfectant on Reduction of Eggshell Bacterial Contamination. *Eur. J. Vet. Sci.* **2012**, *28*, 57–59.
51. Nowaczewski, S.; Szablewski, T.; Cegielska-Radziejewska, R.; Kontecka, H. Microbiological Response of Japanese Quail Eggs to Disinfection and Location in the Setter during Incubation. *Folia Biol.* **2013**, *61*, 119–124. [CrossRef]
52. Aygun, A.; Sert, D. Effects of Prestorage Application of Propolis and Storage Time on Eggshell Microbial Activity, Hatchability, and Chick Performance in Japanese Quail (*Coturnix coturnix* Japonica) Eggs. *Poult. Sci.* **2013**, *92*, 3330–3337. [CrossRef]
53. Lowman, Z.S.; Parkhurst, C.R. Effects of Bac-D on Total Aerobic Bacteria Naturally Found on Broiler Breeder Eggs. *Int. J. Poult. Sci.* **2013**, *12*, 505–508. [CrossRef]
54. Buhr, R.J.; Spickler, J.L.; Ritter, A.R.; Bourassa, D.V.; Cox, N.A.; Richardson, L.J.; Wilson, J.L. Efficacy of Combination Chemicals as Sanitizers of *Salmonella*-Inoculated Broiler Hatching Eggshells. *J. Appl. Poul. Res.* **2013**, *22*, 27–35. [CrossRef]
55. Musgrove, M.T.; Stephens, C.B.; Bourassa, D.V.; Cox, N.A.; Mauldin, J.M.; Berrang, M.E.; Buhr, R.J. Enterobacteri-aceae and *Salmonella* Recovered from Nonsanitized and Sanitized Broiler Hatching Eggs. *J. Appl. Poult. Res.* **2014**, *23*, 516–522. [CrossRef]
56. Hanafy, A.M.; Ahmed, E.G.; Abdel-Ghany, A.M.; Nashaat, M.H. Effect of Disinfectants, Flock Physiological Status, Season and Storage Period of Broiler Breeder Eggs on Field Hatchability and Hatchery Operation Quality under New-Salheya City Situation. *Egypt J. Anim. Prod.* **2015**, *52*, 115–125.
57. Upadhyaya, I.; Yin, H.B.; Nair, M.S.; Chen, C.H.; Upadhyay, A.; Darre, M.J.; Venkitanarayanan, K. Efficacy of Fumi-gation with Trans-Cinnamaldehyde and Eugenol in Reducing *Salmonella enterica* serovar Enteritidis on Embryonated Egg Shells. *Poult. Sci.* **2015**, *94*, 1685–1690. [CrossRef]
58. Yildirim, I.; Aygun, A.; Sert, D. Effects of Preincubation Application of Low and High Frequency Ultrasound on Eggshell Microbial Activity, Hatchability, Supply Organ Weights at Hatch, and Chick Performance in Japanese Quail (*Coturnix coturnix* Japonica) Hatching Eggs. *Poult. Sci.* **2015**, *94*, 1678–1684. [CrossRef]
59. Gottselig, S.M.; Dunn-Horrocks, S.L.; Woodring, K.S.; Coufal, C.D.; Duong, T. Advanced Oxidation Process Sanitiza-tion of Eggshell Surfaces. *Poult. Sci.* **2016**, *95*, 1356–1362. [CrossRef]
60. Rehkopf, A.C.; Byrd, J.A.; Coufal, C.D.; Duong, T. Advanced Oxidation Process Sanitization of Hatching Eggs Re-duces *Salmonella* in Broiler Chicks. *Poult. Sci.* **2017**, *96*, 3709–3716. [CrossRef]
61. Mousa-Balabel, T.M.; Al-Midani, S.A.; Al-Refaay, M.A.; Kerady, S.M. Coliform Bacteria and Hatching Egg Disinfect-ants. *Int. J. Sci. Basic Appl. Res.* **2017**, *33*, 151–163.
62. Fouad, W.; Abdel-Hafez, M.S. Effect of Spraying Hatching Eggs of Japanese Quails by Live Yeast on Physiological Changes in the Embryonic Development, Hatchability and Total Bacterial Count. Egypt. *Poult. Sci. J.* **2017**, *37*, 1303–1321. [CrossRef]

63. Olsen, R.; Kudirkiene, E.; Thøfner, I.; Pors, S.; Karlskov-Mortensen, P.; Li, L.; Papasolomontos, S.; Angastiniotou, C.; Christensen, J. Impact of Egg Disinfection of Hatching Eggs on the Eggshell Microbiome and Bacterial Load. *Poult. Sci.* **2017**, *96*, 3901–3911. [CrossRef] [PubMed]
64. Korowiecka, K.; Trela, M.; Tombarkiewicz, B.; Pawlak, K.; Niedziółka, J.W.; Swadźba, M.; Lis, M.W. Assessment of the Effect of Selected Substances Used for Disinfection of Hatching Eggs on Hatching Results in Chickens. *Sci. Ann. Pol. Soc. Anim. Prod.* **2017**, *13*, 25–35. [CrossRef]
65. Kusstatscher, P.; Cernava, T.; Liebminger, S.; Berg, G. Replacing Conventional Decontamination of Hatching Eggs with a Natural Defense Strategy Based on Antimicrobial, Volatile Pyrazines. *Sci. Rep.* **2017**, *7*, 13253. [CrossRef] [PubMed]
66. Sokovnin, S.Y.; Donnik, I.M.; Shkuratova, I.A.; Krivonogova, A.S.; Isaeva, A.G.; Balezin, M.E.; Vazirov, R.A. The Use of Nanosecond Electron Beam for the Eggs Surface Disinfection in Industrial Poultry. *J. Phys. Conf. Ser.* **2018**, *1115*, 022034. [CrossRef]
67. Fouad, W.; Abdel-Hafez, M.S.; El-Halim, H.A.H.A. Influence of Spraying Garlic Oil on Embryonic Development, Hatchability, Physiological Parameters, Post-Hatch Chick Growth and Bacterial Contamination of Fertile Quail Eggs. *Egypt. Poult. Sci. J.* **2018**, *38*, 877–893. [CrossRef]
68. Li, X.; Anderson, D.; Rathgeber, B.; McLean, N.; MacIsaac, J. Fumigating Broiler Hatching Eggs with Lysozyme Product (Inovapure) to Reduce Eggshell Microbial Load. *Poult. Sci.* **2018**, *97*, 4252–4261. [CrossRef]
69. Chung, H.; Kim, H.; Myeong, D.; Kim, S.; Choe, N.H. Effect of Chlorine Dioxide Gas Application to Egg Surface: Microbial Reduction Effect, Quality of Eggs, and Hatchability. *Korean J. Food Sci. Anim. Resour.* **2018**, *38*, 487–497. [CrossRef]
70. Batkowska, J.; Wlazlo, L.; Drabik, K.; Nowakowicz-Debek, B.; Al-Shammari, K.I.A.; Gryzinska, M. Evaluation of Grapefruit Juice (Citrus paradisi) as an Alternative Disinfectant for Hatching Eggs. *Pak. J. Zool.* **2018**, *50*, 647–653. [CrossRef]
71. Gatea, S.M.; Altaie, S.M.S.; Khafaji, S.S.; Aljanabi, T.K.; Shatti, D.H.; Hussain, M.A. Influence of Spraying Different Solutions at Different Incubation Periods on Hatchability Parameters of Local Iraqi's Eggs. *IOP Conf. Ser. Earth Environ. Sci.* **2019**, *388*, 012034. [CrossRef]
72. Cantu, K.; Archer, G.S.; Tucker, Z.S.; Coufal, C.D. Effectiveness of Duck Hatching Egg Sanitization with the Combi-nation of Hydrogen Peroxide and Ultraviolet Light. *J. Appl. Poult. Res.* **2019**, *28*, 301–306. [CrossRef]
73. Hidayat, M.N.; Thaha, A.H.; Mayanti, R. Effect of the Use of Noni Leaf Extract as a Natural Disinfectant on the Per-centage of Hatchability and Day Old Quail (DOQ) Hatching. *Chal. J. Anim. Husb.* **2020**, *4*, 48–53. [CrossRef]
74. Fouad, W.; Abdelfattah, M.G.; Abdelnabi, M.A. Effect of Spraying Hatching Eggs by Different Levels of Vinegar on Embryological Development, Hatchability and Physiological Performance of Dandarwi Chicks. *Egypt. Poult. Sci. J.* **2019**, *39*, 291–309. [CrossRef]
75. Melo, E.F.; Clímaco, W.L.S.; Triginelli, M.V.; Vaz, D.P.; de Souza, M.R.; Baião, N.C.; Pompeu, M.A.; Lara, L.J.C. An Evaluation of Alternative Methods for Sanitizing Hatching Eggs. *Poult. Sci.* **2019**, *98*, 2466–2473. [CrossRef]
76. Al-Asadi, K.J.T.; Ibrahim, B.M. Effect of the Use of Immersion and Injection Methods for Eggs Hatching of Broiler Breeders in the Aquatic Extracts of Some Plant Seeds as Early Feeding: 2-Subsequent Production Performance. *Plant Arch.* **2020**, *20*, 2123–2130.
77. Toghyani, P.; Shahzamani, S.; Gholami Ahangaran, M.; Ali Mousavi Firouzabadi, S. Comparison of Eucalyptus Ex-tract and Formaldehyde on Hatchability and Survival Rate of Chicks in Disinfection of Fertile Eggs. *Int. J. Pharm. Res. All. Sci.* **2020**, *9*, 105–109.
78. Stegniy, B.T.; Paliy, A.P.; Pavlichenko, O.V.; Stegniy, O.O.; Palii, A.P. Comparative Assessment of the Effect of Dis-infectants on the Level of Biotic Contamination and Hatchability of Chicken Eggs. *J. Vet. Med. Biotechnol. Biosaf.* **2020**, *6*, 17–22. [CrossRef]
79. Patrzałek, M.; Kosecka-Strojek, M.; Lisowska-Łysiak, K.; Trela, M.; Kot, M.; Gawlak, M.; Liszka, D.; Sajewicz, M.; Tombarkiewicz, B.; Pawlak, K.; et al. Preliminary Evaluation of Application of a 3-Dimensional Network Structure of Siloxanes Dergall Preparation on Chick Embryo Development and Microbiological Status of Eggshells. *Poult. Sci.* **2020**, *99*, 1581–1590. [CrossRef] [PubMed]
80. Melo, E.F.; McElreath, J.S.; Wilson, J.L.; Lara, L.J.C.; Cox, N.A.; Jordan, B.J. Effects of a Dry Hydrogen Peroxide Dis-infection System Used in an Egg Cooler on Hatchability and Chick Quality. *Poult. Sci.* **2020**, *99*, 5487–5490. [CrossRef]
81. Cassar, J.R.; Bright, L.M.; Patterson, P.H.; Mills, E.W.; Demirci, A. The Efficacy of Pulsed Ultraviolet Light Processing for Table and Hatching Eggs. *Poult. Sci.* **2021**, *100*, 100923. [CrossRef]
82. Bekhet, G.M.; Sayed, A.A. Oregano-Oil Antagonist Lipopolysaccharide (LPS) Induced Toxicity in Pre- and Post-Hatch Chick Embryo. *J. Appl. Anim. Res.* **2021**, *49*, 211–220. [CrossRef]
83. Branco, J.R.O.; Dallago, B.S.L.; Bernal, F.E.M. Efficiency of Ultraviolet Light for Disinfection of Fertile Broiler Eggs. *Arq. Bras. Med. Vet. Zootec.* **2021**, *73*, 1137–1146. [CrossRef]
84. Koc, S.; Aygun, A. Effects of Ozone on Eggshell Microbial Load, Hatching Traits and Chick Performance in Quail Eggs. *Innoriginal Int. J. Sci.* **2013**, *8*, 47–52.
85. Abo-Samaha, M.I.; Basha, H.A. Effect of Spraying Japanese Quail Eggs with Garlic Oil on Hatching Performance and Hatch Weight. Adv. *Anim. Vet. Sci.* **2021**, *9*, 156–161. [CrossRef]
86. Liu, C.; Zheng, W.; Li, Z.; Zhou, L.; Sun, Y.; Han, S. Slightly Acidic Electrolyzed Water as an Alternative Disinfection Technique for Hatching Eggs. *Poult. Sci.* **2021**, *101*, 101643. [CrossRef]
87. Asad, N.R.; Buarque, L.M.; Asad, O.; Bonacossa De Almeida, C.E.; Felzenszwalb, I.; Bispo Cabral-Neto, J.; Leitão, A.C. Several Pathways of Hydrogen Peroxide Action That Damage the *E. Coli* Genome General Aspects. Genet. *Mol. Biol.* **2004**, *27*, 291–303. [CrossRef]

88. Patil, S.; Valdramidis, V.P.; Karatzas, K.A.G.; Cullen, P.J.; Bourke, P. Assessing the Microbial Oxidative Stress Mechanism of Ozone Treatment through the Responses of *Escherichia Coli* Mutants. *J. Appl. Microbiol.* **2011**, *111*, 136–144. [CrossRef]
89. Dunowska, M.; Morley, P.S.; Hyatt, D.R. The Effect of Virkon®S Fogging on Survival of *Salmonella enterica* and *Staphylococcus Aureus* on Surfaces in a Veterinary Teaching Hospital. *Vet. Microbiol.* **2005**, *105*, 281–289. [CrossRef]
90. Ventura, M.; Wemimont, E. Review of Hydrogen Peroxide Material Safety Data Sheets. In Proceedings of the 38th AIAA/ASME/SAE/ASEE Joint Propulsion Conference & Exhibit, Indianapolis, Indiana, 7–10 July 2002; pp. 1–13.
91. Baysan, A.; Lynch, E. The Use of Ozone in Dentistry and Medicine. Part 2. Ozone and Root Caries. *Prim. Dent. Care* **2006**, *13*, 37–41. [CrossRef]
92. Irshad, M.; Ali Subhani, M.; Ali, S.; Hussain, A. Biological Importance of Essential Oils. In *Essential Oils-Oils of Nature*; El-Shemy, H.A., Ed.; IntechOpen: London, UK, 2019.
93. Stojanović, S.; Najman, S.J.; Bogdanova-Popov, B.; Najman, S.S. Propolis: Chemical Composition, Biological and Pharmacological Activity–a Review. *Acta Med. Median.* **2020**, *59*, 108–113. [CrossRef]
94. Scripnic, E.; Eremia, N. Propolis Extract Use in Incubation Technology for Hens' Eggs Treatment. *Sci. Pap. Ser. D. Anim. Sci.* **2015**, *53*, 330–333.
95. Sasi, M.; Kumar, S.; Kumar, M.; Thapa, S.; Prajapati, U.; Tak, Y.; Changan, S.; Saurabh, V.; Kumari, S.; Kumar, A.; et al. Garlic (Allium sativum L.) Bioactives and Its Role in Alleviating Oral Pathologies. *Antioxidants* **2021**, *10*, 1847. [CrossRef] [PubMed]
96. Chen, C.; Liu, C.H.; Cai, J.; Zhang, W.; Qi, W.L.; Wang, Z.; Liu, Z.B.; Yang, Y. Broad-Spectrum Antimicrobial Ac-tivity, Chemical Composition and Mechanism of Action of Garlic (*Allium sativum*) Extracts. *Food Control* **2018**, *86*, 117–125. [CrossRef]
97. Tariq, S.; Wani, S.; Rasool, W.; Shafi, K.; Bhat, M.A.; Prabhakar, A.; Shalla, A.H.; Rather, M.A. A Comprehensive Re-view of the Antibacterial, Antifungal and Antiviral Potential of Essential Oils and Their Chemical Constituents against Drug-Resistant Microbial Pathogens. *Microb. Pathog.* **2019**, *134*, 103580. [CrossRef] [PubMed]
98. Vadillo-Rodríguez, V.; Cavagnola, M.A.; Pérez-Giraldo, C.; Fernández-Calderón, M.C. A Physico-Chemical Study of the Interaction of Ethanolic Extracts of Propolis with Bacterial Cells. *Coll. Surf. B Bioint.* **2021**, *200*, 111571. [CrossRef]
99. Stähli, A.; Schröter, H.; Bullitta, S.; Serralutzu, F.; Dore, A.; Nietzsche, S.; Milia, E.; Sculean, A.; Eick, S. In Vitro Activity of Propolis on Oral Microorganisms and Biofilms. *Antibiotics* **2021**, *10*, 1045. [CrossRef]
100. Nolan, L.K.; John Barnes, H.; Vaillancourt, J.-P.; Abdul-Aziz, T.; Logue, C.M. Colibacillosis. In *Diseases of Poultry*; Swayne, D.E., Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2013.
101. Hasyim, A.R.; Lase, J.A.; Alwiyah, A.; Suroto, S.; Khairiyah, K.; Hutagalung, M.; Harahap, S.M.; el Ramija, K.; Lestari, D.; Ardiarini, N.; et al. The Effectiveness of Cherry Leaf Extract (*Muntingia calabura* L.) as an Anti-Bacterial Against Hatchability of Kub Chicken Eggs in Artificial Hatchery. *Bul. Peternak.* **2021**, *45*, 214. [CrossRef]
102. Patterson, P.H.; Ricke, S.C.; Sunde, M.L.; Schaefer, D.M. Hatching Eggs Sanitized with Chlorine Dioxide Foam: Egg Hatchability and Bactericidal. *Avian Dis.* **1990**, *34*, 1–6. [CrossRef]

Paper two

Sanitizing Hatching Eggs with Essential Oils: Avian and Microbiological Safety

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Review

Sanitizing Hatching Eggs with Essential Oils: Avian and Microbiological Safety

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Abstract: Increased meat and egg production leads to concomitant changes in poultry practices, including the indiscriminate use of formaldehyde to sanitize hatching eggs. Although this sanitizer aids in the increase in poultry production, its toxic potential for man and for avian embryos represents an obstacle to its long-term use. This review assesses whether essential oils fit into the context of hatching egg contamination, reviewing their antimicrobial efficiency, toxicity to poultry embryos and chicks, and their sanitizing effects on poultry production parameters. Studies have indicated that, because they are safer, most of the essential oils studied can be a potential substitute for formaldehyde for minimizing microbial exposure of hatching eggs and embryos. However, complementary studies on the microbiological profile of embryos and chicks hatched from eggs sanitized with essential oils need to be carried out and the economic feasibility of the candidate products should also be considered.

Keywords: economic gains; egg disinfection; embryological safety; egg microbiology; poultry health; poultry production

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1. Introduction

The large number of healthy embryos that hatch supports the hypothesis that eggs have good microbiological quality. Ensuring embryo safety in the face of microbiological challenges is not easy. The embryo's immature status makes it insecure and defenseless against infection [1]. In this case, the eggshell can have a negative effect because it contains pathogenic microorganisms [2] and has communication routes with the embryo, favoring contact between them. Therefore, the quest for healthier poultry is increasing the need to incubate eggs with minimal microbial loads in poultry hatcheries during all incubation cycles. In this case, sanitizing hatching eggs with liquid or gas is the gold standard method of achieving this goal [3]. The sanitization of hatching eggs is nothing more than an antimicrobial resource intermediated by a simple or complex system (e.g., fumigation, spraying, or immersion) that applies a sanitizing solution to the eggshells to solve poultry losses caused by microorganisms. This step must occur within half an hour after laying or immediately collection [4–6].

In line with the current trend towards ecologically friendly products with minimal impact on animals, the poultry industry needs to gradually adopt sanitizers that respect safety criteria for the protection of avian life. In a previously published review, Oliveira et al. [3] showed that there are various sanitizers for hatching eggs that are available to the poultry industry which are divided into two large groups (synthetic and natural). Among the natural options recommended to the industry, the authors show that essential oils derived from volatile liquids from aromatic plants are antimicrobial and safe to use. The use of essential oils as sanitizers for hatching eggs was reviewed by Oliveira et al. [5].

They reported that essential oils compete with synthetic materials for reasons that are of interest to the poultry industry, including embryo and human safety, the ability to control microorganisms in the eggshell, and increased production rates. These effects can be seen at low concentrations, which may overcome the disadvantages of essential oils where they are more expensive than synthetic compounds that require higher concentrations for effective action. Thus, validating the potential and advantageous characteristics of essential oils in the management of hatching eggs can open an important path for their inclusion in the official list of sanitizers used in poultry routines around the world. This is still a limited field of research. A search carried out in the SCOPUS database showed that, between 1970 and 2022, 89 papers were published evaluating sanitizers for hatching eggs, including research, reviews, conference papers, and book chapters written in English, French, Portuguese, and Russian. Of the 89 papers, only 13 were papers that studied essential oils for hatching eggs, and these came from Brazil (5), Turkey (3), Saudi Arabia (3) and Germany (2) (Figure 1), with 76.92% (10) published between 2019 and 2022 (Figure 2).

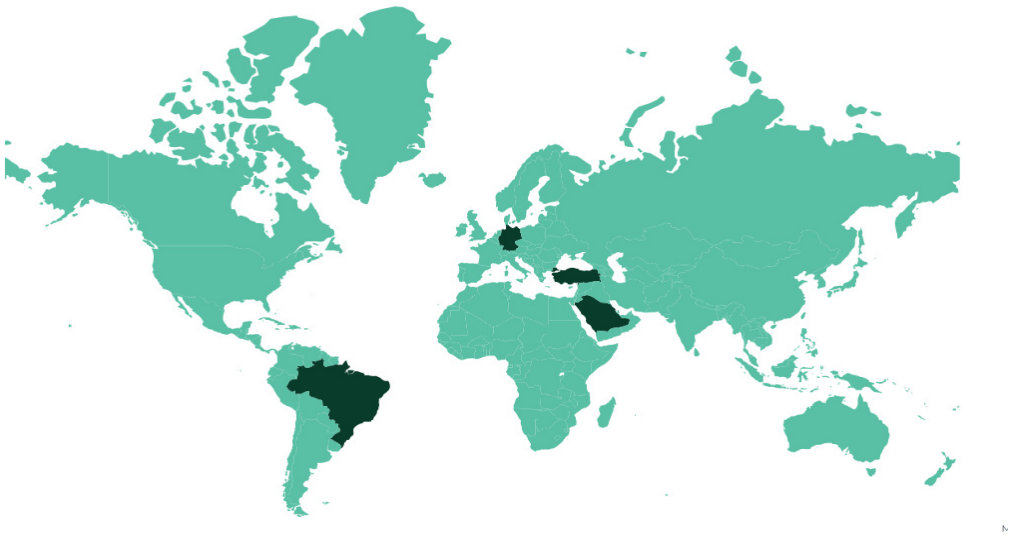

Figure 1. Countries (highlighted in dark green) that most published papers studying essential oils as sanitizers for hatching eggs between 1970 and 2022. Search format performed in SCOPUS: (TITLE-ABS-KEY (sanitizers AND for AND hatching AND eggs) OR TITLE-ABS-KEY (disinfectants AND for AND hatching AND eggs) OR TITLE-ABS-KEY (sanitization AND of AND hatching AND eggs) OR TITLE-ABS-KEY (disinfection AND of AND hatching AND eggs) OR TITLE-ABS-KEY (decontamination AND of AND hatching AND eggs)). Of the 238 papers found, 89 evaluated sanitizers for hatching eggs and of these 13 involved essential oils. The information was collected on 15 February 2023.

The current preventive measure based on formaldehyde to reduce the microbial load of hatching eggs commercially is not friendly to any living organism and its replacement can achieve sustainability in egg sanitization. In this sense, it is important to assess whether essential oils can really fit into the context of decontamination of hatching eggs. Therefore, this study reviewed the antimicrobial efficiency of essential oils and their toxicity in poultry embryos and chicks, as well as their sanitizing effects on poultry production parameters. To better support our discussions and cover gaps in the application of essential oils as sanitizers for hatching eggs, any study involving the direct or indirect relationship between hatching eggs and essential oils, as well as table eggs and essential oils, was reviewed.

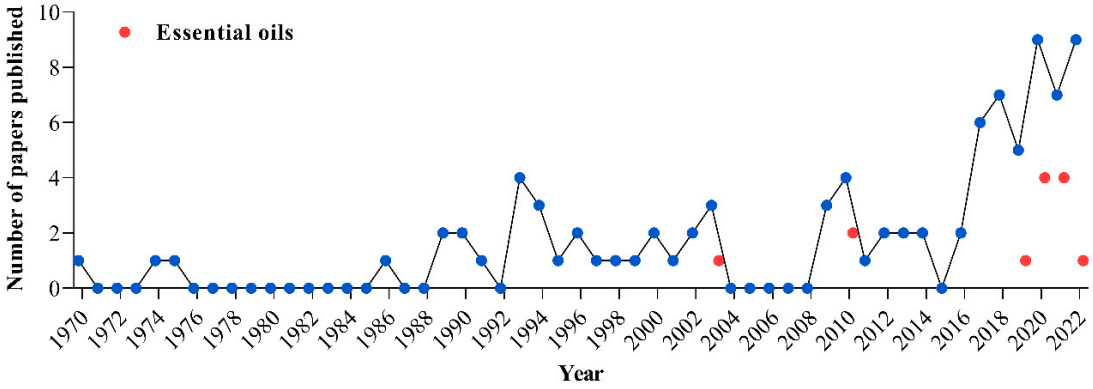

Figure 2. Number of published studies on the use of essential oils as sanitizers for hatching eggs (red ball) compared to the number of published studies that evaluated other sanitizers for hatching eggs (blue ball) in the period 1970 to 2022. Search format performed in SCOPUS: (TITLE-ABS-KEY (sanitizers AND for AND hatching AND eggs) OR TITLE-ABS-KEY (disinfectants AND for AND hatching AND eggs) OR TITLE-ABS-KEY (sanitization AND of AND hatching AND eggs) OR TITLE-ABS-KEY (disinfection AND of AND hatching AND eggs) OR TITLE-ABS-KEY (decontamination AND of AND hatching AND eggs)). Of the 238 papers found, 89 evaluated sanitizers for hatching eggs and of these 13 involved essential oils. The information was collected on 15 February 2023.

2. Paper Search Method

In addition to the search performed via Scopus (mentioned earlier in the introduction), Google Scholar was used to search for the papers reviewed in this study. Several search terms were used to find the research and review papers, book chapters and books that fit the proposed content for each topic, including contamination of hatching eggs, chicken embryo infection, sanitizers for hatching eggs, essential oils, antimicrobial activity of essential oil, essential oil for hatching eggs, eggs and essential oils, essential oil and chicken embryos and formaldehyde for hatching eggs. These terms were also searched in Portuguese when necessary to reach the maximum number of studies. Monographs and dissertations were also considered for review on topics where published studies were scarce. The title, abstract and keywords of the studies in English or Portuguese were read and, if they met the objective of the topic, the study was revised in full. Otherwise, the study was disregarded. This was carried out until each topic was completely written.

3. Eggshell Microorganisms: Risks for Poultry Embryos and Chicks

Even as an immunologically sensitive embryo, poultry already interact with pathogenic microbes originating from any stage prior to hatching [7]. This interaction may be a consequence of horizontal transmission [8] (Figure 3) and puts the poultry's life in danger. Fonseca et al. [9] observed that, by contaminating the eggshell, *Campylobacter jejuni* bacteria can penetrate it, cross the albumen and reach the yolk sac, probably resulting in embryonic mortality. This led the authors to state that the immunity conferred by breeding hens to the egg/embryo may be insufficient and inefficient for certain infections. In addition, although the eggshell is an oval antimicrobial wall formed by the fusion of membranes and mineral layers equivalent to a vital organ of a living organism (it promotes the flow of nutrients, water, oxygen and carbon dioxide to keep the embryo alive) [10], it is not totally resistant to microbial entry. The eggshell is challenged when there are microorganisms trying to move from its surface to the main target (embryo). Oliveira et al. [6] reviewed the types of microorganisms that contaminate eggshells. Among the bacterial and fungal genera cited are *Alcaligenes*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Providencia*, *Pseudomonas*, *Salmonella*, *Clostridium*, *Enterococcus*, *Staphylococcus*, *Streptococcus*, *Aspergillus*, *Candida* and *Penicillium*.

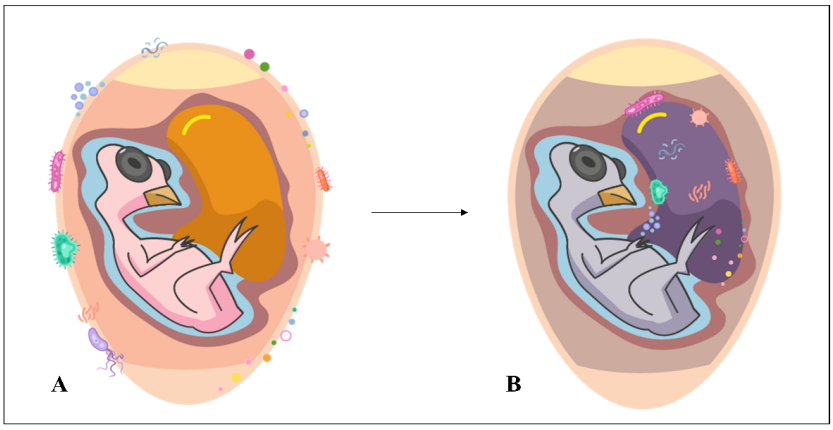

Figure 3. Horizontal contamination in hatching eggs. Microorganisms on the shell (**A**) penetrated and reached the yolk sac (**B**).

Previously published studies have reported the adverse relationship of microorganisms with embryos and/or chicks. Weil and Volentine [11] reported that contamination of the yolk sac of the chicken embryo by *Shigella dysenteriae* can cause lethal infection. Embryos from chickens killed by contamination with avian pathogenic *Escherichia coli* and *Salmonella enterica subsp. enterica serovar* Enteritidis showed signs of congestion and diffuse redness throughout the skin, head and neck, as well as microscopic lesions in the yolk sac, including congestion, inflammation, damaged blood vessels and abnormal endodermal epithelial cells [1]. Fungi of the genus *Aspergillus*, which may be responsible for causing mycoses or mycotoxicosis, have been isolated from dead chicken embryos [12]. Saleemi et al. [13] reported that aflatoxigenic fungal extracts isolated from *Aspergillus* fungi caused high embryonic mortality, weight reduction and severe alterations in the liver (fatty alteration and cell necrosis) and kidneys (congestion and tubular necrosis) of chicks. Karunarathna et al. [14] demonstrated that multidrug-resistant *Escherichia coli* and *Enterococcus* were recovered from the yolk of non-viable chicken embryos at hatching. Contamination by *Enterococcus* spp. can trigger pulmonary hypertension syndrome in chicken embryos and chicks [15]. Mortality of chicken embryos associated with *Enterococcus* contamination was reported by Karunarathna et al. [16]. Multidrug-resistant bacteria that cause yolk sac infection, including *Escherichia coli*, *Salmonella*, and *Staphylococcus*, have been recovered from dead embryos and chicks [17,18]. Far et al. [19] observed that dead ostrich embryos were contaminated with *Pseudomonas* spp., *Klebsiella* spp., *Bacillus* spp., *Citrobacter* spp., *Staphylococcus* spp., *Proteus* spp., *Aeromonas* spp., *Enterobacter* spp., as well as *Escherichia coli* with antimicrobial resistance profile.

The findings mentioned above raise concerns, especially in relation to the health of poultry and humans, since multiresistant microorganisms can spread and cause massive irreversible damage. In addition, the undue, exacerbated use of sanitizers without proven scientific tests and without the prescription of trained professionals can contribute to even worse health and economic instability. Therefore, collective efforts within the poultry industry should focus on antimicrobial interventions that involve the controlled use of broad-spectrum sanitizers focused on hatching egg sanitization.

4. Essential Oils and Their In Vitro Antimicrobial Activity

Essential oils are any aromatic, viscous and volatile oils belonging to plants. *Syzygium aromaticum*, *Allium sativum*, *Ocimum basilicum*, *Thymus vulgaris*, *Lavandula angustifolia*, *Eucalyptus globulus*, *Citrus sinensis*, *Citrus aurantifolia*, *Cinnamomum cassia*, *Rosmarinus officinalis*, *Origanum vulgare*, *Allium cepa*, *Cymbopogon winterianus*, *Cymbopogon flexuosus*, *Piper nigrum*, *Zingiber officinale*, *Protium pallidum*, *Litsea citrata*, *Satureja hortensis*, *Salvia officinalis*, *Mentha piperita*, *Cedrus deodara*, and *Cuminum cymincum* are examples of plant species that provide commercially available essential oils that may have promising futures in poultry nutrition

and production such as egg coating additives and sanitizers for hatching eggs. This is because essential oils have a chemical configuration that triggers their biological properties. For example, hydrocarbons, esters, lactones, alcohols, oxides, phenols, ketones, and aldehydes are present in the chemical composition of essential oils with similar or distinct bioactive functions. Depending on the compound, these functions include antimicrobial, antiviral, antitumoral, antibacterial, stimulant, anesthetic, anti-inflammatory, anti-fungal, antipyretic, and spasmolytic [20]. The content, quality, and effectiveness of essential oil compounds depend on factors such as extraction, which can be by hydro distillation, steam distillation, supercritical CO_2 extraction, ultrasonic extraction, and cold pressing [21–24].

In vitro antimicrobial screenings initially detect the potential viability of essential oils before they are used as in vivo antimicrobial agents. These screenings demonstrated that essential oils are effective against standard Gram-negative and positive bacterial strains and avian isolates, as well as standard and avian-isolated fungi (Table 1). Among the bacteria are *Salmonella enterica subsp. enterica serovar* Enteritidis, *Salmonella enterica subsp. enterica serovar* Typhimurium, *Salmonella enterica subsp. enterica serovar* Infantis and avian pathogenic *Escherichia coli* (APEC), which are important pathogenic bacteria for poultry and public health (Table 1). The antimicrobial effectiveness of essential oils ranges from mild to very strong. In fact, some of them have been shown to be more effective than conventional antibiotics [25,26]. Thymol, eugenol, carvacrol, linalool, citral, limonene, trans-cinnamaldehyde, geraniol and citronellal are some compounds that are part of the composition of some essential oils that can act as protagonists in antimicrobial action (Table 1). The main mechanisms responsible for making the bacterial [27] and fungal [28] cells unfeasible are listed below:

Bacteria:

1. Cell membrane alteration and increased permeability.
2. Stops energy production.
3. Blocks active transport.

Fungi:

1. Cell membrane disruption, alteration, and inhibition of cell wall formation.
2. Dysfunction of fungal mitochondria.
3. Inhibition of efflux pumps.

Essential oils can promote beneficial actions for human health by reducing pain and inflammation, protecting and healing wounds, neutralizing or stopping the development of carcinogens, neutralizing oxidative stress and possessing antiviral, antibacterial, antifungal, cardioprotective, antidiabetic, and insect-repellent properties; among other benefits, they can also potentially treat central-nervous-system-based disorders [29–32]. The safety of a stock of essential oils including *Ocimum basilicum*, *Zingiber officinale*, *Lavandula officinalis*, *Cymbopogon citratus*, *Mentha piperita*, *Rosmarinus officinalis*, *Thymus vulgaris*, *Eugenia caryophyllata*, and *Allium sativum* has been documented and they received the generally recognized as safe (GRAS) seal [33]. However, the intake of essential oils needs to be monitored, as they can, like any other edible food, cause an inappropriate effect.

Table 1. Microorganisms sensitive to essential oils through in vitro screening.

Essential Oil	Majority Element	Analysis	Method	Microorganism	Origin Microorganism	Study
Thymus vulgaris *Origanum vulgare* *Mentha pulegium*	-	B*	Disk diffusion	• *Bacillus cereus* • *Clostridium perfringens* • *Enterococcus faecalis* • *Enterococcus faecium* • *Escherichia coli* • *Listeria monocytogenes* • *Pseudomonas aeruginosa* • *Salmonella enterica subsp. enterica serovar* Enteritidis • *Staphylococcus aureus* • *Staphylococcus epidermidis*	ATCC	[34]
Allium sativum	Diallyl disulfide (44.6%)	B		• *Salmonella enterica subsp. enterica serovar* Typhimurium • *Yersinia enterocolitica* • *Bacillus cereus* • *Staphylococcus aureus*	ATCC and NCTC	[35]
Cinnamomum cassia *Syzygium aromaticum*	Eugenol (72.13%) Eugenol (83.63%)	B	Agar dilution	• *Escherichia coli* • *Staphylococcus aureus* • *Pseudomonas aeruginosa*	ATCC and human clinical isolate	[36]
Thymus vulgaris *Foeniculum vulgare* *Cuminum cyminum*	-	B and F*	Disk diffusion	• *Escherichia coli* • *Staphylococcus aureus* • *Pasteurella multocida* • *Salmonella enterica subsp. enterica serovar* Typhimurium • *Aspergillus fumigatus* • *Candida albicans*	MTCC	[37]
Origanum vulgare *Origanum majorana*	-	B	Disk diffusion and broth microdilution	• *Staphylococcus aureus*	Poultry meat	[38]
Thymus vulgaris *Origanum vulgare*	Thymol (41.60%) Carvacrol (53.4%)	B	Broth microdilution	• *Bacillus cereus* • *Staphylococcus aureus* • *Salmonella enterica subsp. enterica serovar* Infantis • *Escherichia coli*	Clinical isolate and poultry meat isolate	[39]

Table 1. *Cont.*

Essential Oil	Majority Element	Analysis	Method	Microorganism	Origin Microorganism	Study
Lippia rotundifolia *Lippia origanoides*	-	B	Disk diffusion and dilution	• *Staphylococcus aureus* • *Escherichia coli*	Poultry feces	[40]
Thymus schimperi *Rosmarinus officinalis* *Eucalyptus globulus*	Carvacrol (71.02%) α-Pinene (50.83%) 1,8-Cineole (63.00%)	B	Well diffusion	• *Streptococcus pyogenes* • *Staphylococcus epidermidis* • *Salmonella enterica subsp. enterica serovar* Typhimurium • *Shigella* spp. • *Pseudomonas aeruginosa* • *Staphylococcus aureus* • *Escherichia coli* • *Trichophyton* spp. • *Aspergillus* spp.	-	[41]
Pimenta pseudocaryophyllus Citrus Terpenes	- Limonene (28.67%)	B	Disk diffusion	• *Salmonella enterica subsp. enterica serovar* Enteritidis • *Escherichia coli* • *Staphylococcus aureus* • *Listeria innocua* • *Enterococcus faecalis*	ATCC	[42]
Lavandula × *intermedia* *Lavandula angustifolia*	Linalool (57.10%) Linalool (53.97%)	B and F	Well diffusion and broth microdilution	• *Bacillus cereus* • *Bacillus pumilus* • *Enterococcus faecalis* • *Escherichia coli* • *Klebsiella oxytoca* • *Klebsiella pneumoniae* • *Kocuria rhizophila* • *Listeria monocytogenes* • *Proteus mirabilis* • *Pseudomonas aeruginosa* • *Salmonella enterica subsp. enterica serovar* Enteritidis • *Staphylococcus aureus* • *Streptococcus pyogenes* • *Yersinia enterocolitica* • *Candida albicans* • *Candida glabrata* • *Candida kefyr*	ATCC, NCTC, and food and clinical isolates	[43]

Table 1. *Cont.*

Essential Oil	Majority Element	Analysis	Method	Microorganism	Origin Microorganism	Study
Lavandula × *intermedia* *Lavandula angustifolia*	Linalool (57.10%) Linalool (53.97%)	B and F	Well diffusion and broth microdilution	• *Candida krusei* • *Candida tropicalis* • *Cryptococcus neoformans* • *Hansenula anomala* • *Saprochaete capitate* • *Microsporum canis* • *Microsporum gypseum* • *Trichophyton mentagrophytes* • *Trichophyton rubrum* • *Aspergillus fumigatus* • *Aspergillus niger* • *Fusarium oxysporum* • *Penicillium citrinum*	ATCC, NCTC, and food and clinical isolates	[43]
Kaempferia galanga *Cymbopogon flexuosus* *Pogostemon cablin* *Curcuma caesia* *Cymbopogon winterianus* *Clausena heptaphylla* *Cinnamomum tamala* *Ocimum sanctum* *Cinnamomum camphora*	P-Methoxycinnamate (27.84%) Geranial (Citral a) (42.14%) Patchouli alcohol (32.33%) Eucalyptol (15.05%) Citronellal (38.68%) (E)-Anethole (53.49%) Eugenol (72.33%) Eugenol (41.89%) Camphor (49.43%)	B and F	Disk diffusion and broth dilution	• *Staphylococcus aureus* • *Bacillus cereus* • *Bacillus subtilis* • *Salmonella enterica subsp. enterica serovar* Typhimurium • *Escherichia coli* • *Aspergillus niger* • *Aspergillus fumigatus* • *Saccharomyces cerevisiae* *Candida albicans*	ATCC	[44]
Aloysia triphylla *Cinnamomum zeylanicum* *Cymbopogon citratus* *Litsea cubeba* *Mentha piperita*	Limonene (*E*)-Cinamaldeído Neral Geranial Mentol	B and F	Disk diffusion and broth microdilution	• *Salmonella enterica subsp. enterica serovar* Enteritidis • *Salmonella enterica subsp. enterica serovar* Typhimurium • *Saccharomyces cerevisiae*	Poultry	[45]
Syzygium aromaticum	-	B	Disk diffusion and dilution	• Avian pathogenic *Escherichia coli* • *Escherichia coli* • *Salmonella enterica subsp. enterica serovar* Enteritidis • *Salmonella* spp.	Poultry	[46]

Table 1. *Cont.*

Essential Oil	Majority Element	Analysis	Method	Microorganism	Origin Microorganism	Study
Satureja kitaibelii *Origanum vulgare* *Achillea millefolium* *Achillea clypeolata* *Thymus serpyllum*	p-Cymene (24.4%) Germacrene D (21.5%) Camphor (9.8%) 1,8-Cineole (45.1%) Geraniol (63.4%)	B	Broth microdilution	• *Escherichia coli* • *Staphylococcus aureus*	ATCC	[47]
Origanum vulgare	Carvacrol (66.98%)	B	Broth microdilution	• *Salmonella enterica subsp. enterica* serovar Infantis	Intensive poultry farms (boot swabs)	[48]
Melaleuca alternifolia *Rosmarinus officinalis*	Terpinen-4-ol (>30%) 1,8-Cineole (>30%)	B and F	Modified zone of inhibition test with glass cylinders	• *Mycobacterium smegmatis* • *Staphylococcus epidermidis* • *Staphylococcus aureus* • *Methicillin-resistant Staphylococcus aureus* • *Streptococcus pyogenes* • *Pseudomonas aeruginosa* • *Antibiotic-resistant Pseudomonas aeruginosa* • *Bordetella bronchiseptica* • *Klebsiella pneumoniae* • *Candida albicans*	ATCC	[49]
Cinnamomum cassia	Trans-cinnamaldehyde (>30%)					
Cymbopogon flexuosus *Cymbopogon martini* *Eucalyptus citridora* *Pelargonium* spp.	Citral (81.84%) Geraniol (63.79%) Citronellal (76.80%) Geraniol (22.38%)	B and F	Disk diffusion and serial dilution	• *Escherichia coli* • *Escherichia coli* • *Salmonella enterica subsp. enterica serovar* Typhimurium • *Proteus vulgaris* • *Pseudomonas aeruginosa* • *Staphylococcus aureus* • *Streptococcus faecalis* • *Bacillus subtilis* • *Xanthomonas oryzae* • *Xanthomonas malvacearum* • *Aspergillus niger* • *Fusarium oxysporum* • *Fusarium udum* • *Magnaporthe grisea*	NCIM and isolated from blight and blast infected leaves	[50]
Cymbopogon winterianus	Citronellal (34.10%)					

Table 1. *Cont.*

Essential Oil	Majority Element	Analysis	Method	Microorganism	Origin Microorganism	Study
Satureja hortensis	Thymol (41.28%)	B	Disk diffusion and broth microdilution	• *Escherichia coli* • *Salmonella enterica subsp. enterica serovar* Enteritidis	Poultry infections	[51]
Origanum vulgare *Melaleuca alternifolia* *Citrus limonum,* *Cinnamomum cassia,* *Eugenia caryophyllus,* *Eucalyptus globulus, and* *Rosmarinus officinalis*	Carvacrol (65.80%) Terpinen-4-ol (39.60%) - -	B and F	Disk diffusion	• *Staphylococcus aureus* • *Escherichia coli* • *Candida albicans*	ATCC	[52]
Ocimum basilicum	Linalool (65.20%)					
Crithmum maritimum *Cuminum cyminum* *Cupressus arizonica* *Pimpinella anisum*	γ-Terpinene (32.9%) Cumin aldehyde (30.2%) α-Pinene (41.0%) (E)-Anethole (96.7%)	B and F	Well Diffusion and broth microdilution	• *Escherichia coli* • *Listeria* monocytogenes • *Staphylococcus aureus* • *Candida* albicans • Pseudomonas fluorescens	ATCC and DSMZ	[53]
Zingiber officinale *Citrus aurantifolia* *Cymbopogon citratus*	-	B	Disk diffusion	• *Escherichia coli* • Staphylococcus aureus	ATCC	[54]
Origanum vulgare *Thymus vulgaris* *Eugenia caryophyllus* *Cinnamomum cassia*	Carvacrol (68.72%) Thymol (54.60%) Eugenol (86.25%) Trans-cinnamaldehyde (86.57%)	B	Broth microdilution	• *Staphylococcus aureus* • *Listeria monocytogenes* • *Escherichia coli* • *Salmonella enterica subsp. enterica serovar* Typhimurium • *Staphylococcus aureus* • *Listeria monocytogenes* • *Escherichia coli* • *Salmonella* spp.	ATCC and isolated food	[55]
Ocimum basilicum	Estragole (60.98%)	B	Broth microdilution	• *Escherichia coli* • Staphylococcus aureus	ATCC	[56]

*B, Antibacterial; *F, Antifungal; ATCC, American Type of Culture Collection; MTCC, Microbial Type Culture Collection; NCTC, National Collection of Type Cultures; NCIM, National Collection of Industrial Microorganisms; DSMZ, German Collection of Microorganisms and Cell Culture. Only essential oils that inhibited the growth of all bacteria/fungi in each study were cited in the table. When the study used two antimicrobial screening methods, we considered that the oil was efficient when it inhibited microorganisms in at least one of them. The information was collected on 26 April 2023.

5. Antimicrobial Effect of Essential Oils In Vivo (Eggshells)

Sanitization is the basis that sustains microbial control in hatching eggs. By prioritizing sanitization, the poultry industry prevents contamination between hatching eggs themselves and between hatching eggs, humans and poultry. This minimizes or nullifies the risk of pathogenic contamination to poultry and human lives. Thus, sanitizers that combine at least bactericidal or bacteriostatic and fungicidal or fungistatic characteristics are compatible options for sanitizing hatching eggs. As shown in Table 1, microorganisms that can colonize the eggshells [6] showed to be sensitive to the action of different essential oils. This is supported by in vivo tests, which have shown that essential oils reduce the total count of mesophilic aerobic bacteria, enterobacteria, molds and yeasts (Table 2). In addition, essential oil components such as carvacrol, eugenol and trans-cinnamaldehyde at 0.25%, 0.5% and 0.75% when applied to eggs by immersion showed the potential to inactivate *Salmonella enterica subsp. enterica serovar* Enteritidis in eggshells with or without organic matter [57]. More importantly, the ability of trans-cinnamaldehyde to block the migration of this microorganism into the egg contents has been suggested [58]. This evidence may support observed or suggested findings that chicken embryos from eggs sanitized with essential oils or their compounds have a reduced microbial load [59,60].

Table 2. Eggshell microbial counts reduced by actions of essential oils.

Essential Oil or Its Component	Essential Oil Concentration	Eggshell Application	Egg Type	Eggshell Contamination	Eggshell Microbial Load	Study
Carvacrol Eugenol Trans-cinnamaldehyde	0.25, 0.5 and 0.75%	Immersing	Chick	Inoculation	• *Salmonella enterica subsp. enterica serovar* Enteritidis	[57]
Thymus vulgaris	0.25, 0.5 and 1 mg/mL	Immersing	Chick	Inoculation	• *Salmonella enterica subsp. enterica serovar* Enteritidis CICC 21482 • *Salmonella enterica subsp. enterica serovar* Typhimurium CICC 22956	[61]
Cymbopogon winterianus	0.1, 0.15 and 0.2%	Spraying	Chick	Natural	• Molds and yeasts	[62]
Cymbopogon flexuosus *Lippia rotundifolia*	1%	Immersing	Chick	Natural	• Total coliforms • Yeast and filamentous fungi • Aerobic mesophylls	[63]
Syzygium aromaticum	0.39%	Spraying	Chick	Natural	• Total aerobic mesophilic bacteria • Enterobacteriaceae	[64]
Syzygium aromaticum	10–80 µg/g	Vaporizing	Chick	Inoculation Natural	• *Escherichia coli* • *Salmonella enterica subsp. enterica serovar* Typhimurium • *Staphylococcus aureus* • Bacterial counts	[65]
Origanum vulgare *Cuminum cyminum*	0.5%	Immersing	Chick	Natural	• Total bacterial	[66]
Trans-cinnamaldehyde	0.48%	Immersing	Chick	Inoculation	• *Salmonella enterica subsp. enterica serovar* Enteritidis	[58]

Essential oils also contribute to the self-sanitization of eggshell surfaces when used as a bioactive element in coatings applied by spraying or immersion and permanently formed on the surface of eggs [54,67,68]. This was associated with a microbial reduction of eggshells and egg contents [56,67]. Its antimicrobial effect seems to remain active for

longer periods [5,54,59,67], protecting the eggs from microbial recontamination, without the need for additional applications. Frequently reapplication of a sanitizer to control egg contamination is not ideal for two reasons: the cost and because sanitizing during incubation can have undesirable effects on the embryos and reduce hatchability as reported in hatching eggs sprayed with albumin at different incubation periods [69]. In addition to the fact that, in the early stages of incubation, embryos are particularly sensitive to sanitizers such as formaldehyde [70], it is hypothesized that the application of sanitizers during incubation may influence eggshell temperature, particularly if applied by liquid, which interferes with the proper development of the embryo. Therefore, it is recommended to use sanitizers that do not require continuous reapplication, such as those based on essential oils.

6. Toxicity of Essential Oils for Poultry Embryos and Chicks

Before essential oils are effectively used for a specific purpose within the poultry chain, it is advisable to consult scientific studies that prove the limits of the safe use of essential oils. Although essential oils have beneficial antimicrobial residual effects on hatching eggs [5,71], care must be taken to ensure that their contact with embryos and chicks does not cause permanent damage that limits their behavior, physiology, morphology and, above all, their survival. Embryo development with successful hatching is the first positive sign of evaluating a sanitizer. In advance, essential oils should have a positive evaluation, as it has been reported that hatchability rates of hatching eggs sanitized with essential oils can be improved by up to 12.59% [5]. However, so that this preliminary assessment can be better supported, we review the toxic or non-toxic effects of essential oils on embryos and chicks below.

According to de Oliveira [72], spraying of *Melaleuca alternifolia* essential oil at 0.75% on the shells or its delivery in the air chamber of hatching eggs did not affect the viability, heart rate, probability of occurrence of malformation, or the developmental stage of chicken embryos. However, the probability of survival was significantly reduced when this oil was injected into the air chamber. Morphological abnormalities in embryos/chicks from eggs sprayed with essential oils have been reported [72,73], but according to de Oliveira [72], they were within normal limits. Demirci et al. [74] reported that the application of *Origanum onite* essential oil at 250 µg/pellet strongly irritated the chorioallantoic membrane. They stated that this was due to thymol (11.6%) present in the oil composition. Essential oil compounds can negatively affect poultry embryos depending on how they are applied. A dose of 50 µM Citral caused embryonic malformation [75] and a dose of carvacrol (50 µg/kg) impaired the normal development of embryos [76] when injected in ovo. These effects are induced based on concentration [75,76]. On the other hand, Ulucay and Yildirim [77] suggested that embryo respiration and quail chick weight were not affected after egg sanitization with 1% thymol, carvacrol, or cinnamaldehyde. Thus, the chemical composition and route of application of essential oils are factors that can have a significant influence on embryo safety.

Syzygium aromaticum essential oil at 0.39%, when applied to hatching eggs, did not cause alterations or lesions in the trachea of day-old chicks (Figure 4) showing that the application of this compound to hatching eggshells in pre-incubation and without re-application during incubation had a protective effect and probably did not cause any tracheal tissue disturbance that compromised the respiratory system of day-old chicks. Furthermore, in the histological analysis of tissues and organs (large and small intestines, pectoral muscle, proventriculus and gizzard, liver and gallbladder, and heart), no microscopical changes were detected (Figure 5). The lack of morphological changes in the tissue samples supports the absence or negligible topical toxicity of *Syzygium aromaticum* essential oil in ensuring the hatching of healthy chicks.

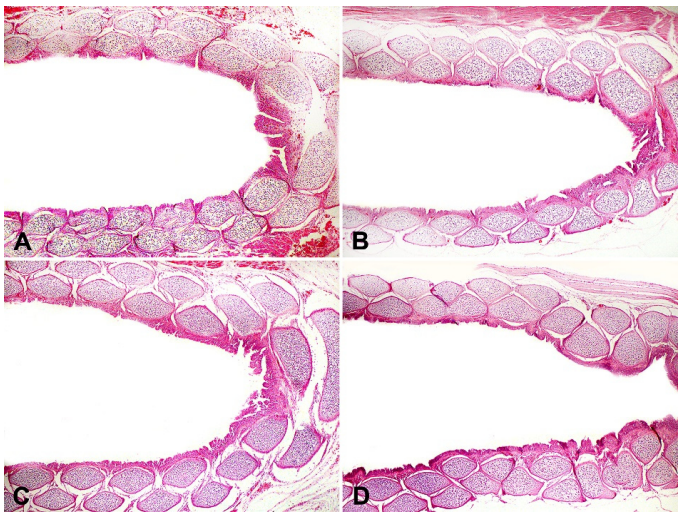

Figure 4. One-day-old chicks. Tracheas showing no histological changes (H&E objective 4×). (**A**) Chick from non-sanitized eggs. (**B**) Chick from eggs sanitized with *Syzygium aromaticum* essential oil. (**C**) Chick from eggs sanitized with grain alcohol. (**D**) Chick from eggs sanitized with paraformaldehyde. No significant difference among treatments tested.

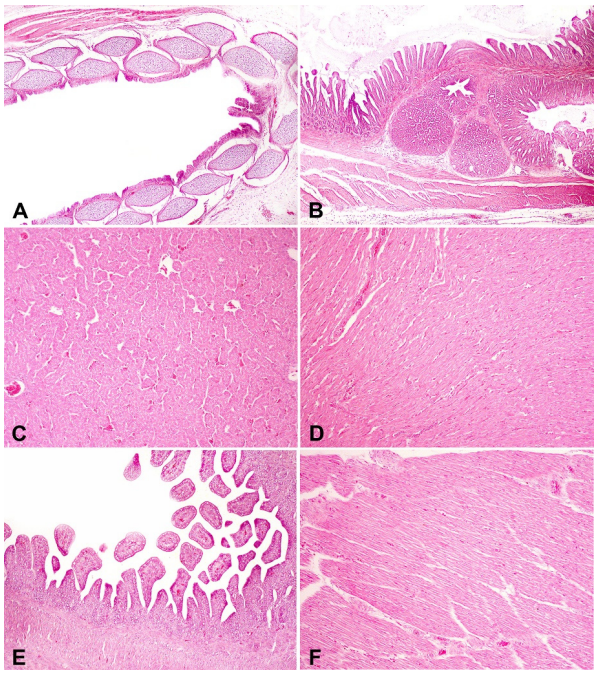

Figure 5. One-day-old chicks. Organs showing no histological changes. (**A**) Trachea, chick from eggs sanitized with paraformaldehyde (H&E objective 4×). (**B**) Proventriculus and gizzard, chick from non-sanitized eggs (H&E objective 4×). (**C**) Liver and gallbladder, chick from eggs sanitized with grain alcohol (H&E objective 10×). (**D**) Heart, chick from eggs sanitized with paraformaldehyde (H&E objective 10×). (**E**) Intestine, chick from eggs sanitized with *Syzygium aromaticum* essential oil (H&E objective 10×). (**F**) Chest, skeletal muscle, chick from eggs sanitized with grain alcohol (H&E objective 10×). No significant difference among treatments tested.

Other published studies have reinforced that most essential oils do not have negative effects on embryos and chicks, even when injected directly into the developing embryo (Table 3).

Table 3. Effects of direct or non-direct application of essential oils on embryos or chicks.

Essential Oil or Its Component	Essential Oil Concentration	Egg Application Method	Application Target	Egg Type	Authors' Findings for Embryos and Chicks	Study
Origanum vulgare *Cuminum cyminum*	0.2 and 0.4% or 0.5%	Sanitizing	Eggshell	Chick	• Improved hatch time and chick body weight. • Heavy and well-developed embryos. • No brain and spinal cord malformations in the embryos.	[66,71,78]
Juniperus excelsa	10% ratio of 9 (oil):1 (ethyl alcohol)	Micropipetting	Blastodisc	Chick	• Antiangiogenic action.	[79]
Cymbopogon winterianus	0.1, 0.15 and 0.2%	Sanitizing	Eggshell	Chick	• No influence on chick quality and weight.	[62]
Syzygium aromaticum	0.39%	Sanitizing	Eggshell	Chick	• No weight changes and atrophy or hypertrophy in large and small intestine, pectoral muscle, proventriculus and gizzard, liver and gallbladder and heart of embryos and day-old chicks.	[60]
Origanum vulgare	0.5%	Sanitizing	Embryo	Chick	• No embryonic malformations. • Restored the antioxidant balance of the embryos.	[80]
Commercial blend	0.2 mL ratio of 2 (saline): 1 (Commercial blend)	Injecting	Amnion	Chick	• Chick length reduction. • Improvement of intestinal morphometric properties of broiler chickens. • No adverse effect on growth performance.	[81]
Thymus vulgaris	0.03 mL/egg	Injecting	Embryo	Chick	• Improved the initial weight of chicks.	[82]
Rosmarinus officinalis	1 µL or 3 µL/egg	Injecting	Air chamber	Quail	• Embryo protection (better embryonic development) and higher birth weight.	[83]
Trans-cinnamaldehyde	0.48%	Washing	Eggshell	Chick	• No effect on yolk sac, embryo and tibia weight. • No change in embryo and tibia length.	[84]

7. Comparing Essential Oils and Formaldehyde for Sanitizing Hatching Eggs

Formaldehyde is still preferably used in the practice of sanitizing hatching eggs [3,85,86]. Antimicrobial effectiveness and cost are two of the main reasons why formaldehyde remains in use in the poultry industry. Even its strong toxicity to poultry embryos [66,85,87] and humans [88–90] has not managed to have it removed from the practice of sanitizing hatching eggs. However, researchers are strongly committed to continuing to alert the poultry industry that, from a health point of view, formaldehyde is not compatible with a sustainable and safe poultry chain.

The sanitization of hatching eggs with natural sanitizers is based on a sanitary practice of microbial control of eggshells without synthetic chemical treatments, which aims to contribute to the production of healthy chicks free of pathogenic microorganisms using exclusively substances derived from plants and friends of living organisms [5,64,91,92]. Comparing natural sanitizers made from essential oils with synthetic sanitizers made from formaldehyde, there should be conscious support for the transition from sanitization systems that involve aggressive products to those that use green and responsible products. In addition to the antibiotic profile capable of significantly reducing the microbial count

of hatching eggshells, one of the main advantages of using essential oils as sanitizers for hatching eggs is the productive results promoted in terms of hatchability, which, on average, are not inferior to those of sanitization with formaldehyde (Table 4). Thus, the application of essential oils to hatching eggs does not require additional or different practices to promote the production of the same number of poultry than is routine in the conventional poultry sector. The prioritized use of synthetic chemicals in hatching egg management can be minimized by replacing them with essential oils.

Table 4. Comparison between the efficiency of essential oils and formaldehyde after application in hatching eggs.

Compounds	Bacterial Count (log) [a]	Hatchability (%) [a]	Significance [b]	Most Efficient	Study
Origanum onites Formaldehyde	<0.47 <0.06	>1.98 >1.89	* TBC [ns] Hatchability	Essential oil	[93]
Thymus vulgaris Formaldehyde	<1.68 <1.81	>6.95 >9.70	*	Formaldehyde	[94]
Syzygium aromaticum Paraformaldehyde	<1.19 <1.26	>10.66 >7.84	ns	Similar	[64]
Origanum vulgare *Cuminum cyminum* Formaldehyde	<6.33 <6.13 <3.03	>12.05 >11.70 <2.01	*	Essential oils	[66]

[a] Comparison of essential oils and formaldehyde with non-sanitized eggs; [b] Comparison between essential oil and formaldehyde; * Significant; [ns] non-significant, TBC, Total bacteria count.

8. Conclusions

In general, we have found that essential oil sanitizers are effective in reducing the microbial load on eggshells. From a safety point of view, the direct application of essential oils in developing poultry can generate toxic effects on the survival and integrity of these animals, but this seems to be mainly associated with specific components of the composition of essential oils and/or factors intrinsic to the application protocols, such as method, time, location and concentration. This raises the hypothesis that the residual contact of essential oils applied on hatching eggshells with the embryo is minimal and gradual, as most of the effects found when these compounds were applied to eggshells were beneficial for the embryo and chick. The dosage and concentration of the essential oils in contact with the embryos need to be studied and adjusted, especially if applied directly so that all harms are converted into benefits. The effectiveness of essential oils is comparable to formaldehyde, but they are less toxic. Complementary studies on the microbiological profile of embryos and chicks hatched from eggs sanitized with essential oils need to be carried out. In addition, the economic viability of essential oils before their possible effective use in the sanitization of hatching eggs needs to be investigated to know which essential oils adapt to small- and large-scale applications.

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References

1. Rezaee, M.S.; Liebhart, D.; Hess, C.; Hess, M.; Paudel, S. Bacterial Infection in Chicken Embryos and Consequences of Yolk Sac Constitution for Embryo Survival. *Vet. Pathol.* **2021**, *58*, 71–79. [CrossRef]
2. Fardows, J.; Shamsuzzaman, S.M. Detection of Potential Pathogenic Aerobic Bacteria from Egg Shell and Egg Contents of Hen Collected from Poultry. *Bangladesh Med. Res. Counc. Bull.* **2015**, *41*, 67–72. [CrossRef] [PubMed]
3. Oliveira, G.d.S.; McManus, C.; Salgado, C.B.; dos Santos, V.M. Effects of Sanitizers on Microbiological Control of Hatching Eggshells and Poultry Health during Embryogenesis and Early Stages after Hatching in the Last Decade. *Animals* **2022**, *12*, 2826. [CrossRef]
4. Araújo, W.A.G.; Albino, L.F.T. *Incubação Comercial [Commercial Incubation]*; Transworld Research Network: Trivandrum, India, 2011.
5. Oliveira, G.d.S.; dos Santos, V.M.; Nascimento, S.T. Essential Oils as Sanitisers for Hatching Eggs. *Worlds Poult. Sci. J.* **2021**, *77*, 605–617. [CrossRef]
6. Oliveira, G.d.S.; dos Santos, V.M.; McManus, C. Propolis: Effects on the Sanitisation of Hatching Eggs. *Worlds Poult. Sci. J.* **2022**, *78*, 261–272. [CrossRef]
7. Oliveira, G.d.S.; McManus, C.; dos Santos, V.M. Garlic as Active Principle of Sanitiser for Hatching Eggs. *Worlds Poult. Sci. J.* **2022**, *78*, 1037–1052. [CrossRef]
8. Jahantigh, M. Bacteriological Study of Dead-in-Shell Embryos of Ostrich. *Iran J. Vet. Res.* **2010**, *11*, 88–90.
9. Fonseca, B.B.; Beletti, M.E.; Melo, R.T.; Mendonça, E.P.; Vieira, C.U.; Levenhagen, M.A.; Rossi, D.A. Transfer, Viability and Colonisation of *Campylobacter jejuni* in the Chicken Vitellus and in Embryos. *Br. Poult. Sci.* **2011**, *52*, 279–286. [CrossRef]
10. Hincke, M.; Gautron, J.; Rodriguez-Navarro, A.B.; McKee, M.D. The Eggshell: Structure and Protective Function. In *Improving the Safety and Quality of Eggs and Egg Products: Egg Chemistry, Production and Consumption*; Woodhead Publishing: Sawston, UK, 2011; pp. 151–182.
11. Weil, A.J.; Volentine, J.A. Infection of the Developing Chick Embryo with *Dysentery Bacilli*. *Proc. Soc. Exp. Biol. Med.* **1940**, *44*, 160–161. [CrossRef]
12. Kwanashie, C.N.; Kazeem, H.M.; Umoh, J.U.; Abdu, P.A. *Aspergillus* Species Associated with Dead-in-Shell Chick Embryo in Some Hatcheries in Northwest Nigeria. *Eurasian J. Vet. Sci.* **2014**, *30*, 11–13. [CrossRef]
13. Saleemi, M.K.; Khan, M.Z.; Khan, A.; Hassan, Z.U.; Khan, W.A.; Rafique, S.; Fatima, Z.; Sultan, A. Embryotoxic and Histopathological Investigations of In-Ovo Inoculation of Aflatoxigenic Fungal Extracts in Chicken Embryos. *Pak. Vet. J.* **2015**, *35*, 403–408.
14. Karunarathna, R.; Ahmed, K.A.; Liu, M.; Yu, C.; Popowich, S.; Goonewardene, K.; Gunawardana, T.; Kurukulasuriya, S.; Gupta, A.; Ayalew, L.E.; et al. Non-Viable Chicken Embryos: An Overlooked Niche Harbouring a Significant Source of Multidrug Resistant Bacteria in the Poultry Production. *Int. J. Vet. Sci. Med.* **2020**, *8*, 9–17. [CrossRef] [PubMed]
15. Kizerwetter-Świda, M.; Binek, M. Bacterial Microflora of the Chicken Embryos and Newly Hatched Chicken. *J. Anim. Feed Sci.* **2008**, *17*, 224–232. [CrossRef]
16. Karunarathna, R.; Popowich, S.; Wawryk, M.; Chow-Lockerbie, B.; Ahmed, K.A.; Yu, C.; Liu, M.; Goonewardene, K.; Gunawardana, T.; Kurukulasuriya, S.; et al. Increased Incidence of Enterococcal Infection in Nonviable Broiler Chicken Embryos in Western Canadian Hatcheries as Detected by Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry. *Avian. Dis.* **2017**, *61*, 472–480. [CrossRef] [PubMed]
17. Nasrin, S.; Islam, M.; Khatun, M.; Akhter, L.; Sultana, S. Characterization of Bacteria Associated with Omphalitis in Chicks. *Bangladesh Vet.* **2012**, *29*, 63–68. [CrossRef]
18. Amer, M.M.; Elbayoumi, K.M.; Amin Girh, Z.M.S.; Mekky, H.M.; Rabie, N.S. A Study On Bacterial Contamination of Dead in Shell Chicken Embryos and Culled One Day Old Chicks. *Int. J. Pharm. Clin. Res.* **2017**, *7*, 5–11.
19. Far, R.; Peighambari, S.M.; Sadrzadeh, A.; Badouei, A. Bacterial Contamination of Dead-in-Shell Embryos in Ostrich Hatcheries and Antimicrobial Resistance Patterns of Isolated *Escherichia coli*. *Iran. J. Vet. Med.* **2013**, *7*, 169–175.
20. Ni, Z.J.; Wang, X.; Shen, Y.; Thakur, K.; Han, J.; Zhang, J.G.; Hu, F.; Wei, Z.J. Recent Updates on the Chemistry, Bioactivities, Mode of Action, and Industrial Applications of Plant Essential Oils. *Trends Food Sci. Technol.* **2021**, *110*, 78–89. [CrossRef]
21. Tu, X.F.; Hu, F.; Thakur, K.; Li, X.L.; Zhang, Y.S.; Wei, Z.J. Comparison of Antibacterial Effects and Fumigant Toxicity of Essential Oils Extracted from Different Plants. *Ind. Crops Prod.* **2018**, *124*, 192–200. [CrossRef]
22. Hatami, T.; Johner, J.C.F.; Zabot, G.L.; Meireles, M.A.A. Supercritical Fluid Extraction Assisted by Cold Pressing from Clove Buds: Extraction Performance, Volatile Oil Composition, and Economic Evaluation. *J. Supercrit. Fluids* **2019**, *144*, 39–47. [CrossRef]
23. Shukla, A.; Naik, S.N.; Goud, V.V.; Das, C. Supercritical CO_2 Extraction and Online Fractionation of Dry Ginger for Production of High-Quality Volatile Oil and Gingerols Enriched Oleoresin. *Ind. Crops Prod.* **2019**, *130*, 352–362. [CrossRef]
24. Jadhav, N.L.; Garule, P.A.; Pinjari, D.V. Comparative Study of Ultrasound Pretreatment Method with Conventional Hydrodistillation Method for Extraction of Essential Oil from *Piper betle* L. (Paan). *Indian Chem. Eng.* **2022**, *64*, 132–140. [CrossRef]

25. El Atki, Y.; Aouam, I.; El Kamari, F.; Taroq, A.; Nayme, K.; Timinouni, M.; Lyoussi, B.; Abdellaoui, A. Antibacterial Activity of Cinnamon Essential Oils and Their Synergistic Potential with Antibiotics. *J. Adv. Pharm. Technol. Res.* **2019**, *10*, 63–67. [CrossRef] [PubMed]
26. Selles, S.M.A.; Kouidri, M.; Belhamiti, B.T.; Ait Amrane, A. Chemical Composition, In Vitro Antibacterial and Antioxidant Activities of *Syzygium aromaticum* Essential Oil. *J. Food Meas. Charact.* **2020**, *14*, 2352–2358. [CrossRef]
27. Swamy, M.K.; Akhtar, M.S.; Sinniah, U.R. Antimicrobial Properties of Plant Essential Oils against Human Pathogens and Their Mode of Action: An Updated Review. *Evid.-Based Complement. Altern. Med.* **2016**, *2016*, 3012462. [CrossRef]
28. Nazzaro, F.; Fratianni, F.; Coppola, R.; De Feo, V. Essential Oils and Antifungal Activity. *Pharmaceuticals* **2017**, *10*, 86. [CrossRef]
29. Elshafie, H.S.; Camele, I. An Overview of the Biological Effects of Some Mediterranean Essential Oils on Human Health. *BioMed Res. Int.* **2017**, *2017*, 9268468. [CrossRef]
30. Haro-González, J.N.; Castillo-Herrera, G.A.; Martínez-Velázquez, M.; Espinosa-Andrews, H. Clove Essential Oil (*Syzygium aromaticum* L. Myrtaceae): Extraction, Chemical Composition, Food Applications, and Essential Bioactivity for Human Health. *Molecules* **2021**, *26*, 6387. [CrossRef]
31. Soares, G.A.B.E.; Bhattacharya, T.; Chakrabarti, T.; Tagde, P.; Cavalu, S. Exploring Pharmacological Mechanisms of Essential Oils on the Central Nervous System. *Plants* **2022**, *11*, 21. [CrossRef]
32. Ezeorba, T.P.C.; Chukwudozie, K.I.; Ezema, C.A.; Anaduaka, E.G.; Nweze, E.J.; Okeke, E.S. Potentials for Health and Therapeutic Benefits of Garlic Essential Oils: Recent Findings and Future Prospects. *Pharmacol. Res. Mod. Chin. Med.* **2022**, *3*, 100075. [CrossRef]
33. Code of Federal Regulations (CFR). Part 182.20 Essential Oils, Oleoresins (Solvent-Free), and Natural Extractives (including Distillates). Available online: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=182.20. (accessed on 16 May 2023).
34. Silva, N.; Alves, S.; Gonçalves, A.; Amaral, J.S.; Poeta, P. Antimicrobial Activity of Essential Oils from Mediterranean Aromatic Plants against Several Foodborne and Spoilage Bacteria. *Food Sci. Technol. Int.* **2013**, *19*, 503–510. [CrossRef] [PubMed]
35. Chekki, R.Z.; Snoussi, A.; Hamrouni, I.; Bouzouita, N. Chemical composition, antibacterial and antioxidant activities of Tunisian garlic (*Allium sativum*) essential oil and ethanol extract. *Med. J. Chem.* **2014**, *3*, 947–956. [CrossRef]
36. Murbach Teles Andrade, B.F.; Nunes Barbosa, L.; da Silva Probst, I.; Fernandes Júnior, A. Antimicrobial Activity of Essential Oils. *J. Essent. Oil Res.* **2014**, *26*, 34–40. [CrossRef]
37. Bhaisare, D.B.; Thyagarajan, D.; Churchil, R.R.; Punniamurthy, N. In Vitro Antimicrobial Efficacy of Certian Herbal Seeds Essential Oils against Important Poultry Microbes. *Indian J. Anim. Res.* **2016**, *50*, 561–564. [CrossRef]
38. de Marques, J.L.; Volcão, L.M.; Funck, G.D.; Kroning, I.S.; da Silva, W.P.; Fiorentini, Â.M.; Ribeiro, G.A. Antimicrobial Activity of Essential Oils of *Origanum vulgare* L. and *Origanum majorana* L. against *Staphylococcus aureus* Isolated from Poultry Meat. *Ind. Crops Prod.* **2015**, *77*, 444–450. [CrossRef]
39. Gavaric, N.; Mozina, S.S.; Kladar, N.; Bozin, B. Chemical Profile, Antioxidant and Antibacterial Activity of Thyme and Oregano Essential Oils, Thymol and Carvacrol and Their Possible Synergism. *J. Essent. Oil-Bear. Plants* **2015**, *18*, 1013–1021. [CrossRef]
40. Souza, D.S.; Almeida, A.C.; Andrade, V.A.; Marcelo, N.A.; Azevedo, I.L.; Martins, E.R.; Figueiredo, L.S. Antimicrobial Activity of *Lippia origanoides* and *Lippia rotundifolia* Oils against Enterobacteria Isolated from Poultry. *Arq. Bras. Med. Vet. Zootec.* **2015**, *67*, 940–944. [CrossRef]
41. Mekonnen, A.; Yitayew, B.; Tesema, A.; Taddese, S. In vitro Antimicrobial Activity of Essential Oil of *Thymus schimperi*, *Matricaria chamomilla*, *Eucalyptus globulus*, and *Rosmarinus officinalis*. *Int. J. Microbiol.* **2016**, *2016*, 9545693. [CrossRef]
42. Ambrosio, C.M.S.; de Alencar, S.M.; de Sousa, R.L.M.; Moreno, A.M.; Da Gloria, E.M. Antimicrobial Activity of Several Essential Oils on Pathogenic and Beneficial Bacteria. *Ind. Crops Prod.* **2017**, *97*, 128–136. [CrossRef]
43. Blažeković, B.; Yang, W.; Wang, Y.; Li, C.; Kindl, M.; Pepeljnjak, S.; Vladimir-Knežević, S. Chemical Composition, Antimicrobial and Antioxidant Activities of Essential Oils of *Lavandula* × *intermedia* 'Budrovka' and *L. angustifolia* Cultivated in Croatia. *Ind. Crops Prod.* **2018**, *123*, 173–182. [CrossRef]
44. Munda, S.; Dutta, S.; Pandey, S.K.; Sarma, N.; Lal, M. Antimicrobial Activity of Essential Oils of Medicinal and Aromatic Plants of the North East India: A Biodiversity Hot Spot. *J. Essent. Oil-Bear. Plants* **2019**, *22*, 105–119. [CrossRef]
45. Ebani, V.V.; Nardoni, S.; Bertelloni, F.; Tosi, G.; Massi, P.; Pistelli, L.; Mancianti, F. In Vitro Antimicrobial Activity of Essential Oils against *Salmonella Enterica Serotypes* Enteritidis and Typhimurium Strains Isolated from Poultry. *Molecules* **2019**, *24*, 900. [CrossRef] [PubMed]
46. Kammon, A. In Vitro Antimicrobial Activity of Clove Oil against Gram Negative Bacteria Isolated from Chickens. *Approaches Poult. Dairy Vet. Sci.* **2019**, *6*, 542–546. [CrossRef]
47. Aćimović, M.; Zorić, M.; Zheljazkov, V.D.; Pezo, L.; Čabarkapa, I.; Jeremić, J.S.; Cvetković, M. Chemical Characterization and Antibacterial Activity of Essential Oil of Medicinal Plants from Eastern Serbia. *Molecules* **2020**, *25*, 5482. [CrossRef] [PubMed]
48. Di Vito, M.; Cacaci, M.; Barbanti, L.; Martini, C.; Sanguinetti, M.; Benvenuti, S.; Tosi, G.; Fiorentini, L.; Scozzoli, M.; Bugli, F.; et al. *Origanum vulgare* Essential Oil vs. A Commercial Mixture of Essential Oils: In vitro Effectiveness on *Salmonella* spp. from Poultry and Swine Intensive Livestock. *Antibiotics* **2020**, *9*, 763. [CrossRef]
49. Abers, M.; Schroeder, S.; Goelz, L.; Sulser, A.; St. Rose, T.; Puchalski, K.; Langland, J. Antimicrobial Activity of the Volatile Substances from Essential Oils. *BMC Complement. Med. Ther.* **2021**, *21*, 124. [CrossRef]

50. Mangalagiri, N.P.; Panditi, S.K.; Jeevigunta, N.L.L. Antimicrobial Activity of Essential Plant Oils and Their Major Components. *Heliyon* **2021**, *7*, e06835. [CrossRef]
51. Seyedtaghiya, M.H.; Fasaei, B.N.; Peighambari, S.M. Antimicrobial and Antibiofilm Effects of *Satureja hortensis* Essential Oil against *Escherichia coli* and *Salmonella* Isolated from Poultry. *Iran. J. Microbiol.* **2021**, *13*, 74–80. [CrossRef]
52. Blejan, E.I.; Popa, D.E.; Costea, T.; Cioacă, A.; Olariu, L.; Ghica, M.; Georgescu, M.; Stancov, G.; Arsene, A.L. The in vitro Antimicrobial Activity of Some Essential Oils from Aromatic Plants. *Farmacia* **2021**, *69*, 290–298. [CrossRef]
53. Campana, R.; Tiboni, M.; Maggi, F.; Cappellacci, L.; Cianfaglione, K.; Morshedloo, M.R.; Frangipani, E.; Casettari, L. Comparative Analysis of the Antimicrobial Activity of Essential Oils and Their Formulated Microemulsions against Foodborne Pathogens and Spoilage Bacteria. *Antibiotics* **2022**, *11*, 447. [CrossRef]
54. Oliveira, G.D.S.; McManus, C.; Pires, P.G.D.S.; dos Santos, V.M. Combination of Cassava Starch Biopolymer and Essential Oils for Coating Table Eggs. *Front. Sustain. Food Syst.* **2022**, *6*, 957229. [CrossRef]
55. Milagres de Almeida, J.; Crippa, B.L.; Martins Alencar de Souza, V.V.; Perez Alonso, V.P.; da Motta Santos Júnior, E.; Siqueira Franco Picone, C.; Prata, A.S.; Cirone Silva, N.C. Antimicrobial Action of Oregano, Thyme, Clove, Cinnamon and Black Pepper Essential Oils Free and Encapsulated against Foodborne Pathogens. *Food Control* **2023**, *144*, 109356. [CrossRef]
56. de Araújo, M.V.; da Oliveira, G.S.; McManus, C.; Vale, I.R.R.; Salgado, C.B.; da Pires, P.G.S.; de Campos, T.A.; Gonçalves, L.F.; Almeida, A.P.C.; dos Martins, G.S.; et al. Preserving the Internal Quality of Quail Eggs Using a Corn Starch-Based Coating Combined with Basil Essential Oil. *Processes* **2023**, *11*, 1612. [CrossRef]
57. Upadhyaya, I.; Upadhyay, A.; Kollanoor-Johny, A.; Baskaran, S.A.; Mooyottu, S.; Darre, M.J.; Venkitanarayanan, K. Rapid Inactivation of *Salmonella* Enteritidis on Shell Eggs by Plant-Derived Antimicrobials. *Poult. Sci.* **2013**, *92*, 3228–3235. [CrossRef] [PubMed]
58. Allen, J.; Balasubramanian, B.; Rankin, K.; Shah, T.; Donoghue, A.M.; Upadhyaya, I.; Sartini, B.; Luo, Y.; Upadhyay, A. Trans-Cinnamaldehyde Nanoemulsion Wash Inactivates *Salmonella* Enteritidis on Shelled Eggs without Affecting Egg Color. *Poult. Sci.* **2023**, *102*, 102523. [CrossRef] [PubMed]
59. Upadhyaya, I.; Yin, H.B.; Nair, M.S.; Chen, C.H.; Upadhyay, A.; Darre, M.J.; Venkitanarayanan, K. Efficacy of Fumigation with Trans-Cinnamaldehyde and Eugenol in Reducing *Salmonella Enterica Serovar* Enteritidis on Embryonated Egg Shells. *Poult. Sci.* **2015**, *94*, 1685–1690. [CrossRef]
60. Oliveira, G.S.; Nascimento, S.T.; Dos Santos, V.M.; Dallago, B.S.L. Spraying Hatching Eggs with Clove Essential Oil Does Not Compromise the Quality of Embryos and One-Day-Old Chicks or Broiler Performance. *Animals* **2021**, *11*, 2045. [CrossRef]
61. Cui, H.; Ma, C.; Li, C.; Lin, L. Enhancing the Antibacterial Activity of Thyme Oil against *Salmonella* on Eggshell by Plasma-Assisted Process. *Food Control* **2016**, *70*, 183–190. [CrossRef]
62. de Andrade, M. *Avaliação do óleo Essencial de Citronela (Cymbopogon winterianus) Como Agente Alternativo na Desinfecção de ovos Incubáveis [Evaluation of Citronella Essential Oil (Cymbopogon winterianus) as an Alternative Agent in the Disinfection of Hatching Eggs]*; Graduation Completion Work; Federal Technological University of Paraná: Curitiba, Paraná, Brazil, 28 June 2018.
63. Nogueira, W.C.L.; Pena, A.C.S.; de Souza, C.N.; Azevedo, I.L.; Fariafilho, D.E.; Almeida, A.C. Disinfection of Fertile Eggs of Free-Range Poultry with Essential Oils. *Rev. Bras. Saude Prod. Anim.* **2019**, *20*, e0822019. [CrossRef]
64. Oliveira, G.D.S.; Nascimento, S.T.; dos Santos, V.M.; Silva, M.G. Clove Essential Oil in the Sanitation of Fertile Eggs. *Poult. Sci.* **2020**, *99*, 5509–5516. [CrossRef]
65. Phothisuwan, S.; Preechatiwong, W.; Matan, N. Enhancement of Antibacterial Activity of Essential Oil Vapor Released from a Paper Egg Tray in Combination with UV-C Radiation against Pathogenic Bacteria on Chicken Eggs. *J. Food Process. Preserv.* **2020**, *44*, e14794. [CrossRef]
66. Bekhet, G.; Khalifa, A.Y.Z. Essential Oil Sanitizers to Sanitize Hatching Eggs. *J. Appl. Anim. Res.* **2022**, *50*, 695–701. [CrossRef]
67. Oliveira, G.d.S.; McManus, C.C.; Salgado, C.B.; Pires, P.G.d.S.; dos Santos, V.M. Rice Flour Coating Supplemented with Rosemary Essential Oil to Preserve the Internal, Microbiological and Sensory Quality of Quail Eggs. *Acta Aliment.* **2023**, *52*, 294–304. [CrossRef]
68. Oliveira, G.D.S.; McManus, C.; Dos Santos, V.M. Essential Oils and Propolis as Additives in Egg Coatings. *Worlds Poult. Sci. J.* **2022**, *78*, 1053–1066. [CrossRef]
69. Gatea, S.M.; Altaie, S.M.S.; Khafaji, S.S.; Aljanabi, T.K.; Shatti, D.H.; Hussain, M.A. Influence of Spraying Different Solutions at Different Incubation Periods on Hatchability Parameters of Local Iraqi's Eggs. *IOP Conf. Ser. Earth Environ. Sci.* **2019**, *388*, 012034. [CrossRef]
70. Harry, E.G.; Binstead, J.A. Studies on Disinfection of Eggs and Incubators. V: The Toxicity of Formaldehyde to the Developing Embryo. *Br. Vet. J.* **1961**, *117*, 532–539. [CrossRef]
71. Zeweil, H.S.; Rizk, R.E.; Bekhet, G.M.; Ahmed, M.R. Comparing the Effectiveness of Egg Disinfectants against Bacte-ria and Mitotic Indices of Developing Chick Embryos. *J. Basic Appl. Zool.* **2015**, *70*, 1–15. [CrossRef]
72. de Oliveira, S.Z. Toxicidade do óleo Essencial de *Melaleuca* a Organismos não-alvo [Toxicity of Melaleuca Essential Oil to Non-target Organism]. Master's Dissertation, Federal Technological University of Paraná, Curitiba, Paraná, Brazil, 28 February 2023.
73. Tebrün, W.; Motola, G.; Hafez, M.H.; Bachmeier, J.; Schmidt, V.; Renfert, K.; Reichelt, C.; Brüggemann-Schwarze, S.; Pees, M. Preliminary Study: Health and Performance Assessment in Broiler Chicks Following Application of Six Different Hatching Egg Disinfection Protocols. *PLoS ONE* **2020**, *15*, e0232825. [CrossRef]

74. Demirci, F.; Paper, D.H.; Franz, G.; Hüsnü Can Başer, K. Investigation of the *Origanum onites* L. Essential Oil Using the Chorioallantoic Membrane (CAM) Assay. *J. Agric. Food Chem.* **2004**, *52*, 251–254. [CrossRef]
75. Ali, R.; Elzahraa Mostafa, D.F.; Aboulqasem, H.; Ali, R.A.; Aboulqasem, H.E. The Effect of Co-Treatment with Retinoic Acid on Rescuing Citral Induced Morphological Anomalies during Chick Embryo Development. *Assiut. Univ. J. Zool.* **2019**, *1*, 42–63.
76. Zhang, X.; Peng, Y.; Wu, C. Chicken Embryonic Toxicity and Potential in vitro Estrogenic and Mutagenic Activity of Carvacrol and Thymol in Low Dose/Concentration. *Food Chem. Toxicol.* **2021**, *150*, 112038. [CrossRef]
77. Ulucay, I.O.; Yildirim, I. Hatching Traits of Quail (*Coturnix Coturnix Japonica*) Eggs Disinfected with Carvacrol, Cinnamaldehyde or Thymol. *J. Appl. Anim. Res.* **2010**, *38*, 139–142. [CrossRef]
78. Zeweil, H.; Rizk, R.; Bekhet, G.; Ahmed, R. Effect of Egg Disinfection on Hatching Performance for Bandarah Chicken Strain. *Egypt. Poult. Sci. J.* **2013**, *33*, 289–307.
79. Goze, A.; Gose, I.; Alim, A.; Saygin, H.; Alim, B.A. Investigation of Effects of Essential Oil from Berries of *Juniperus excelsa* Bieb. Subsp *Excelsa* (Cupressaceae) on Angiogenesis in Shell-Less Chick Embryo (CAM) Culture. *J. Essent. Oil-Bear. Plants* **2015**, *18*, 1100–1107. [CrossRef]
80. Bekhet, G.M.; Sayed, A.A. Oregano-Oil Antagonist Lipopolysaccharide (LPS) Induced Toxicity in Pre- and Post-Hatch Chick Embryo. *J. Appl. Anim. Res.* **2021**, *49*, 211–220. [CrossRef]
81. Oladokun, S.; Macisaac, J.; Rathgeber, B.; Adewole, D. Essential Oil Delivery Route: Effect on Broiler Chicken's Growth Performance, Blood Biochemistry, Intestinal Morphology, Immune, and Antioxidant Status. *Animals* **2021**, *11*, 3386. [CrossRef]
82. Yaseen, A.; Gaafar, K.; Abou-elkhaire, R. Response of Broiler Chicken to in Ovo Administration of Nano Encapsulated Thyme Oil. *J. Curr. Vet. Res.* **2022**, *4*, 166–172. [CrossRef]
83. Aberbour, A.; Touazi, L.; Benberkane, A.; Aissanou, S.; Sherasiya, A.; Iguer-Ouada, M.; Hornick, J.L.; Moula, N. The Effect of In Ovo Administration of Rosemary Essential Oil on Hatchability, Relative Hatching Weight, and Embryo Mortality Rate in Japanese Quail (*Coturnix Coturnix Japonica*). *Animals* **2023**, *13*, 1217. [CrossRef]
84. Allen, J.; Balasubramanian, B.; Donoghue, A.M.; Upadhyaya, I.; Luo, Y.; Upadhyay, A. Effect of Trans-Cinnamaldehyde Nanoemulsion Wash on Chicken Embryo Development in Fertilized Eggs. *Poult. Sci.* **2023**, *102*, 102812. [CrossRef] [PubMed]
85. Cadirci, S. Disinfection of Hatching Eggs by Formaldehyde Fumigation—A Review. *Eur. Poult. Sci.* **2009**, *73*, 116–123.
86. Melo, E.F.; Clímaco, W.L.S.; Triginelli, M.V.; Vaz, D.P.; de Souza, M.R.; Baião, N.C.; Pompeu, M.A.; Lara, L.J.C. An Evaluation of Alternative Methods for Sanitizing Hatching Eggs. *Poult. Sci.* **2019**, *98*, 2466–2473. [CrossRef] [PubMed]
87. Hayretdağ, S.; Kolankaya, D. Investigation of the Effects of Pre-Incubation Formaldehyde Fumigation on the Tracheal Epithelium of Chicken Embryos and Chicks. *Turk. J. Vet. Anim. Sci.* **2008**, *32*, 263–267.
88. Qu, M.; Lu, J.; He, R. Formaldehyde from Environment. In *Formaldehyde and Cognition*; He, H., Ed.; Springer Science + Business Media B.V.: Berlin, Germany, 2017; Volume 1, pp. 1–19.
89. Dan, S.; Pant, M.; Kaur, T.; Pant, S. Toxic effect of formaldehyde: A systematic review. *Int. Res. J. Mod. Eng. Technol. Sci.* **2020**, *2*, 179–189.
90. Bernardini, L.; Barbosa, E.; Charão, M.F.; Brucker, N. Formaldehyde Toxicity Reports from In Vitro and in vivo Studies: A Review and Updated Data. *Drug Chem. Toxicol.* **2020**, *45*, 972–984. [CrossRef] [PubMed]
91. Fouad, W.; Abdel-Hafez, M.S. Effect of Spraying Hatching Eggs of Japanese Quails by Live Yeast on Physiological Changes in the Embryonic Development, Hatchability and Total Bacterial Count. *Egypt. Poult. Sci. J.* **2017**, *37*, 1303–1321.
92. Toghyani, P.; Shahzamani, S.; Gholami Ahangaran, M.; Ali Mousavi Firouzabadi, S. Comparison of Eucalyptus Extract and Formaldehyde on Hatchability and Survival Rate of Chicks in Disinfection of Fertile Eggs. *Int. J. Pharm. Res. Allied Sci.* **2020**, *9*, 105–109.
93. Copur, G.; Arslan, M.; Duru, M.; Baylan, M.; Canogullari, S.; Aksan, E. Use of Oregano (*Origanum onites* L.) Essential Oil as Hatching Egg Disinfectant. *Afr. J. Biotechnol.* **2010**, *8*, 2531–2538.
94. Shahein, E.H.A.; Sedeek, E.K. Role of Spraying Hatching Eggs with Natural Disinfectants on Hatching Characteristics and Eggshell Bacterial Counts. *Egypt. Poult. Sci. J.* **2014**, *34*, 213–230. [CrossRef]

Paper three

A Mini-Review of the Main Effects of Essential Oils from *Citrus aurantifolia*, *Ocimum basilicum*, and *Allium sativum* as Safe Antimicrobial Activity in Poultry

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Review

A Mini-Review of the Main Effects of Essential Oils from *Citrus aurantifolia*, *Ocimum basilicum*, and *Allium sativum* as Safe Antimicrobial Activity in Poultry

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Simple Summary: Increasing the use of natural antimicrobials and introducing them into the poultry production cycle is desirable. Essential oils are promoted as one of the main alternatives to intensive conventional antibiotic therapy in poultry farming worldwide. Studies show that when applied in specific forms and concentrations, several essential oils or their components have demonstrated the ability to protect both humans and poultry from serious diseases, including those associated with microorganisms. For example, in theory and practice, salmonellosis is widely recognised as a significant concern for human and avian health. In this context, acquiring knowledge about essential oils that can potentially prevent or combat microbial outbreaks in poultry farming, leading to serious infectious complications for avian and human health, is crucial. This review aimed to compile information on the application of *Citrus aurantifolia* (CAEO), *Ocimum basilicum* (OBEO), and *Allium sativum* (ASEO) essential oils as antimicrobials in poultry farming.

Abstract: Poultry production is accompanied by the use of antimicrobial agents because no production step is free of microorganisms. In the absence of antimicrobial treatments with synthetic drugs, essential oils are among the most cited natural alternatives used to prevent and treat microbial contamination in poultry. Although there are several studies on the antimicrobial properties of essential oils, there is still no review that simultaneously compiles information on the leading antimicrobial role of essential oils from *Citrus aurantifolia* (CAEO), *Ocimum basilicum* (OBEO), and *Allium sativum* (ASEO) in poultry. Awareness of the antimicrobial role of these substances opens the door to encouraging their use in natural antimicrobial protocols and discouraging harmful synthetics in poultry. This review aimed to compile information on applying CAEO, OBEO, and ASEO as antimicrobials in poultry farming. The available literature suggests that these essential oils can proportionately align with the poultry industry's demands for microbiologically safe food products.

Keywords: antimicrobials; essential oils; health protection; microbiological safety; microorganisms; natural product

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1. Introduction

In contaminated poultry environments, which include contact surfaces, soil, air, and water, among others, the chances of contaminating poultry and their products are high. This scenario can trigger a sequence of interconnected events. Poultry can develop severe disease symptoms or die when they are exposed to contamination. For example, poultry contaminated with *Escherichia coli* may present an infectious condition associated with

movement restrictions and nutrient deprivation [1], leading to death. However, regardless of the severity of the symptoms—whether intense, mild or even absent, poultry can become asymptomatic carriers, with contamination maintained in a latent state until the final product (meat or egg) is obtained. This process can compromise the productivity and viability of commercialisation. Thus, the impacts of microorganisms such as *Salmonella* spp., *Campylobacter* spp., *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus* spp., and *Fusarium* spp. on broilers or laying hens cannot go unnoticed, as these poultry animals sustain the needs of populations internationally.

Sanitisation and other protocols that use effective and safe antimicrobials interrupt the different cycles of contamination that compromise the productivity and quality of poultry food products, ensuring food availability and keeping consumers safe from contaminated food. These cycles start from the contaminated environment and then reach humans, as shown below:

- Environment → poultry → table eggs → humans.
- Environment → poultry → hatching eggs → embryos → poultry → meat → humans.
- Environment → poultry → meat → humans.
- Environment → hatching eggs → embryos → poultry → meat → humans.
- Environment → hatching eggs → embryos → poultry → table eggs → humans.
- Environment → table eggs → humans.
- Environment → meat → humans.

Essential oils comprise blends of aromatic compounds extracted from different parts of plants, such as fruits, roots, rhizomes, leaves, flowers, bark, buds, twigs, wood, and seeds, known for their distinctive fragrances [2]. Certain essential oils have been investigated for microbiological control in poultry [3–6]. Among these, the essential oils of *Citrus aurantifolia* (CAEO), *Ocimum basilicum* (OBEO), and *Allium sativum* (ASEO) stand out [7–9], mainly for their natural and antimicrobial characteristics, which shape their sustainable properties for probable productive gains and benefits to human and environmental health. Therefore, the strategic and conscious administration of these essential oils in the poultry sector involves innovative protocols for solving microbiological problems, favouring sustainable practices. This review compiles information on applying CAEO, OBEO, and ASEO as antimicrobials in poultry farming. The following issues were addressed: which bacteria and fungi can be inhibited using essential oils; and the primary uses of essential oil antimicrobial properties for different purposes in poultry.

2. The Choice of Essential Oils and Methodology for Collecting Studies in the Available Database

Due to the usage requirements and selection criteria, there was no single and definitive reason for choosing these CAEO, OBEO, and ASEO essential oils to study in poultry production. However, at least eight reasons were decisive in selecting essential oils:

→ Authors' research materials.
→ Potential innovative applications in poultry production stages.
→ Specific chemical composition.
→ Meet the specific requirements of the study objective.
→ Functions that serve the industrial field.
→ Commercial availability and practical application.
→ Advantageous competitive efficiency compared with different conventional products.
→ Economic viability, potential benefit, and minimal risks.

All scientific documents cited in this study were found on Google Scholar: https://scholar.google.com/ (accessed on 17 October 2023).

The keywords or sentences used to search scientific documents were the following:

→ *Citrus aurantifolia* (CA).
→ CAEO.
→ In vitro antimicrobial (or antibacterial/antifungal) effects of CAEO.

→ Toxicity (or safety) of CAEO.
→ CAEO and poultry.
→ Antimicrobial effect of CAEO in poultry.
→ *Ocimum basilicum* (OB).
→ OBEO.
→ In vitro antimicrobial (or antibacterial/antifungal) effect of OBEO.
→ Toxicity (or safety) of OBEO.
→ OBEO and poultry.
→ Antimicrobial effects of OBEO in poultry.
→ *Allium sativum* (AS).
→ ASEO.
→ In vitro antimicrobial (or antibacterial/antifungal) effects of ASEO.
→ Toxicity (or safety) of ASEO.
→ ASEO and poultry.
→ Antimicrobial effects of ASEO in poultry.

The criteria used to review the documents found in full were as follows:

→ Research, review and conference papers, book chapters and monographs.
→ Written in English or Portuguese.
→ Published in any year.
→ Title, abstract, and keywords that meet the idea proposed for each topic of this study.

3. *Citrus aurantifolia* (CA), *Ocimum basilicum* (OB), and *Allium sativum* (AS) Plants and Their Essential Oils

Plants present an incredible bank of functional compounds. Each plant has its own individual characteristics, regardless of the species or variety. They are easily influenced by a considerable list of environmental factors, including density, photoperiod, and temperature [10,11]. Three of the most popular plants are CA, OB, and AS. These plants provide essential oils highly valued by the scientific community, industry, and consumers.

CA is a perennial fruit tree with an average height of 5 m belonging to the Rutaceae family [12]. Its leaves are greenish with an elliptical to oblong-ovate shape and can reach 9 cm in length [12,13]. It produces a greenish-yellow sour fruit with a smooth surface, approximately 4 cm long and 4 cm in diameter, weighing 41 g [13,14]. The fruits are harvested in substantial quantities 4 to 8 years after planting the seedlings [13]. The CA tree produces fruit annually and adapts well to different climates and soil conditions, requiring low input costs and good market demand [15]. CAEO is an aromatic liquid mainly extracted from the peels and leaves of CA through various extraction protocols, which include maceration, solvent (Soxhlet), hydrodistillation, and steam distillation [16–20]. This oil can yield 18.02% when extracted by Soxhlet [21], a density of 0.86 g/cm^3, and a refractive index of 1.48 at 20 °C [19]. Its colouration can range from colourless to greenish-yellow or yellowish-green [19]. The main component of CAEO is limonene, representing up to 98.3% of the essential oil [22]. In addition to limonene, at least 17 other compounds can make up the complex chemical arrangement of CAEO [16]. Linalool, β-Pinene, γ-Terpinene, citronellal, and citronellol are other compounds that comprise a significant proportion of this essential oil [16,23].

OB is an annual aromatic herb with heights and weights ranging from approximately 26 to 59 cm and 76 to 203 g, respectively, and belonging to the Lamiaceae family [24–26]. Its leaves are oval, pointed, and opposite, and in most varieties, they are green, reaching approximately 11 cm in length and 8 cm in width [24,26]. The flowers are predominantly white but can be other colours, such as pink and purple. Each herb can have up to approximately 50 inflorescences, reaching about 19 cm in length [24]. OB can be grown without problems in various environmental conditions [25]. OBEO is a liquid storehouse of active compounds that can be extracted from OB's leaves, stems, and flowers via hydrodistillation and steam distillation [27–29]. This oil can have a yield of 2.26% when extracted by hydrodistillation [30], a density of 1.20 g/cm^3, a viscosity of 14.37 g^{-1}·cm·s, and a

refractive index of 1.64 at 20 °C [31]. Its colouration is usually yellowish [30]. OBEO can be chemically formed by just 2 compounds or up to 23 [32], depending on the conditions in which the plant from which it originated was cultivated [32]. Estragole (60.98%) and Linalool (41.2%) constitute the significant chemical portion of OBEO [28,33]. Other compounds, such as methyl chavicol and methyl-eugenol, can also be detected in significant concentrations [27,28].

AS is a bulbous perennial herb belonging to the Lilliceae family [34] capable of reaching 117 cm in height [35]. Each bulb can contain up to 42 white cloves and can weigh up to 257 g [35]. A plant can produce 13 leaves [35] characterised as greenish, elongated, and flattened from the bulb to pointed at the tip [36]. AS grows well in heavy clay soils enriched with humus and water [36]. ASEO is a yellowish concentrate of natural chemical compounds [37] that can be extracted from the clove, aerial part, and bulb of AS through steam distillation [38–40]. This oil can yield 22.5% when extracted by Soxhlet [41], a density of 1.03 g/cm^3, and a refractive index of 1.47 at 20 °C [37]. Diallyl disulfide or allicin commonly constitute a major part of the chemical composition of ASEO [39,40]. Other identified compounds that contributed significantly to the total content of this oil are 2-methoxy-4-vinylphenol, decene, allyl propyl disulfide, allyl methyl trisulfide, di-2-propenyl-trisulfide, diallyl tetrasulfide, and siloxane [38–40].

4. The Antimicrobial Efficacy of Essential Oils from *Citrus aurantifolia* (CAEO), *Ocimum basilicum* (OBEO), and *Allium sativum* (ASEO), and Their Applications in Poultry

CAEO, OBEO, and ASEO open up a network of possibilities to be applied as antibacterial and antifungal agents thanks to in vitro results that support their effectiveness (Figure 1).

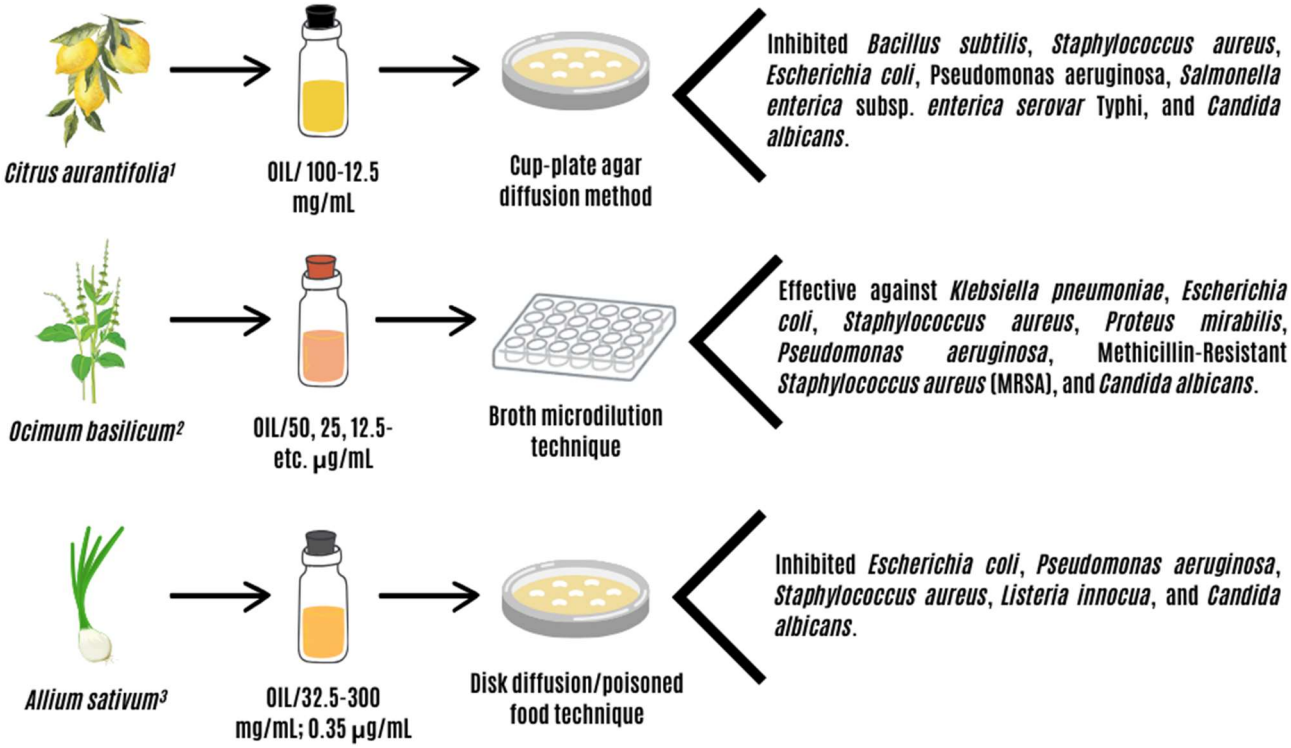

Figure 1. Findings on the antimicrobial efficiency of essential oils from *Citrus aurantifolia* (CAEO), *Ocimum basilicum* (OBEO), and *Allium sativum* (ASEO). Sources: [1] [42]; [2] [43]; [3] [44,45].

The mechanisms of action against microorganisms that justify the antimicrobial characteristics of these essential oils have already been studied and described [46–48]. Musdja et al. [46] reported that CAEO causes cumulative bacterial cell destruction events. It starts by damaging the membrane, followed by the leakage of proteins, nucleic acids, and K^+

and Ca^{2+} ions. Dysfunctions induced by this essential oil yield bacterial cells of irregular sizes with debris [49]. As the main compound of CAEO, limonene has demonstrated its antibacterial potential due to, among other things, enzyme inhibition and regulation, the disruption of translation in protein synthesis, and the inhibition of cell wall synthesis [50]. Similar events were also found when investigating the mechanism of action of limonene against fungi [51]. This demonstrates this compound's significant contribution to the antimicrobial potential of CAEO. When in contact with bacteria, OBEO can compromise cell structure and integrity, resulting in the leakage of macromolecular substances and intracellular ionic electrolytes [48]. Although this mechanism of action against bacterial survival is not linked to just one compound, the significant participation of linalool in this antimicrobial process was emphasised by Li et al. [48]. The antifungal potential of OBEO may be related to the inhibition of yeast transformation into hyphae [52]. Bacteria exposed to ASEO show an increase in the permeability of their cell membrane, and the entire environment responsible for their survival and metabolism is impaired, making their survival impossible [47]. Fungi exposed to ASEO are killed because their cell membranes and organelles (e.g., mitochondria) are damaged [45]. Allicin is one of the representative points of origin for the antimicrobial capacity of AS essential oil [53]. In situations where allicin is present in low concentrations in AS oil, other compounds, such as Diallyl disulfide, can effectively reinforce the antimicrobial function of ASEO [54].

Natural antimicrobials with different colours, smells, compositions, biological effects, toxicity levels, and countless applications can be developed from trees, shrubs, herbs, and other plant varieties worldwide. Some of these antimicrobials are CAEO, OBEO, and ASEO, which have been suggested for use in poultry production to ensure that poultry food products (meat and eggs) have minimal microbial loads. This should not incur sudden changes in appearance, ensure a long shelf life, and ensure that the food can be safely consumed for an extended period. The ability of these three essential oils to provide microbial control in eggs and poultry meat, alone or in combination with other substances or formulations, has been tested; a summary of the findings is reported in Table 1.

Table 1. Antimicrobial effects of essential oils from *Citrus aurantifolia* (CAEO), *Ocimum basilicum* (OBEO), and *Allium sativum* (ASEO) in poultry products.

Essential Oil	Concentration	Application Method	Poultry Product	Food Image	Findings	Reference
CAEO	200 and 400 mg/mL	Spraying	Chicken meat		Reduced counts of *Escherichia coli* (−7.90 log), *Salmonella enterica* subsp. *enterica serovar* Typhi (−5.30 log) and *Salmonella enterica* subsp. *enterica serovar* Typhimurium (−3.10 log).	[8]
CAEO	1%	Immersion	Egg		Reduced the total number of aerobic mesophilic bacteria in the shell (−0.92 log) and content (−1.04 log).	[55]
OBEO	0.20%	Vacuum packaging	Chicken thighs		Reduced the population of anaerobic bacteria (−1.34 log) and lactic acid bacteria (−0.62 log) and bacteria from the Enterobacteriaceae family (−1.55 log).	[56]
OBEO	2.5 and 5.0 mg/mL	Immersion	Chicken meat		Reduced the number of *Salmonella enterica* subsp. *enterica serovar* Enteritidis (−1.15–2.46 log).	[7]

Table 1. *Cont.*

Essential Oil	Concentration	Application Method	Poultry Product	Food Image	Findings	Reference
OBEO	300 mg/mL	Immersion	Egg		Reduced the number of aerobic mesophilic bacteria, Enterobacteriaceae, moulds, and yeasts in the shell (−1.71 log, −1.51 log and −1.20 log, respectively) and content (−1.50 log, −1.83 log and −1.42 log, respectively).	[33]
ASEO	4% and 8%	Coated	Chicken nuggets		Reduced total viable counts (approximately −2.00 log) and psychrotrophic bacteria (approximately −1.00 log).	[9]
ASEO	0.5%, 1%, and 2%	Film packaging	Chicken breast fillet		Reduced total viable count, *Staphylococcus aureus* and psychrotrophic bacteria (−1.80 log, approximately −1.50 log, approximately, −1.00 log, respectively).	[57]
ASEO	100 mg/mL	Immersion	Egg		Reduced the load of aerobic mesophilic bacteria, Enterobacteriaceae and moulds and yeasts in the shell (−1.18 log, −1.27 log and −1.34 log, respectively) and content (−1.18 log, −1.27 log and −1.34 log, respectively).	[40]

The antimicrobial effects of essential oils have also been used in dietary interventions for poultry from the initial stage of life after hatching. This approach aims to balance the diet beyond the conventional standard, offering nutritional support that beneficially promotes the development, survival, productivity, and, mainly, the health of poultry. Elnaggar and El-Tahawy [58] observed that providing diets containing OBEO (0.5 g and 1 g of oil/kg of feed) resulted in significant improvements in the growth, feed conversion, economic efficiency, production index, immune response, carcass characteristics, and general health of broiler chickens when compared with the non-supplemented control group. Additionally, there were reductions in total bacterial counts, *Salmonella* spp., *Escherichia coli*, and *Proteus* spp. in the digestive system of broiler chickens compared with the control group. Similarly, one study demonstrated that the strategic introduction of 0.05% OBEO oil into the diet of broiler chickens increased the concentration of beneficial bacteria (e.g., *Lactobacillus*) and reduced harmful bacteria (e.g., *Escherichia coli*) in the intestine and cecum, which was reflected in an improved feed conversion ratio [59]. Abd El-Latif et al. [60] also observed that the provision of poultry feed plus 100 mg of ASEO/kg of feed improved the performance of broiler chickens and stimulated innate immunity. In microbiological terms, the intestinal health of broiler chickens benefited when they consumed a diet with nanoencapsulated ASEO in the same concentrations [61]. As indicated by Elbaz et al. [62], dietary supplementation with ASEO (200 mg of oil/kg diet) improved growth performance and enhanced carcass characteristics, nutrient digestion, blood lipid metabolism, and intestinal microbiota. We found no studies that evaluated the use of CAEO in poultry nutrition.

5. Conclusions and Future Perspectives

Existing findings reinforce the concept that essential oils can proportionately align with the poultry industry's demands for microbiologically safe food products. However, antimicrobial measures should not be limited exclusively to final poultry products. Such measures must be used throughout the production chain. For example, healthy chickens for consumption are likely to come from properly handled healthy embryos/chicks [63].

Proper management needs to involve antimicrobial protocols as a form of prevention. These protocols must be implemented from the beginning of the production process, with sanitisation processes of hatching eggs. After hatching, providing a diet that includes natural antimicrobials is essential, maintaining this approach until the final product is consumed. Based on knowledge of the safety and antimicrobial efficacy of CAEO, OBEO, and ASEO, it is hypothesised that these are potent alternatives for safe microbiological control in all sectors of the poultry chain. During the poultry breeding phase, microbiological control is strongly associated with the preservation of health. Poultry professionals and researchers need to fill knowledge gaps with investigations aimed at producing state-of-the-art antimicrobials based on CAEO, OBEO, and ASEO that can promote healthy poultry farming, explicitly targeting the pathogenic microbiota of poultry products and ensure the integrity of products from the poultry sector and human health. Advancing improvements in poultry management and understanding the need for green antimicrobials in poultry farming is a big step towards achieving these goals.

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References

1. Nolan, L.K.; John Barnes, H.; Vaillancourt, J.-P.; Abdul-Aziz, T.; Logue, C.M. Colibacillosis. In *Diseases of Poultry*; Swayne, D.E., Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2013.
2. Ebrahimzadeh, S.; Biswas, D.; Roy, S.; McClements, D.J. Incorporation of essential oils in edible seaweed-based films: A comprehensive review. *Trends Food Sci. Technol.* **2023**, *135*, 43–56. [CrossRef]
3. Kačániová, M.; Terentjeva, M.; Puchalski, C.; Petrová, J.; Hutková, J.; Kántor, A.; Mellen, M.; Čuboň, J.; Haščík, P.; Kluz, M.; et al. Microbiological quality of chicken thighs meat after application of essential oils combination, EDTA and vaccum packing. *Slovak J. Food Sci.* **2016**, *10*, 107–113. [CrossRef]
4. Oliveira, G.D.S.; Nascimento, S.T.; dos Santos, V.M.; Silva, M.G. Clove Essential Oil in the Sanitation of Fertile Eggs. *Poult. Sci.* **2020**, *99*, 5509–5516. [CrossRef]
5. Yousefi, M.; Khorshidian, N.; Hosseini, H. Potential Application of Essential Oils for Mitigation of Listeria monocytogenes in Meat and Poultry Products. *Front. Nutr.* **2020**, *7*, 577287. [CrossRef]
6. Oliveira, G.D.S.; McManus, C.; Pires, P.G.D.S.; dos Santos, V.M. Combination of Cassava Starch Biopolymer and Essential Oils for Coating Table Eggs. *Front. Sustain. Food Syst.* **2022**, *6*, 957229. [CrossRef]
7. Stojanović-Radić, Z.; Pejčić, M.; Joković, N.; Jokanović, M.; Ivić, M.; Šojić, B.; Škaljac, S.; Stojanović, P.; Mihajilov-Krstev, T. Inhibition of *Salmonella* Enteritidis growth and storage stability in chicken meat treated with basil and rosemary essential oils alone or in combination. *Food Control* **2018**, *90*, 332–343. [CrossRef]
8. Irokanulo, E.O.; Oluyomi, B.W.; Nwonuma, C.O. Effect of citrus fruit (*Citrus sinensis, Citrus limon* and *Citrus aurantifolia*) rind essential oils on preservation of chicken meat artificially infected with bacteria. *Afr. J. Food Agric. Nutr. Dev.* **2021**, *21*, 18950–18964. [CrossRef]
9. Raeisi, S.; Ojagh, S.M.; Pourashouri, P.; Salaün, F.; Quek, S.Y. Shelf-life and quality of chicken nuggets fortified with encapsulated fish oil and garlic essential oil during refrigerated storage. *J. Food Sci. Technol.* **2021**, *58*, 121–128. [CrossRef] [PubMed]

10. Atif, M.J.; Amin, B.; Ghani, M.I.; Ali, M.; Zhang, S.; Cheng, Z. Effect of photoperiod and temperature on garlic (*Allium sativum* L.) bulbing and selected endogenous chemical factors. *Environ. Exp. Bot.* **2020**, *180*, 104250. [CrossRef]
11. Kholiya, S.; Punetha, A.; Venkatesha, K.T.; Kumar, D.; Upadhyay, R.K.; Padalia, R.C. Essential oil yield and composition of *Ocimum basilicum* L. at different phenological stages, plant density and post-harvest drying methods. *South Afr. J. Bot.* **2022**, *151*, 919–925. [CrossRef]
12. Enejoh, O.S.; Ogunyemi, I.O.; Bala, M.S.; Oruene, I.S.; Suleiman, M.M.; Ambali, S.F. Ethnomedical Importance of *Citrus Aurantifolia* (Christm) Swingle. *Pharm. Innov. J.* **2015**, *4*, 1–6.
13. Lal, G.; Dayal, H. Effect of integrated nutrient management on yield and quality of acid lime (*Citrus aurantifolia* Swingle). *Afr. J. Agric. Res.* **2014**, *9*, 2985–2991. [CrossRef]
14. Cruz-Valenzuela, M.R.; Tapia-Rodríguez, M.R.; Vazquez-Armenta, F.J.; Silva-Espinoza, B.A.; Ayala-Zavala, J.F. Lime (*Citrus aurantifolia*) Oils. In *Essential Oils in Food Preservation, Flavor and Safety*; Elsevier: Amsterdam, The Netherlands, 2016; pp. 531–537. [CrossRef]
15. Ladaniya, M.S.; Marathe, R.A.; Das, A.K.; Rao, C.N.; Huchche, A.D.; Shirgure, P.S.; Murkute, A.A. High density planting studies in acid lime (*Citrus aurantifolia* Swingle). *Sci. Hortic.* **2020**, *261*, 108935. [CrossRef]
16. Lemes, R.S.; Alves, C.C.F.; Estevam, E.B.B.; Santiago, M.B.; Martins, C.H.G.; dos Santos, T.C.D.; Crotti, A.E.; Miranda, M.L. Chemical composition and antibacterial activity of essential oils from *Citrus aurantifolia* leaves and fruit peel against oral pathogenic bacteria. *An. Acad. Bras. Cienc.* **2018**, *90*, 1285–1292. [CrossRef] [PubMed]
17. Ibrahim, F.A.; Usman, L.A.; Akolade, J.O.; Idowu, O.A.; Abdulazeez, A.T.; Amuzat, A.O. Antidiabetic Potentials of *Citrus aurantifolia* Leaf Essential Oil. *Drug. Res.* **2019**, *69*, 201–206. [CrossRef] [PubMed]
18. Lin, L.Y.; Chuang, C.H.; Chen, H.C.; Yang, K.M. Lime (*Citrus aurantifolia* (Christm.) swingle) essential oils: Volatile compounds, antioxidant capacity, and hypolipidemic effect. *Foods* **2019**, *8*, 398. [CrossRef] [PubMed]
19. Hasibuan, R.; Gultom, E. The effect of method, type of solvent and extraction time towards the yield of oil on essential oil extraction from lime peel (*Citrus aurantifolia*). IOP Conf. *Ser. Mater. Sci. Eng.* **2021**, *1122*, 012108. [CrossRef]
20. de Sousa, A.D.; Barros Gomes, P.R.B.; Souza, R.D.; Mouchrek Filho, V.E.; Mouchrek, C.N.; Everton, G.O.; de Almeida, N.R.; de Araújo, J.P. Composição Química e Atividade Larvicida de Óleos Essenciais de *Citrus aurantifolia* (Christm.) Swingle bark Contra Larvas de Aedes aegypti [Chemical Composition and Larvicidal Activity of Essential Oil from *Citrus aurantifolia* (Christm.) Swingle bark Against Aedes aegypti Larvae]. *Ver. Virt. Quím.* **2003**, *15*, 722–727. [CrossRef]
21. de Lima, N.P.; da Nóbrega, G.A.S. Extração do Óleo Essencial das Folhas e Cascas do Limão Tahiti (*Citrus aurantifolia*) para Produção de Saneantes [Extraction of Essential Oil from the Leaves and Peels of Tahiti Lemon (*Citrus aurantifolia*) for the Production of Sanitizing Products]. Diploma Thesis, Federal Rural University of the Semi-Arid, Mossoró, Brazil, 2021.
22. Allam, O.G.; Ramadan, M.M.; Ismail, S.A.; Hebeish, A. Preparation and application of lemon peel oil (*Citrus aurantifolia*) to improve microbial resistance of wool and viscose fabrics. *Egypt. J. Chem.* **2022**, *65*, 511–521. [CrossRef]
23. Akolade, J.O.; Nasir-Naeem, K.O.; Swanepoel, A.; Yusuf, A.A.; Balogun, M.; Labuschagne, P. CO_2-assisted production of polyethylene glycol/lauric acid microparticles for extended release of *Citrus aurantifolia* essential oil. *J. CO_2 Util.* **2020**, *38*, 375–384. [CrossRef]
24. Nurzyńska-Wierdak, R. Comparing the growth and flowering of selected basil (*Ocimum basilicum* L.) varieties. *Acta Agrobot.* **2007**, *60*, 127–131. [CrossRef]
25. Purushothaman, B.; Prasannasrinivasan, R.; Suganthi, P.; Ranganathan, B.; Gimbun, J.; Shanmugam, K. A comprehensive review on *Ocimum basilicum*. *J. Nat. Remedies* **2018**, *18*, 71–85. [CrossRef]
26. Shahrajabian, M.H.; Sun, W.; Cheng, Q. Chemical components and pharmacological benefits of Basil (*Ocimum basilicum*): A review. *Int. J. Food Prop.* **2020**, *23*, 1961–1970. [CrossRef]
27. Perveen, K.; Bokhari, N.A.; Al-Rashid, S.A.I.; Al-Humaid, L.A. Chemical Composition of Essential Oil of *Ocimum basilicum* L. and Its Potential in Managing the Alternaria Rot of Tomato. *J. Essent. Oil-Bear. Plants* **2020**, *23*, 1428–1437. [CrossRef]
28. Anwar, F.; Alkharfy, K.M.; Mehmood, T.; Bakht, M.A. Variation in Chemical Composition and Effective Antibacterial Potential of *Ocimum basilicum* L. Essential Oil Harvested from Different Regions of Saudi Arabia. *Pharm. Chem. J.* **2021**, *55*, 187–193. [CrossRef]
29. Abdoul-Latif, F.M.; Elmi, A.; Merito, A.; Nour, M.; Risler, A.; Ainane, A.; Bignon, J.; Ainane, T. Essential Oils of *Ocimum basilicum* L. and *Ocimum americanum* L. from Djibouti: Chemical Composition, Antimicrobial and Cytotoxicity Evaluations. *Processes* **2022**, *10*, 1785. [CrossRef]
30. El Mokhtari, K.; El Brouzi, A.; El Kouali, M.; Talbi, M. Extraction and composition of the essential oil of *Ocimum basilicum* collected in Morocco. *J. Anal. Sci. Appl. Biotech.* **2019**, *1*, 45–48. [CrossRef]
31. Khair-ul-Bariyah, S. Comparison of the Physical Characteristics and GC/MS of the Essential Oils of *Ocimum basilicum* and *Ocimum sanctum*. *Int. J. Sci. Res. Knowl.* **2013**, *1*, 363–372. [CrossRef]
32. Luong, N.X.; Nhan, N.T.; Thuy, N.T.; Vinh, T.X. Chemical composition, antimicrobial and antioxidant activitites of essential oil from (*Ocimum Basilicum* L.) collected in thanh hoa province. *J. Sci.* **2022**, *12*, 66–75.
33. de Araújo, M.V.; Oliveira, G.D.S.; McManus, C.; Vale, I.R.R.; Salgado, C.B.; Pires, P.G.d.S.; de Campos, T.A.; Gonçalves, L.F.; Almeida, A.P.C.; Martins, G.D.S.; et al. Preserving the Internal Quality of Quail Eggs Using a Corn Starch-Based Coating Combined with Basil Essential Oil. *Processes* **2023**, *11*, 1612. [CrossRef]
34. Wilson, A.; Pandya, D.; Mankad, A.; Solanki, H. A Review on *Allium sativum* (L.) as medicinal plant. *Int. J. Res. Anal. Rev. (IJRAR)* **2019**, *6*, 140–147.

35. Esho, K.B. Performance of genetic parameters in garlic (*Allium Sativum* L.). *Res. J. Agr. Env. Sci.* **2015**, *2*, 1–9.
36. Tellez, T.M.E.; Aguilar, E.S.V.; Rojas, M.A.T.; Guineo, D.O.D.; Quevedo, R.; Díaz, O.; Bastías, J. Garlic (*Allium sativum* L) and its beneficial properties for health: A Review. *Agroind. Sci.* **2020**, *10*, 103–115. [CrossRef]
37. Boukeria, S.; Kadi, K.; Kalleb, R.; Benbott, A.; Bendjedou, D.; Yahia, A. Phytochemical and physicochemical characterisation of *Allium sativum* L. and Allium cepa L. Essential oils. *J. Mater. Environ. Sci.* **2016**, *7*, 2362–2368.
38. Akinyemi, A.J.; Faboya, L.; Olayide, I.; Anadozie, S.; Awonegan, A.P.; Anadozie, S.; Oluwasola, T. Antioxidant and Anti-Acetylcholinesterase Activities of Essential Oils from Garlic (*Allium Sativum*) Bulbs. *Int. J. Plant Res.* **2018**, *31*, 2. [CrossRef]
39. Nazzaro, F.; Polito, F.; Amato, G.; Caputo, L.; Francolino, R.; D'acierno, A.; Fratianni, F.; Candido, V.; Coppola, R.; De Feo, V. Chemical Composition of Essential Oils of Bulbs and Aerial Parts of Two Cultivars of *Allium sativum* and Their Antibiofilm Activity against Food and Nosocomial Pathogens. *Antibiotics* **2022**, *11*, 724. [CrossRef] [PubMed]
40. Vale, I.R.R.; Oliveira, G.d.S.; McManus, C.; de Araújo, M.V.; Salgado, C.B.; Pires, P.G.d.S.; de Campos, T.A.; Gonçalves, L.F.; Almeida, A.P.C.; Martins, G.D.S.; et al. Whey Protein Isolate and Garlic Essential Oil as an Antimicrobial Coating to Preserve the Internal Quality of Quail Eggs. *Coatings* **2023**, *13*, 1369. [CrossRef]
41. Gafar, M.K.; Itodo, A.U.; Warra, A.A.; Abdullahi, L. Extraction and physicochemical determination of garlic (*Allium sativum* L) oil. *Int. J. Food Nut. Sci.* **2012**, *1*, 4–7.
42. Mohamed, S.G.; Badri, A.M. Antimicrobial Activity of *Syzygium aromaticum* and *Citrus aurantifolia* Essential Oils Against Some Microbes in Khartoum, Sudan. *EC Microbiol* **2017**, *12*, 253–259.
43. Eid, A.M.; Jaradat, N.; Shraim, N.; Hawash, M.; Issa, L.; Shakhsher, M.; Nawahda, N.; Hanbali, A.; Barahmeh, N.; Taha, B.; et al. Assessment of anticancer, antimicrobial, antidiabetic, anti-obesity and antioxidant activity of *Ocimum basilicum* seeds essential oil from Palestine. *BMC Complement Med. Ther.* **2023**, *23*, 221. [CrossRef]
44. Yasin, G.; Jasim, S.A.; Mahmudiono, T.; Al-Shawi, S.G.; Shichiyakh, R.A.; Shoukat, S.; Kadhim, A.J.; Iswanto, A.H.; Saleh, M.M.; Fenjan, M. Investigating the effect of garlic (*Allium sativum*) essential oil on foodborne pathogenic microorganisms. *Food Sci. Technol.* **2022**, *42*, e03822. [CrossRef]
45. Li, W.R.; Shi, Q.S.; Dai, H.Q.; Liang, Q.; Xie, X.B.; Huang, X.M.; Zhao, G.Z.; Zhang, L.X. Antifungal activity, kinetics and molecular mechanism of action of garlic oil against Candida albicans. *Sci. Rep.* **2016**, *6*, 22805. [CrossRef] [PubMed]
46. Musdja, M.Y.; Pariwidjayanti, A.M.; Agusta, A. Activity Test and Inhibitory Mechanism of Essential Oil of Lime Leaf (*Citrus Aurantifolia*, Swingle) Against Some of Bacterial Pathogens. *Adv. Intell. Syst. Res.* **2018**, *149*, 42–50. [CrossRef]
47. Zhang, H.; Wang, J. Constituents of the essential oils of garlic and citronella and their vapor-phase inhibition mechanism against *S. aureus*. *Food Sci. Technol. Res.* **2019**, *25*, 65–74. [CrossRef]
48. Li, C.; Zhang, C.; Chen, X.; Cui, H.; Lin, L. The interference mechanism of basil essential oil on the cell membrane barrier and respiratory metabolism of Listeria monocytogenes. *Front. Microbiol* **2022**, *13*, 855905. [CrossRef]
49. Ojha, S.; Pandey, A.K.; Singh, P. *Citrus Aurantifolia* Essential Oil Composition, Bioactivity, and Antibacterial Mode of Action on *Salmonella Enterica*, a Foodborne Pathogen. *Food Sci. Eng.* **2023**, *4*, 75–88. [CrossRef]
50. Gupta, A.; Jeyakumar, E.; Lawrence, R. Strategic approach of multifaceted antibacterial mechanism of limonene traced in *Escherichia coli*. *Sci. Rep.* **2021**, *11*, 13816. [CrossRef]
51. Cai, R.; Hu, M.; Zhang, Y.; Niu, C.; Yue, T.; Yuan, Y.; Wang, Z. Antifungal activity and mechanism of citral, limonene and eugenol against Zygosaccharomyces rouxii. *LWT* **2019**, *106*, 50–56. [CrossRef]
52. Gucwa, K.; Milewski, S.; Dymerski, T.; Szweda, P. Investigation of the antifungal activity and mode of action of *Thymus vulgaris*, *Citrus limonum*, *Pelargonium graveolens*, *Cinnamomum cassia*, *Ocimum basilicum*, and *Eugenia caryophyllus* essential oils. *Molecules* **2018**, *23*, 1116. [CrossRef]
53. Fujisawa, H.; Watanabe, K.; Suma, K.; Origuchi, K.; Matsufuji, H.; Seki, T.; Ariga, T. Antibacterial potential of garlic-derived allicin and its cancellation by sulfhydryl compounds. *Biosci. Biotechnol. Biochem.* **2009**, *73*, 1948–1955. [CrossRef]
54. Torpol, K.; Wiriyacharee, P.; Sriwattana, S.; Sangsuwan, J.; Prinyawiwatkul, W. Antimicrobia activity of garlic (*Allium sativum* L.) and holy basil (*Ocimum sanctum* L.) essential oils applied by liquid vs. vapour phases. *Int. J. Food Sci. Technol.* **2018**, *53*, 2119–2128. [CrossRef]
55. Oliveira, G.D.S.; McManus, C.; Salgado, C.B.; Pires, P.G.D.S.; de Figueiredo Sousa, H.A.; da Silva, E.R.; Dos Santos, V.M. Antimicrobial Coating Based on Tahiti Lemon Essential Oil and Green Banana Flour to Preserve the Internal Quality of Quail Eggs. *Animals* **2023**, *13*, 2123. [CrossRef] [PubMed]
56. Kačániová, M.; Terentjeva, M.; Arpášová, H.; Hleba, L.; Ivanišová, E.; Petrová, J.; Kántor, A.; Čuboň, J.; Haščík, P. Effect of *Thymus Serpyllum* and *Ocimum Basilicum* Essential Oils on the Shelf-Life of Chicken's Meat during Refrigerated Storage. *Sci. Pap. Anim. Sci. Biotechnol. Lucr. Stiintifice Zooteh. Si Biotehnol.* **2016**, *49*, 103–108.
57. Kamkar, A.; Molaee-aghaee, E.; Khanjari, A.; Akhondzadeh-basti, A.; Noudoost, B.; Shariatifar, N.; Sani, M.A.; Soleimani, M. Nanocomposite active packaging based on chitosan biopolymer loaded with nano-liposomal essential oil: Its characterisations and effects on microbial, and chemical properties of refrigerated chicken breast fillet. *Int. J. Food Microbiol.* **2021**, *342*, 109071. [CrossRef] [PubMed]
58. Elnaggar, A.S.; El-Tahawy, W.S. Productive performance, physiological and immunological response of broiler chicks as affected by dietary aromatic plants and their essential oils. *Egypt. Poult. Sci. J.* **2018**, *38*, 773–795. [CrossRef]

59. Vlaicu, P.A.; Untea, A.E.; Panaite, T.D.; Saracila, M.; Turcu, R.P.; Dumitru, M. Effect of basil, thyme and sage essential oils as phytogenic feed additives on production performances, meat quality and intestinal microbiota in broiler chickens. *Agriculture* **2023**, *13*, 874. [CrossRef]
60. Abd El-Latif, A.S.; Saleh, N.S.; Allam, T.S.; Ghazy, E.W. The effects of rosemary (*Rosemarinus afficinalis*) and garlic (*Allium sativum*) essential oils on performance, hematological, biochemical and immunological parameters of broiler chickens. *Br. J. Poult. Sci.* **2013**, *2*, 16–24. [CrossRef]
61. Amiri, N.; Afsharmanesh, M.; Salarmoini, M.; Meimandipour, A.; Hosseini, S.; Ebrahimnejad, H. Nanoencapsulation (in vitro and in vivo) as an efficient technology to boost the potential of garlic essential oil as alternatives for antibiotics in broiler nutrition. *Animal* **2021**, *15*, 100022. [CrossRef]
62. Elbaz, A.M.; Ashmawy, E.S.; Salama, A.A.; Abdel-Moneim, A.E.; Badri, F.B.; Thabet, H.A. Effects of garlic and lemon essential oils on performance, digestibility, plasma metabolite, and intestinal health in broilers under environmental heat stress. *BMC Vet. Res.* **2022**, *18*, 430. [CrossRef]
63. Oliveira, G.D.S.; McManus, C.; Salgado, C.B.; dos Santos, V.M. Effects of Sanitizers on Microbiological Control of Hatching Eggshells and Poultry Health during Embryogenesis and Early Stages after Hatching in the Last Decade. *Animals* **2022**, *12*, 2826. [CrossRef]

Paper four

Obtaining Microbiologically Safe Hatching Eggs from Hatcheries: Using Essential Oils for Integrated Sanitization Strategies in Hatching Eggs, Poultry Houses and Poultry

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Review

Obtaining Microbiologically Safe Hatching Eggs from Hatcheries: Using Essential Oils for Integrated Sanitization Strategies in Hatching Eggs, Poultry Houses and Poultry

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Abstract: Essential oils are liquids containing non-toxic compounds that are unfavorable to the growth of microorganisms. They are sold globally at affordable or very high prices, depending on the availability and type of plant, the scale of production, the extraction method, costs associated with logistics and electricity consumption, among other variables. Each year, the quantity of research dedicated to the antimicrobial potential of essential oils in poultry farming is expanding. Researchers consensually relay that this increase is due to the growing resistance of microorganisms to traditional antimicrobials and concerns about the toxicity of these products. This review proposes an analysis of the antimicrobial feasibility of using essential oils to address microbial challenges in poultry farms, aiming to ensure the production and supply of microbiologically safe hatching eggs. Based on the findings in the literature, in addition to following other necessary precautions in the daily routines of poultry farming practices, developing an antimicrobial control program with essential oils that integrates poultry facilities, poultry and hatching eggs, adapted to the particularities of each context seems to be extremely effective.

Keywords: egg microbiology; microbial reduction; natural antimicrobials; poultry industry; poultry microbiology; poultry safety

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1. Introduction

Microbial communities are not limited to just colonizing poultry facilities, they also colonize poultry until the moment of slaughter and beyond [1]. Microbial colonization comes from the environment, maternal transmission, transmission between poultry during the consumption of feed and water, as well as human transmission [2–6]. Microbiological damage that compromises the health and well-being of poultry can occur much sooner than expected, resulting in widespread complications such as production cessation and significant repair expenses, requiring immediate solutions.

Poultry farms that follow quality sanitary principles make it possible to raise poultry both in confined and unconfined environments, with due restrictions on contamination of their products, including hatching eggs. Poultry feeding and watering systems, egg collection systems, ventilation and refrigeration systems, materials and equipment storage rooms and egg storage rooms, as well as parking areas, transport trucks and circulation spaces of people and vehicles, must comply with high sanitary standards. Typically, professional staff at poultry farms perform a variety of tasks to mitigate the risk of uncontrolled contamination in poultry facilities and prevent the development of avian infections [7]. Adopting appropriate work attire, following procedures using microbiologically safe materials, and strictly controlling access to facilities, are some measures adopted. However, concern about the abusive use of synthetic antimicrobials in the poultry industry, aiming to

maintain high sanitary standards, has led researchers to recommend updating prevention strategies [7–11].

The global dissemination of essential oils as sanitizers in poultry farming [7,8,12–15] promotes the innovative "Healthy Sanitization of Poultry Farms" concept. This paradigm aims to implement effective avian health control protocols, adapted to ideal spatial conditions, with the responsibility of reducing microbial levels in the air and on contaminated surfaces and preserving the integrity of animal, human and environmental health, considering possible failures during handling and repetitive daily work. Furthermore, it is a concept that aims to encompass standards established by regulatory authorities or government bodies to ensure the supply of hatching eggs with acceptable microbiological parameters. Furthermore, the synergy between indirect antimicrobial treatment (in the poultry farming environment, as mentioned above) and direct antimicrobial treatment (in the poultry itself) [7–9,16] can be a way to maximize the antimicrobial benefits in poultry products. Notably, the administration of antimicrobials formulated with essential oils through diets or water to poultry provided impressive results in the production of eggs without conventional antibiotic residues and with lower microbial loads in the shell [17,18].

This review proposes an analysis of the antimicrobial feasibility of using essential oils to address microbial challenges in poultry farms, aiming to ensure the production and supply of microbiologically safe hatching eggs.

2. Paper Search Strategy

For this review, papers (research and review), book chapters and conference papers available on Google Scholar written in Portuguese or English up to 2 January 2024 were examined. The search terms were organized into six distinct groups, covering investigations on topics such as "microbial contamination in poultry farms", "poultry antimicrobial management", "essential oils", "antimicrobial function of essential oils", "application of essential oils in poultry farming" and "essential oils and poultry products". The papers were thoroughly researched until each topic was comprehensively understood. Papers meeting the criteria defined for each group were chosen for inclusion, while those that did not meet the specified criteria were excluded.

3. Poultry Farms Are an Ideal Environment for Undesirable Microorganisms

Floors, fans, vents, feed loaders, feeders, drinkers, and wall crevices of poultry farms can be persistently contaminated with *Salmonella* spp., *Campylobacter* spp., *Escherichia coli* and/or *Staphylococcus aureus* [4]. Likewise, feed contaminated with *Salmonella* spp. and *Escherichia coli* can be fed to poultry, contributing to systemic contamination of the farm [5]. Furthermore, fungal contamination by *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus*, *Mucor* spp., *Penicillium* spp. and/or *Fusarium* spp. Can be observed in water lines, cooling pad water, fans, and floors of broiler farms [19]. These factors compromise the quality of water and air in poultry facilities. Bacterial (e.g., by *Salmonella* spp. and *Escherichi coli*) and fungal (e.g., by *Aspergillus* spp.) contamination in poultry farms harms the poultry health and the quality and viability of poultry products.

In a study carried out by Kemmett et al. [20], the bacterium *Escherichia coli* was identified in several pathological changes present in broiler chickens during the first week of life, including pericarditis, perihepatitis, abnormal liver color, ascites, cellulitis, and abnormal yolk sac. These changes are particularly concerning, as it suggests that approximately 70% of poultry mortality in the first week can be attributed to these complications [20]. Muna et al. [21] reported that young broilers contaminated with *Salmonella* spp., mainly *Salmonella enterica* subsp. *enterica* serovar Enteritidis and *Salmonella enterica* subsp. *enterica* serovar Typhimurium developed septicemia due to systemic changes and injuries in vital organs, such as the liver, intestine, spleen, heart, and brain. These changes include hepatomegaly, splenomegaly, inflammation of the intestinal mucosa, necrotic foci in the spleen, liver, and brain, as well as degeneration of the myocardial muscle fiber [21]. An outbreak of fungal infections of the respiratory tract of poultry naturally caused by *Aspergillus* spp. was reported

in a poultry house [22]. These infections have resulted in significant complications, such as alveolar emphysema, atelectasis, thrombosis, and pneumonic lung with granulomatous tissue and granulomatous encephalitis [22]. These complications, in turn, contributed to the mortality of 200 approximately two-week-old broiler chickens [22].

In production systems, eggs can be horizontally contaminated by *Salmonella enterica* subsp. *enterica* serovar Typhimurium, present in poultry feces [3]. Thus, the concern arises because the eggshell is an access portal for microorganisms and is close to internal structures. In addition to *Salmonella* spp., pathogens from other genera such as *Clostridium*, *Enterococcus*, *Staphylococcus*, *Alcaligenes*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Pseudomonas*, *Shigella*, *Aspergillus*, *Candida*, *Fusarium* and *Penicillium* can also lodge in the eggshell (reviewed by Oliveira et al. [23]), exposing the embryo to a more intense microbial load during critical stages of development, where the embryo's period of vulnerability is more evident. Due to this concern, some studies have explored the severity of microbial infections during embryonic development in poultry [24–26]. Embryonic mortality appears to be the most common consequence [27], becoming a detriment to the productive balance of the poultry chain.

It is important to clarify that the proliferation and dissemination of high rates of microbial contamination and mortality on poultry farms is not something expected and common on farms that adopt a rigorous and correct routine in health management at all stages of the production chain.

4. Essential Oils and Their Bacterial and Fungal Functions

In the industrial processing of natural products, large volumes of essential oils can be extracted from aromatic plants. Conventional and green processes can extract these oils from plants, but the conventional process by steam distillation stands out among them all [28]. These essential oils, volatile liquids, have aromas similar to those of the original plant and are loaded with functional components. Studies on the chemical analysis of essential oils have revealed that oil can contain more than 20 functional compounds [29]. The heterogeneity of the chemical composition of essential oils requires chemical analysis to determine the essential oils suitable for use as an antimicrobial agent. Depending on the essential oil, the main compound may be a monoterpene, phenol, aldehyde, ketone, alcohol, hydrocarbons, or another compound (Table 1). Phenols, alcohols, and aldehydes were found to be the most effective against Gram-negative and Gram-positive bacteria, while hydrocarbons were the least effective [30]. This finding agrees with El-Baroty et al. [31], who stated that antimicrobial activity gradually decreases from phenols (with greater activity) to hydrocarbons (with lower activity).

Table 1. Main compound of different essential oils.

Essential Oil	Main Compound	Reference
Cymbopogon winterianus	Citronellal (41.80%)	
Eucalyptus paniculata	α-pinene (55.47%)	[32]
Lavandula angustifolia	1,8-cineole (46.78%)	
Origanum vulgare	Carvacrol (69.1%)	
Thymus vulgaris	Thymol (45.5%)	[33]
Lippia sidoides	Thymol (77.2%)	
Allium sativum	Diallyl disulfide (40%)	
Carapa guianensis	Limmonoids (2–5%)	
Syzygium aromaticum	Eugenol (92.3%)	
Zingiber officinale	Zingiberene (33%)	[34]
Citrus sinensis	Limonene (95.48%)	
Mentha piperita	Menthol (55%)	
Piper nigrum	α-pinene (30%)	

Table 1. *Cont.*

Essential Oil	Main Compound	Reference
Citrus aurantium	Limonene (84.3%)	[35]
Cinnamomum camphora	1,8-cineole (54.0%)	
Curcuma longa	Turmerone (31.4%)	
Morinda citrifolia	Octanoic acid (78.9%)	
Plectranthus amboinicus	Carvacrol (17.9%)	
Petroselinum crispum	Myristicin (40.3%)	
Pittosporum senacia	Myrcene (62.2%)	
Syzygium coriaceum	(E)-β-ocimene (24.4%)	
Syzygium samarangense	β-pinene (21.3%)	
Origanum majorana	Terpinene-4-ol (22%)	[36]
Rosmarinus officinalis	1,8-cineole (40%)	
Trachyspermum ammi	Thymol (45.17%)	
Matricaria recutita	E-β-farnesene (34.61%)	
Ocimum basilicum	Estragole (87.58%)	
Cinnamomum cassia	E-cinnamaldehyde (82.05%)	[37]
Coriandrum sativum	Linalool (43.67%)	
Eugenia caryophyllus	Eugenol (84.58%)	
Cymbopogon citratus	Citral (75.16%)	
Lavandula stoechas	Camphor (32.54%)	
Origanum compactum	Carvacrol (57.21%)	
Cymbopogon martinii	Geraniol (81.05%)	
Cinnamomum zeylanicum	Cinnamaldehyde (68.31%)	[38]
Melaleuca alternifolia	Terpinen-4-ol (42.65%)	
Thymus satureioides	Borneol (32.33%)	
Rosemary officinalis	1,8-cineole (49.49%)	

Therefore, the chemical composition of essential oils may explain their antimicrobial functions, including effectiveness against bacteria and fungi isolated or not from poultry (Table 2). This occurs because the interaction of these compounds with the cell wall and membrane of microorganisms promotes an increase in the permeability of these structures, resulting in leakage or alteration of microbial homeostasis [39,40]. Although some essential oils have been tested effectively to combat microorganisms on poultry farms, recent studies have warned that the effectiveness of these oils depends on the dose [13,15]. *Zingiber Officinalis* essential oil reduced the bacterial growth of *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 11622) strains in a dose-dependent manner (400–5 µg/mL), as evidenced by Galgano et al. [41]. In agreement, Boukhatem et al. [42] reported that *Eucalyptus globulus* essential oil also inhibited, depending on the dose (20, 40 and 60 µL/disc), the growth of foodborne and/or food spoilage pathogens such as *Enterobacter sakazakii*, *Klebsiella ornithinolytica*, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Candida albicans*, *Candida parapsilosis*, *Saccharomyces cerevisiae*, *Trichosporon* spp. and *Aspergillus niger*. Therefore, it is essential to carefully evaluate essential oils when programming an antimicrobial formulation that meets the specific demands of each poultry farm sector. For example, the appropriate sanitizing formula for a poultry house may not be the same as that recommended for application to poultry or for sanitizing hatching eggs. Furthermore, the formulation must simultaneously act to reduce Gram-negative and Gram-positive bacteria, as well as fungi, to levels that are considered safe. Carrying out in vitro antimicrobial tests is an initial direction for developing antimicrobial formulations in poultry farming. This is because the results obtained in vitro generally reflect directly on in vivo tests [12].

Table 2. Summary of essential oils' antibacterial and antifungal capacity against bacteria and fungi isolated or not from poultry.

Essential Oil	MIC	SIM	SM	Reference
Lippia origanoides	40 µL/mL	*Escherichia coli* *Staphylococcus aureus*	Isolated	[43]
Lippia rotundifolia	80 µL/mL 160 µL/mL	*Escherichia coli* *Staphylococcus aureus*	Isolated	[43]
Litsea cubeba	17.72 mg/mL 8.86 mg/mL 1.11 mg/mL 17.72 mg/mL	*Salmonella* Typhimurium *Yersinia enterocolitica* *Listeria monocytogenes* *Enterococcus durans* *Enterococcus faecium* *Enterococcus faecalis*	Isolated	[44]
Origanum vulgare	2.37 mg/mL 0.59 mg/mL 1.18 mg/mL 2.37 mg/mL	*Salmonella* Typhimurium *Yersinia enterocolitica* *Listeria monocytogenes* *Enterococcus durans* *Enterococcus faecium* *Enterococcus faecalis*	Isolated	[44]
Origanum majorana	4.47 mg/mL 17.88 mg/mL	*Salmonella* Typhimurium *Yersinia enterocolitica* *Listeria monocytogenes* *Enterococcus durans* *Enterococcus faecium*	Isolated	[44]
Thymus vulgaris	2.34 mg/mL	*Salmonella* Typhimurium *Yersinia enterocolitica* *Listeria monocytogenes* *Enterococcus durans* *Enterococcus faecium* *Enterococcus faecalis*	Isolated	[44]
Cinnamomum zeylanicum *Cymbopogon citratus* *Litsea cubeba* *Ocimum basilicum* *Mentha piperita* *Pelargonium graveolens* *Syzygium aromaticum*	2.52 mg/mL 1.118 mg/mL 1.106 mg/mL 9.15 mg/mL 1.14 mg/mL 17.8 mg/mL 1.318 mg/mL	*Escherichia coli*	Isolated	[45]
Cymbopogon winterianus *Clausena heptaphylla* *Cinnamomum tamala* *Ocimum sanctum*	50–500 µL/mL	*Staphylococcus aureus* *Bacillus cereus* *Bacillus subtilis* *Salmonella* Typhimurium *Escherichia coli*	ATCC	[46]
Cinnamon Clove White thyme	0.1%	*Escherichia coli* *Salmonella* Typhimurium	CECT	[47]
Satureja hortensis	0.07 µL/mL 0.31 µL/mL	*Escherichia coli* *Salmonella* Enteritidis	Isolated	[48]
Syzygium aromaticum	50–0.39%	*Escherichia coli* *Pseudomonas aeruginosa* *Staphylococcus aureus*	ATCC	[12]

Table 2. *Cont.*

Essential Oil	MIC	SIM	SM	Reference
Citrus latifolia	10 mL (pure)	*Bacillus cereus* *Bacillus subtilis* *Escherichia coli* *Salmonella* Enteritidis *Salmonella* Typhimurium *Staphylococcus aureus*	ATCC	[49]
Melaleuca alternifolia *Origanum vulgare* *Pelargonium graveolens* *Eucaliptus globulus* *Cymbopogon citratus* *Citrus paradis* *Thymus vulgaris* *Cinnamomum cassia* *Citrus bergamia* *Cymbopogon winterianus* *Lippia sidoides* *Rosmarinus officinalis* *Syzygium aromaticum* *Mentha spicata* *Cinnamomum glaucescens* *Ocimum gratissimum* *Citrus limonum* *Citrus sinensis* *Citrus aurantifolia*	10 µL (pure)	*Salmonella* Typhimurium *Staphylococcus aureus*	ATCC	[33]
Zingiber officinale *Cymbopogon citratus* *Citrus aurantifolia*	1%	*Escherichia coli* *Staphylococcus aureus*	ATCC ATCC	[50]
Piper nigrum *Petroselinum crispum* *Ocimum basilicum*	10 mL (pure)	*Bacillus cereus* *Bacillus subtilis* *Escherichia coli* *Salmonella* Enteritidis *Salmonella* Typhimurium *Staphylococcus aureus*	ATCC	[51]
Allium sativum	500–100 mg/mL	*Escherichia coli* *Staphylococcus aureus*	ATCC	[52]
Ocimum basilicum	300–100 mg/mL	*Escherichia coli* *Staphylococcus aureus*	ATCC	[53]
Citrus aurantifolia	1%	*Escherichia coli* *Staphylococcus aureus*	ATCC	[54]
Rosmarinus officinalis	1%	*Escherichia coli* *Staphylococcus aureus*	ATCC ATCC	[55]
Litsea cubeba	13.29 mg/mL 1.33 mg/mL 13.29 mg/mL 1.77 mg/mL 13.29 mg/mL	*Candida albicans* *Candida guilliermondii* *Candida tropicalis* *Candida parapsilosis* *Candida krusei* *Saccharomyces cerevisiae*	Isolated	[44]

Table 2. *Cont.*

Essential Oil	MIC	SIM	SM	Reference
Origanum vulgare	1.89 mg/mL 0.95 mg/mL 3.79 mg/mL 1.89 mg/mL 4.73 mg/mL	*Candida albicans* *Candida guilliermondii* *Candida tropicalis* *Candida parapsilosis* *Candida krusei* *Saccharomyces cerevisiae*	Isolated	[44]
Origanum majorana	13.41 mg/mL	*Candida albicans* *Candida guilliermondii* *Candida tropicalis* *Candida parapsilosis* *Candida krusei* *Saccharomyces cerevisiae*	Isolated	[44]
Thymus vulgaris	14.05 mg/mL 0.94 mg/mL 14.05 mg/mL 1.87 mg/mL 1.41 mg/mL	*Candida albicans* *Candida guilliermondii* *Candida tropicalis* *Candida parapsilosis* *Candida krusei* *Saccharomyces cerevisiae*	Isolated	[44]
Cymbopogon winterianus *Clausena heptaphylla* *Cinnamomum tamala* *Ocimum sanctum*	50–500 µL/mL	*Aspergillus niger* *Aspergillus fumigatus* *Saccharomyces cerevisiae* *Candida albicans*	ATCC	[46]
Cinnamomum cassia *Melaleuca alternifolia* *Eucalyptus globulus* *Eugenia caryophyllus*	40 µL (pure)	*Candida albicans*	ATCC	[56]
Thyme	0.2%	*Aspergillus niger* *Aspergillus flavus* *Aspergillus fumigatus* *Candida albicans*	Isolated	[57]
Anise	0.5%	*Aspergillus niger* *Aspergillus flavus* *Aspergillus fumigatus* *Candida albicans*	Isolated	[57]
Cinnamon	0.1%	*Aspergillus niger* *Aspergillus flavus* *Aspergillus fumigatus* *Candida albicans*	Isolated	[57]

MIC, minimum inhibitory concentration; SIM, sensitive isolated microorganism; SM, source of the microorganism; ATCC, American type of culture collection; and CECT, Spanish type of culture collection.

5. Managing Poultry Farms with Essential Oils to Obtain Microbiologically Safe Hatching Eggs

5.1. Poultry House

Improving the relationship between poultry farms and the application of essential oils can mark substantial poultry production progress, as the antimicrobial efficacy of these oils can effectively align with management practices in poultry production sheds. An efficient sanitization plan for poultry sheds using essential oils must cover all structural and non-structural elements necessary to guarantee high-quality poultry production. Essential oils as sanitizers have proven efficiency in poultry sheds. An investigation into daily aerosol air sanitization in a poultry house during broiler farming revealed that sanitization for 60 min with a formulation containing different compounds, including 0.3% thyme, eucalyptus, and fir essential oils, in a dose of 50 mL/m^3 of the room reduced the bacterial load in the

air by 99%, without presenting toxicity to chickens [14]. In addition to broilers exposed to sanitization having a higher average body weight, than those not exposed, their blood tests indicated a significant increase in the amount of haemoglobin, lysozyme levels and bactericidal activity [14]. The nebulization of 0.5 mL of an aqueous solution of *Mentha piperita* or *Thymus vulgaris* essential oil at a concentration of 1:500 to 1:250 in poultry houses was proven effective in reducing the bacterial and fungal load in the air, drinkers, walls and/or litter [7,8]. Similarly, the combined application every three hours of *Pinus silvestris* and *Eucalyptus polybractea* essential oils at a concentration of 1:500 proved to be an efficient protocol for improving bacterial and fungal quality in the poultry air environment [16].

5.2. Poultry

Hatching eggs, subjected to an effective sanitization process, do not absolutely guarantee that the poultry resulting from hatching will be free of pathogens. Furthermore, even with inefficiently clean and sanitized poultry environments, this poultry can still be colonized by microorganisms present in the environment in which they live. As a result of this scenario, poultry constitutes a potential source of contamination for derived products. The main concern is centered on assessing the microbiological quality of poultry, aiming to ensure that it does not pose harmful risks to the final product or consumers. Given this need, it is recommended to subject poultry to antimicrobial therapies to guarantee both their microbiological quality and that of their final products within acceptable parameters. Studies have reported interesting results from antimicrobial treatments with essential oils via feed or water in poultry (Figure 1). Denli et al. [9] demonstrated that laying hen diets plus 150 mg/kg of *Origanum vulgare* essential oil reduced the contamination of total coliforms by 0.61 $\log_{10}$ CFU/mL and *Escherichia coli* by 1.09 $\log_{10}$ CFU/mL in eggshells. An antimicrobial treatment for layers via water-drinkers using cinnamaldehyde essential oil (diluted in a proportion of 1:8000 in drinking water) reduced the bacterial count in the cecum and eggshells [18]. Laying hens (89%) naturally infected with *Mycoplasma synoviae* (pathogen normally transmitted from breeding poultry to eggs) recovered after consuming diets supplemented with 100 mg/kg of *Melaleuca alternifolia* essential oil [17]. These authors reinforced the importance of these poultry eggs being free of conventional antibiotic residues [17]. Dietary supplementation with a blend of essential oils (containing 25% thymol and 25% carvacrol as active components, 37% silicon dioxide as caking inhibitor, and 13% glycerides as stabilizing agents; 120 mg/kg of feed) significantly reduced mortality associated with necrotic enteritis, inhibited the transport of Enterobacteriaceae in the liver and improved the intestinal integrity of broiler chickens [58]. On the other hand, diets containing 150 ppm of *Lippia origanoides* essential oil improved the feed conversion rate of layers [59].

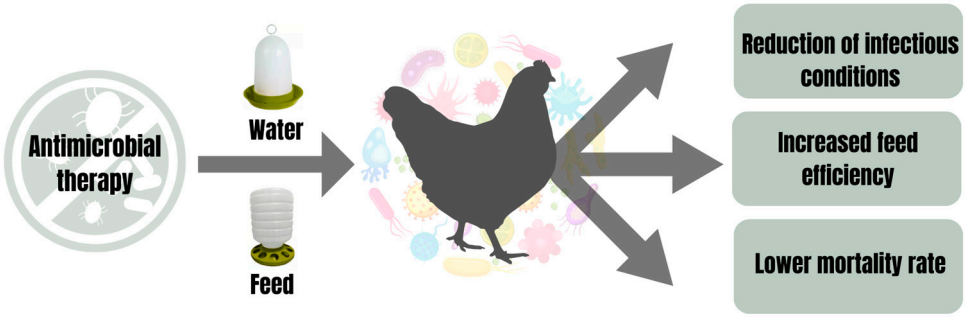

Figure 1. Benefits of antimicrobial therapy via water or feed in poultry.

The effects of supplying feed and water with essential oils on digestibility, feed consumption, feed conversion and, mainly, on maintaining the integrity of the intestinal health of poultry were also investigated. Barbarestani et al. [60] reported that providing feed supplemented with 600 mg of *Lavandula angustifolia* essential oil per kg of feed improved the growth performance of broilers. These improvements were mainly attributed to promoting

intestinal microbiota balance, improving intestinal structure, and increasing antioxidant capacity. Abdel-Wareth and Lohakare [61] observed that the inclusion of *Mentha piperita* essential oil in the diet of laying hens at different concentrations (0, 74, 148, 222 and 296 mg/kg of feed) resulted in notable improvements in the feed intake and feed conversion ratio. Furthermore, there was a linear increase in the digestibility of crude protein, ether extract and phosphorus. These findings were directly correlated with significant improvements in the poultry laying performance. Providing drinking water for broilers enriched with 0.4 mL/L of *Lavandula angustifolia* essential oil [62] or 400 mg/L of *Satureja khuzistanica* essential oil [63] resulted in significant improvements in performance indices, including feed conversion. This improvement was also observed when laying hens received drinking water containing 0.2 to 0.3 mL/L of a mixture of essential oils from *Origanum vulgare*, *Mentha piperita* and *Pimpinella anisum* [64]. Diet supplemented with 15 mg/kg of e *Origanum vulgare* essential oil plus 2.4 g/kg of attapulgite demonstrated significant benefits on the height of ileal villi and the composition of the intestinal microbiota of broilers [65].

5.3. Hatching Eggs

Sanitizing eggs for hatching is also a poultry standard to ensure eggs have fewer pathogens. A bibliographical survey by Oliveira et al. [6] reported that the sanitization of hatching eggs proved viable to reduce the microbial load of the eggshell in 85–86% of protocols carried out at the experimental level. Sanitization offers an immediate reduction in the microbial load of the shell and internal contents of the eggs, lower chances of recontaminated eggs, a better hatchability rate, microbiologically safer embryos and chicks, and healthier and more viable poultry [12,13,66–70]. However, in some cases, sanitization did not reduce microbial contamination of the eggshell and/or caused complications such as malformations and failure to hatch [71–74]. Most of these complications require corrections in the sanitization protocol, as they may be due to poor application and the level of toxicity of the sanitizers.

Plants naturally provide many of the active ingredients for preparing sanitizers. Some of the sanitizers currently available and tested for a commercial application are based on essential oils produced by plants. In poultry farming, microbiological tests are progressively carried out to evaluate the viability of essential oils in sanitizing hatching eggs [12,15,75]. This intensifies practices that use ecological principles for antimicrobial protection in the poultry sector and de-intensifies environmental externalities caused by environmentally harmful practices. Many trees, including *Citrus aurantifolia*, *Ocimum basilicum*, and *Allium sativum*, harbor essential oils of interest to global research centers thanks to dedicated researchers who consistently share experimental results that advance the characterization of these essential oils [11,76–78]. Because they are (1) active against bacteria and fungi, (2) safe for humans and animals (dose-dependent), (3) sourced from readily available plants, and they have (4) positive cost–benefit ratio and (5) application versatility, essential oils need to be continually validated to redefine poultry farming, seeking to move it away from its conventional approach and cultivate an image deeply rooted in sustainability, where natural and ecologically responsible practices are the main guide.

Antimicrobial therapy on poultry farms with essential oils requires a comprehensive approach. In addition to focusing on microbial control of the air, physical structure, materials, and poultry, it is necessary to integrate the sanitization of hatching eggs. This is a therapeutic complement to the cleaning activities that must be included in the management plan of poultry farms, aiming, through methods such as spraying, to reinforce the natural antimicrobial barrier of eggshells (Figure 2) [79]. This therapy may involve the use of essential oils to obtain a series of benefits that favor poultry production within appropriate microbiological quality standards (Figure 2) [15]. Mustafa et al. [80] observed that spraying *Lavandula angustifolia* essential oil significantly reduced the total count of aerobic bacteria on the eggshell surface of hatching eggs by 1.42 $\log_{10}$. Before the eggs hatched, this reduction was still significantly 0.52 $\log_{10}$ [80]. Likewise, Oliveira et al. [12] highlighted that after 1 h of spraying on hatching eggs, *Syzygium aromaticum* essential oil (0.39%) significantly re-

duced the total count of aerobic mesophilic bacteria and Enterobacteriaceae in eggshells by 1.19 $\log_{10}$. In addition to essential oils (1%) demonstrating the ability to reduce the bacterial load in eggshells after collection, a significant fungal reduction of 0.55 $\log_{10}$ and 0.45 $\log_{10}$ was also evidenced after immersing the eggs for 10 s in the essential oil of *Cymbopogon flexuosus* and *Lippia rotundifolia*, respectively [81].

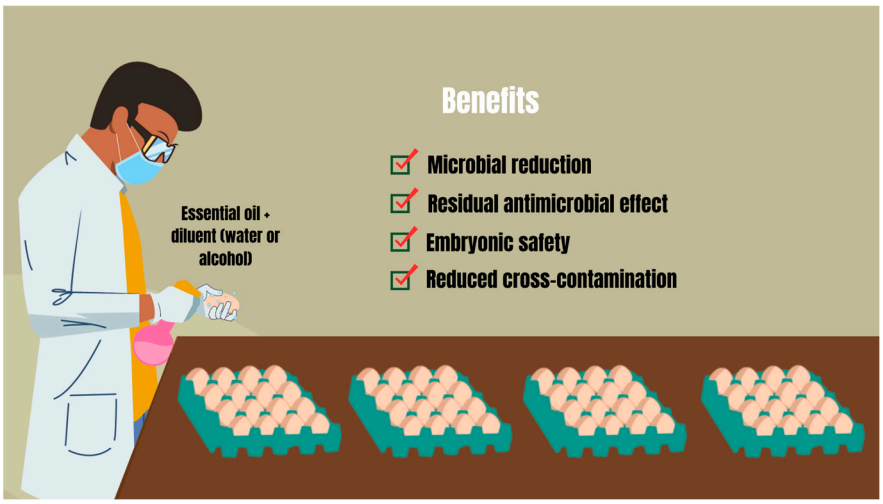

Figure 2. Spraying hatching eggs with essential oils and some of their benefits for poultry production. Source: Adapted from Oliveira et al. [6].

Eggs subjected to sanitization with essential oils of *Citrus aurantifolia*, *Ocimum basilicum* and *Allium sativum* demonstrated significantly lower mean counts for total aerobic mesophilic bacteria (2.41 $\log_{10}$ CFU/mL) and Enterobacteriaceae (0.34 $\log_{10}$ CFU/mL) compared to non-sanitized eggs (5.12 ± 0.10 and 3.25 ± 0.75 $\log_{10}$ CFU/mL, respectively) (Table 3). The sanitizer based on *Allium sativum* essential oil demonstrated the greatest efficiency in reducing the bacterial load of the eggshell, resulting in a significant reduction of 3.25 log for total aerobic mesophilic bacteria and Enterobacteriaceae (Table 3). The three essential oils are comparable to formaldehyde (Table 3; unpublished data). Therefore, the essential oils used to date to sanitize eggs meet the recommendations of previous studies to balance microbiological efficiency with environmental responsibility and health preservation, as they are biodegradable, healthy, available, and efficient antimicrobial materials without serious impacts on the environment.

Table 3. The bacterial count of eggshells sanitized with *Citrus aurantifolia*, *Ocimum basilicum* and *Allium sativum* [1] essential oils *.

Treatments	Concentration Sanitizer	Application Method	Number of Eggs	TAMB ($\log_{10}$ CFU/mL)	Enterobacteriaceae ($\log_{10}$ CFU/mL)
Non-sanitized eggs	-	Spraying	12	5.12 ± 0.10 [a]	3.25 ± 0.75 [a]
Grain alcohol	93.8%	Spraying	12	4.51 ± 0.33 [a]	3.00 ± 0.37 [ab]
Formaldehyde	1.5%	Spraying	12	2.39 ± 0.49 [bc]	1.37 ± 1.19 [bc]
Citrus aurantifolia	1% [2]	Spraying	12	2.28 ± 0.50 [bc]	0.00 ± 0.00 [c]
Ocimum basilicum	1% [2]	Spraying	12	3.09 ± 0.23 [b]	1.02 ± 0.89 [c]
Allium sativum	1% [2]	Spraying	12	1.87 ± 0.54 [c]	0.00 ± 0.00 [c]
p value	-	-	-	<0.0001	<0.0001

[a–c] Different letters in the same column indicate significant differences among means ($p < 0.05$). [1] Bacterial counting using the eggshell washing method. [2] Oils at a concentration of 300 mg/mL of DMSO were used. Abbreviation: TAMB, total aerobic mesophilic bacteria. * unpublished data.

The hatchability rates of an incubation cycle may be associated with the toxicity and antimicrobial profile of the compounds used to sanitize hatching eggs [12,82]. Oliveira

et al. [12] reported that the greatest hatchability success was observed in eggs sprayed with *Syzygium aromaticum* essential oil at 0.6 mg/mL (92.37 ± 3.25%) and paraformaldehyde (94.44 ± 4.54%), which were statistically similar. However, the grain alcohol treatment resulted in a lower hatching success (85.00 ± 2.20%) compared to the paraformaldehyde treatment while the propolis treatment resulted in an approximate 43–48% lower hatchability than the other treatments. It was demonstrated that day-old chicks from eggs sprayed with 0.39% *Syzygium aromaticum* essential oil did not exhibit morphological changes in their tissues [15]. The authors suggested that this indicates no or negligible topical toxicity of *Syzygium aromaticum* essential oil to ensure the hatching of healthy chicks [15]. Bekhet and Khalifa [82] showed that immersing eggs in a solution of 0.5% *Origanum vulgare* or *Cuminum cyminum* essential oil showed a better hatchability rate (96.21 ± 0.56% and 95.76 ± 0.94%, respectively) than eggs sanitized with alcohol (88.66 ± 1.54%), formaldehyde (82.05 ± 0.56%) and non-sanitized eggs (84.06 ± 1.54%). However, due to their oily nature, the use of essential oils in high concentrations can be disadvantageous [13], as this can result in the formation of an artificial layer that occludes the pores and potentially affects gas exchange of embryos until hatching, leading to reduced hatchability rates [83]. This argument is supported by results from several studies on table egg coatings that have proven the efficient contribution of essential oils in minimizing water and gas loss from eggs [52–56,84]. No negative effects were reported on the timing or hatch window of chicks from eggs sanitized with 0.39% *Syzygium aromaticum* essential oil, 0.2–0.4% *Cuminum cyminum*, or 0.2–0.4% *Origanum vulgare* [69,85].

6. Conclusions

In summary, the antimicrobial effects of essential oils bring significant benefits to poultry farming, contributing to the reduction of pathogen load in poultry houses and promoting positive effects on digestibility and feed consumption, improving feed conversion and the health of the poultry intestinal tract. Additionally, they help reduce the microbiota on eggshells and improve hatchability rates. It is important to highlight that essential oils are an antimicrobial treatment option that has been accepted for administration in ovo. This in ovo delivery device is a carefully researched topic in poultry farming mainly to overcome challenges of post-hatch poultry vaccination, to improve poultry production efficiency and to protect or treat poultry from pathogenic microbial infections [86,87]. Therefore, future studies also need to focus especially on the use of essential oils to prevent the growth of pathogenic bacteria in the embryonic development microenvironment and their effects on productivity. Implementing interconnected therapies using essential oils via feed, drinking water and sanitation (depending on the production stage) can be an effective strategy to combat primary and secondary contamination on poultry farms, generating synergistic effects and optimizing the results of systemic treatment. This approach needs to involve the integrated application of several therapies with essential oils, from entry into the farm until the transport of eggs to the hatcheries, contributing to maintaining an environment with safe microbiological levels throughout the process. Ensuring the availability of microbiologically safe eggs for the hatchery represents the first step to generating healthy chicks destined for farms. It is proposed to use essential oils as a microbial control agent in the poultry sector, suggesting their integrated application as follows: sanitization of poultry sheds with *Thymus vulgaris* essential oil in a proportion of 1:500–1:250 and incorporation of 150 mg/kg of *Origanum vulgare* in poultry feed. After laying, sanitize the hatching eggs with *Syzygium aromaticum* essential oil 0.39%.

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References

1. Schäfer, D.F.; Steffens, J.; Barbosa, J.; Zeni, J.; Paroul, N.; Valduga, E.; Junges, A.; Backes, G.T.; Cansian, R.L. Monitoring of contamination sources of *Listeria monocytogenes* in a poultry slaughterhouse. *LWT Food Sci. Technol.* **2017**, *86*, 393–398. [CrossRef]
2. Cox, N.A.; Richardson, L.J.; Maurer, J.-J.; Berrang, M.E.; Fedorka-Cray, P.J.; Buhr, R.J.; Byrd, J.A.; Lee, M.D.; Hofacre, C.L.; O'Kane, P.M. Evidence for Horizontal and Vertical Transmission in Campylobacter Passage from Hen to Her Progeny. *J. Food Prot.* **2012**, *75*, 1896–1902. [CrossRef] [PubMed]
3. Pande, V.V.; Devon, R.L.; Sharma, P.; McWhorter, A.R.; Chousalkar, K.K. Study of *Salmonella* Typhimurium infection in laying hens. *Front. Microbiol.* **2016**, *7*, 203. [CrossRef] [PubMed]
4. Castañeda-Gulla, K.; Sattlegger, E.; Mutukumira, A.N. Persistent contamination of *Salmonella*, *Campylobacter*, *Escherichia coli*, and *Staphylococcus aureus* at a broiler farm in New Zealand. *Can. J. Microbiol.* **2020**, *66*, 171–185. [CrossRef] [PubMed]
5. Ezemba, C.C.; Obi, C.P.; Eleanya, L.C.; Udoye, I.; Ezemba, A.S.; Osuala, O.J.; Archibong, E.J. The incidence of *Salmonella* and *E. coli* in poultry feeds. *J. Curr. Biomed. Res.* **2021**, *1*, 45–56.
6. Oliveira, G.d.S.; McManus, C.; Salgado, C.B.; dos Santos, V.M. Effects of Sanitizers on Microbiological Control of Hatching Eggshells and Poultry Health during Embryogenesis and Early Stages after Hatching in the Last Decade. *Animals* **2022**, *12*, 2826. [CrossRef] [PubMed]
7. Witkowska, D.; Sowińska, J. The effectiveness of peppermint and thyme essential oil mist in reducing bacterial contamination in broiler houses. *Poult. Sci.* **2013**, *92*, 2834–2843. [CrossRef]
8. Witkowska, D.; Sowińska, J.; Żebrowska, J.; Mituniewicz, E. The antifungal properties of peppermint and thyme essential oils misted in broiler houses. *Braz. J. Poult. Sci.* **2016**, *18*, 629–638. [CrossRef]
9. Denli, M.; Vural, A.; Alp, S.Y. The Influence of Oregano Essential Oil on Egg Quality and Egg Shell Contamination of Laying Hens Kept in Furnished Cages. *Sci. Pap. Ser. D Anim. Sci.* **2019**, *62*, 48–52.
10. Toghyani, P.; Shahzamani, S.; Gholami Ahangaran, M.; Ali Mousavi Firouzabadi, S. Comparison of *Eucalyptus* Extract and Formaldehyde on Hatchability and Survival Rate of Chicks in Disinfection of Fertile Eggs. *Int. J. Pharm. Res. Allied Sci.* **2020**, *9*, 105–109.
11. Oliveira, G.D.S.; McManus, C.; Sousa, H.A.D.F.; Santos, P.H.G.D.S.; dos Santos, V.M. A Mini-Review of the Main Effects of Essential Oils from *Citrus aurantifolia*, *Ocimum basilicum*, and *Allium sativum* as Safe Antimicrobial Activity in Poultry. *Animals* **2024**, *14*, 382. [CrossRef]
12. Oliveira, G.D.S.; Nascimento, S.T.; dos Santos, V.M.; Silva, M.G. Clove Essential Oil in the Sanitation of Fertile Eggs. *Poult. Sci.* **2020**, *99*, 5509–5516. [CrossRef]
13. Oliveira, G.d.S.; dos Santos, V.M.; Nascimento, S.T. Essential Oils as Sanitisers for Hatching Eggs. *Worlds Poult. Sci. J.* **2021**, *77*, 605–617. [CrossRef]
14. Ponomarenko, G.V.; Kovalenko, V.L.; Balatskiy, Y.O.; Ponomarenko, O.V.; Paliy, A.P.; Shulyak, S.V. Bactericidal efficiency of preparation based on essential oils used in aerosol disinfection in the presence of poultry. *Regul. Mech. Biosyst.* **2021**, *12*, 635–641. [CrossRef]
15. Oliveira, G.D.S.; McManus, C.; de Araújo, M.V.; de Sousa, D.E.R.; de Macêdo, I.L.; de Castro, M.B.; dos Santos, V.M. Sanitizing hatching eggs with essential oils: Avian and microbiological safety. *Microorganisms* **2023**, *11*, 1890. [CrossRef]
16. Bakutis, B.; Baliukoniene, V.; Mickiene, R. The use of essential oils to improve of environment quality in poultry houses. In *Animal Hygiene and Sustainable Livestock Production, Proceedings of the XVth International Congress of the International Society for Animal Hygiene, Vienna, Austria, 3–7 July 2011*; Tribun EU: Brno, Czech Republic, 2011; Volume 2, pp. 643–645.
17. Puvača, N.; Lika, E.; Tufarelli, V.; Bursić, V.; Pelić, D.L.; Nikolova, N.; Petrović, A.; Prodanović, R.; Vuković, G.; Lević, J.; et al. Influence of Different Tetracycline Antimicrobial Therapy of Mycoplasma (*Mycoplasma synoviae*) in Laying Hens Compared to Tea Tree Essential Oil on Table Egg Quality and Antibiotic Residues. *Foods* **2020**, *9*, 612. [CrossRef]
18. Patil, V.; Hedau, M.; Kaore, M.; Badar, S.; Kadam, M.; Chaudhari, S.; Rawool, D.; Barbuddhe, S.; Vergis, J.; Kurkure, N. Potential of cinnamaldehyde essential oil as a possible antimicrobial against fowl typhoid in layers. *Trop. Anim. Health Prod.* **2023**, *55*, 126. [CrossRef] [PubMed]

19. Ibrahim, A.N.; Khalefa, H.S.; Aboul-Ella, H.; Mubarak, S.T. Efficacy of disinfection on airborne and waterborne fungal load in broiler chicken houses. *J. Anim. Health Prod.* **2021**, *9*, 296–302. [CrossRef]
20. Kemmett, K.; Williams, N.J.; Chaloner, G.; Humphrey, S.; Wigley, P.; Humphrey, T. The contribution of systemic *Escherichia coli* infection to the early mortalities of commercial broiler chickens. *Avian Pathol.* **2014**, *43*, 37–42. [CrossRef] [PubMed]
21. Muna, E.A.; Salih, M.H.; Zakia, A.M.; Halima, M.O.; Abeer, A.M.; Ameera, M.M.; Ali, H.O.; Idris, S.B. Pathology of Broiler Chicks Naturally Infected with *Salmonella enteritidis* (*S. Enteritidis*) & *Salmonella typhimurium* (*S. Typhimurium*) during an Outbreak in Sudan. *J. Sci. Res. Rep.* **2016**, *10*, 1–8. [CrossRef]
22. Hamid, F.F.A.; Reduan, M.F.H.; Jasni, S.; Chung, E.L.T.; Nordin, M.L.B.; Jesse, F.F.A.; Rajdi, N.I.Z.M.; Kamaruzaman, I.N.A.B.; Shaharulnizim, N. Aspergillosis concurrent with secondary bacterial infection in broiler chicks: A case report. *Comp. Clin. Path.* **2021**, *30*, 341–345. [CrossRef]
23. Oliveira, G.d.S.; dos Santos, V.M.; McManus, C. Propolis: Effects on the Sanitisation of Hatching Eggs. *Worlds Poult. Sci. J.* **2022**, *78*, 261–272. [CrossRef]
24. Kabir, S.M.L. Avian colibacillosis and salmonellosis: A closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *Int. J. Environ. Res. Public Health* **2010**, *7*, 89–114. [CrossRef]
25. Kalita, N.; Pathak, N.; Saikia, G.; Ahmed, M. Prevalence and pathology of dead-in-shell embryos of Vanaraja egg. *Indian J.* **2013**, *37*, 104–105.
26. Hananeh, W.M.; Al-Natour, M.Q.; Alaboudi, A.R.; Abo-Shehada, M.N.; Ismail, Z.A.B. Congenital abnormalities in dead-in-shell chicks associated with mixed bacterial infections. *Heliyon* **2021**, *7*, e06272. [CrossRef] [PubMed]
27. Ozaki, H.; Murase, K.Y. Virulence of *Escherichia coli* Isolates Obtained from Layer Chickens with Colibacillosis Associated with Pericarditis, Perihepatitis, and Salpingitis in Experimentally Infected Chicks and Embryonated Eggs. *Avian Dis.* **2018**, *62*, 233–236. [CrossRef] [PubMed]
28. Stratakos, A.C.; Koidis, A. Methods for extracting essential oils. In *Essential Oils in Food Preservation, Flavor and Safety*; Elsevier: Amsterdam, The Netherlands, 2016; pp. 31–38. [CrossRef]
29. Jiang, Y.; Wu, N.; Fu, Y.J.; Wang, W.; Luo, M.; Zhao, C.J.; Zu, Y.G.; Liu, X.L. Chemical composition and antimicrobial activity of the essential oil of Rosemary. *Environ. Toxicol. Pharmacol.* **2011**, *32*, 63–68. [CrossRef] [PubMed]
30. Reichling, J.; Suschke, U.; Schneele, J.; Geiss, H.K. Antibacterial activity and irritation potential of selected essential oil components—Structure-activity relationship. *Nat. Prod. Commun.* **2006**, *1*, 1003–1012. [CrossRef]
31. El-Baroty, G.S.; El-Baky, H.H.A.; Farag, R.S.; Saleh, M.A. Characterization of Antioxidant and Antimicrobial Compounds of Cinnamon and Ginger Essential Oils. *Afr. J. Biochem. Res.* **2010**, *6*, 167–174.
32. Silveira, S.M.; Cunha, A., Jr.; Scheuermann, G.N.; Secchi, F.L.; Verruck, S.K.M. Composição química e atividade antibacteriana dos óleos essenciais de *Cymbopogon winterianus* (citronela), *Eucalyptus paniculata* (eucalipto) e *Lavandula angustifolia* (lavanda) [Chemical composition and antibacterial activity of essential oils from Cymbopogon winterianus (citronella), *Eucalyptus paniculata* (eucalyptus) and *Lavandula angustifolia* (lavender)]. *Rev. Inst. Adolfo Lutz* **2012**, *71*, 471–480.
33. Penteado, A.L.; Eschionato, R.A.; de Souza, D.R.C.; Queiroz, S.D.N. Avaliação in vitro de atividade antimicrobiana de óleos essenciais contra *Salmonella* Typhimurium e *Staphylococcus aureus* [In Vitro Evaluation Of Antimicrobial Activity Of Essential Oils Against *Salmonella* Typhimurium and *Staphylococcus aureus*]. *Hig. Aliment.* **2021**, *35*, e1060. [CrossRef]
34. Parreira, D.S.; Alcántara-de la Cruz, R.; Leite, G.L.D.; Ramalho, F.D.S.; Zanuncio, J.C.; Serrão, J.E. Quantifying the harmful potential of ten essential oils on immature *Trichogramma pretiosum* stages. *Chemosphere* **2018**, *199*, 670–675. [CrossRef] [PubMed]
35. Jugreet, B.S.; Mahomoodally, M.F. Essential oils from 9 exotic and endemic medicinal plants from Mauritius shows in vitro antibacterial and antibiotic potentiating activities. *S. Afr. J. Bot.* **2020**, *132*, 355–362. [CrossRef]
36. Reis, J.B.; Figueiredo, L.A.; Castorani, G.M.; Veiga, S.M.O.M. Avaliação Da Atividade Antimicrobiana Dos Óleos Essenciais Contra Patógenos Alimentares [Evaluation of antimicrobial activity of essential oils against food pathogens]. *Braz. J. Health Rev.* **2020**, *3*, 342–363. [CrossRef]
37. Ribeiro, S.O.; Fontaine, V.; Mathieu, V.; Zhiri, A.; Baudoux, D.; Stévigny, C.; Souard, F. Antibacterial and cytotoxic activities of ten commercially available essential Oils. *Antibiotics* **2020**, *9*, 717. [CrossRef]
38. Hayatgheib, N.; Fournel, C.; Calvez, S.; Pouliquen, H.; Moreau, E. In vitro antimicrobial effect of various commercial essential oils and their chemical constituents on *Aeromonas salmonicida* subsp. *salmonicida*. *J. Appl. Microbiol.* **2020**, *129*, 137–145. [CrossRef]
39. D'Agostino, M.; Tesse, N.; Frippiat, J.P.; Machouart, M.; Debourgogne, A. Essential Oils and Their Natural Active Compounds Presenting Antifungal Properties. *Molecules* **2019**, *24*, 3713. [CrossRef]
40. Álvarez-Martínez, F.J.; Barrajón-Catalán, E.; Herranz-López, M.; Micol, V. Antibacterial plant compounds, extracts and essential oils: An updated review on their effects and putative mechanisms of action. *Phytomedicine* **2021**, *90*, 153626. [CrossRef]
41. Galgano, M.; Capozza, P.; Pellegrini, F.; Cordisco, M.; Sposato, A.; Sblano, S.; Camero, M.; Lanave, G.; Fracchiolla, G.; Corrente, M.; et al. Antimicrobial Activity of Essential Oils Evaluated In Vitro against *Escherichia coli* and *Staphylococcus aureus*. *Antibiotics* **2022**, *11*, 979. [CrossRef]
42. Boukhatem, M.N.; Boumaiza, A.; Nada, H.G.; Rajabi, M. *Eucalyptus globulus* essential oil as a natural food preservative: Antioxidant, antibacterial and antifungal properties in vitro and in a real food matrix (orangina fruit juice). *Appl. Sci.* **2020**, *10*, 5581. [CrossRef]

43. Souza, D.S.; Almeida, A.C.; Andrade, V.A.; Marcelo, N.A.; Azevedo, I.L.; Martins, E.R.; Figueiredo, L.S. Antimicrobial Activity of *Lippia origanoides* and *Lippia rotundifolia* Oils against Enterobacteria Isolated from Poultry. *Arq. Bras. Med. Vet. Zootec.* **2015**, *67*, 940–944. [CrossRef]
44. Ebani, V.V.; Nardoni, S.; Bertelloni, F.; Giovanelli, S.; Rocchigiani, G.; Pistelli, L.; Mancianti, F. Antibacterial and antifungal activity of essential oils against some pathogenic bacteria and yeasts shed from poultry. *Flav. Frag. J.* **2016**, *31*, 302–309. [CrossRef]
45. Ebani, V.V.; Najar, B.; Bertelloni, F.; Pistelli, L.; Mancianti, F.; Nardoni, S. Chemical Composition and In Vitro Antimicrobial Efficacy of Sixteen Essential Oils against *Escherichia coli* and *Aspergillus fumigatus* Isolated from Poultry. *Vet. Sci.* **2018**, *5*, 62. [CrossRef]
46. Munda, S.; Dutta, S.; Pandey, S.K.; Sarma, N.; Lal, M. Antimicrobial Activity of Essential Oils of Medicinal and Aromatic Plants of the North East India: A Biodiversity Hot Spot. *J. Essent. Oil-Bear. Plants* **2019**, *22*, 105–119. [CrossRef]
47. Valdivieso-Ugarte, M.; Plaza-Diaz, J.; Gomez-Llorente, C.; Lucas Gómez, E.; Sabés Alsina, M.; Gil, Á. In vitro examination of antibacterial and immunomodulatory activities of cinnamon, white thyme, and clove essential oils. *J. Funct. Foods* **2021**, *81*, 104436. [CrossRef]
48. Seyedtaghiya, M.H.; Fasaei, B.N.; Peighambari, S.M. Antimicrobial and Antibiofilm Effects of *Satureja hortensis* Essential Oil against *Escherichia coli* and *Salmonella* Isolated from Poultry. *Iran. J. Microbiol.* **2021**, *13*, 74–80. [CrossRef] [PubMed]
49. Fazio, M.L.S.; Bazan, J.R.; Geromel, M.R. Ação De Óleos Essenciais Cítricos Sobre Algumas Bactérias [Action of Citrus Essential Oils on Some Bacteria]. *Rev. InterCiência-IMES Catanduva* **2020**, *1*, 11–16.
50. Oliveira, G.D.S.; McManus, C.; Pires, P.G.D.S.; dos Santos, V.M. Combination of Cassava Starch Biopolymer and Essential Oils for Coating Table Eggs. *Front. Sustain. Food Syst.* **2022**, *6*, 957229. [CrossRef]
51. Campos, R.C.; Peres, C.D.; de Almeida, V.S.; Virgolin, L.B.; Geromel, M.R.; Fazio, M.L.S. Ação Antimicrobiana De Óleos Essenciais De Pimenta Preta, Salsa E Manjericão Doce [Antimicrobial Action of Essential Oils from Black Pepper, Parsley and Sweet Basil]. In *Segurança Alimentar e Nutricional [Food and Nutrition Security]*, 2nd ed.; Treptow, T.C., Ed.; Atena: Ponta Grossa, Brazil, 2022; pp. 167–172. [CrossRef]
52. Vale, I.R.R.; Oliveira, G.d.S.; McManus, C.; de Araújo, M.V.; Salgado, C.B.; Pires, P.G.d.S.; de Campos, T.A.; Gonçalves, L.F.; Almeida, A.P.C.; Martins, G.D.S.; et al. Whey Protein Isolate and Garlic Essential Oil as an Antimicrobial Coating to Preserve the Internal Quality of Quail Eggs. *Coatings* **2023**, *13*, 1369. [CrossRef]
53. de Araújo, M.V.; Oliveira, G.d.S.; McManus, C.; Vale, I.R.R.; Salgado, C.B.; Pires, P.G.d.S.; de Campos, T.A.; Gonçalves, L.F.; Ameida, A.P.C.; Martins, G.d.S.; et al. Preserving the Internal Quality of Quail Eggs Using a Corn Starch-Based Coating Combined with Basil Essential Oil. *Processes* **2023**, *11*, 1612. [CrossRef]
54. Oliveira, G.d.S.; McManus, C.; Salgado, C.B.; Pires, P.G.d.S.; de Figueiredo Sousa, H.A.; da Silva, E.R.; dos Santos, V.M. Antimicrobial Coating Based on Tahiti Lemon Essential Oil and Green Banana Flour to Preserve the Internal Quality of Quail Eggs. *Animals* **2023**, *13*, 2123. [CrossRef] [PubMed]
55. Oliveira, G.d.S.; McManus, C.; Salgado, C.B.; Pires, P.G.d.S.; dos Santos, V.M. Rice flour coating supplemented with rosemary essential oil to preserve the internal, microbiological, and sensory quality of quail eggs. *Acta Aliment.* **2023**, *52*, 294–304. [CrossRef]
56. Oliveira, A.F.M.; da Silva, F.L.; da Silva, R.T.; Morais, F.M.; dos Santos, R.R.L.; da Silva, L.L.W.V.; Morais, C.C. Atividade antifúngica de óleos essenciais frente a cepa de *Candida albicans* [Antifungal activity of essential oils against Candida albicans strain]. *Res. Soc. Dev.* **2022**, *11*, e04111435696. [CrossRef]
57. Abed, A.H.; Radwan, I.A.; El-Aziz, M.M.A.; Ali, A. Antifungal activity of natural essential oils against molds and yeasts associated with respiratory problems in broiler chickens. *Adv. Anim. Vet. Sci.* **2021**, *9*, 348–355. [CrossRef]
58. Du, E.; Guo, Y. Dietary Supplementation of Essential Oils and Lysozyme Reduces Mortality and Improves Intestinal Integrity of Broiler Chickens with Necrotic Enteritis. *Anim. Sci. J.* **2021**, *92*, e13499. [CrossRef]
59. Ramirez, S.Y.; Peñuela-Sierra, L.M.; Ospina, M.A. Effects of oregano (*Lippia origanoides*) essential oil supplementation on the performance, egg quality, and intestinal morphometry of Isa Brown laying hens. *Vet. World* **2021**, *14*, 595–602. [CrossRef]
60. Yarmohammadi Barbarestani, S.; Jazi, V.; Mohebodini, H.; Ashayerizadeh, A.; Shabani, A.; Toghyani, M. Effects of dietary lavender essential oil on growth performance, intestinal function, and antioxidant status of broiler chickens. *Livest. Sci.* **2020**, *233*, 103958. [CrossRef]
61. Abdel-Wareth, A.A.A.; Lohakare, J.D. Productive performance, egg quality, nutrients digestibility, and physiological response of bovans brown hens fed various dietary inclusion levels of peppermint oil. *Anim. Feed Sci. Technol.* **2020**, *267*, 114554. [CrossRef]
62. Adaszyńska-Skwirzyńska, M.; Szczerbińska, D.; Zych, S. The use of lavender (*Lavandula angustifolia*) essential oil as an additive to drinking water for broiler chickens and its in vitro reaction with enrofloxacin. *Animals* **2021**, *11*, 1535. [CrossRef]
63. Khosravinia, H. Mortality, production performance, water intake and organ weight of the heat stressed broiler chicken given savory (*Satureja khuzistanica*) essential oils through drinking water. *J. Appl. Anim. Res.* **2016**, *44*, 273–280. [CrossRef]
64. Karadagoglu, O.; Ozsoy, B.; Olmez, M.; Aydin, O.D.; Sahin, T. The Effects of Drinking Water Supplemented with Essential Oils on Performance, Egg Quality and Egg Yolk Fatty Acid Composition in Laying Hens. *Acta Vet. Eurasia* **2018**, *44*, 85–92.
65. Skoufos, I.; Tzora, A.; Giannenas, I.; Tontis, D.; Bartzanas, T.; Kittas, C.; Panagakis, P. Effects of oregano essential oil and attapulgite on growth performance, intestinal microbiota and morphometry in broilers. *S. Afr. J. Anim. Sci.* **2016**, *46*, 77–88. [CrossRef]
66. Shafey, T.M.; Hussein, E.O.S.; Al-Batshan, H.A. Effects of Ultrasonic Waves on Eggshell Strength and Hatchability of Layer-Type Breeder Eggs. *S. Afr. J. Anim. Sci.* **2013**, *43*, 56–63. [CrossRef]

67. Shahein, E.H.A.; Sedeek, E.K. Role of Spraying Hatching Eggs with Natural Disinfectants on Hatching Characteristics and Eggshell Bacterial Counts. *Egypt. Poult. Sci. J.* **2014**, *34*, 213–230. [CrossRef]
68. Cantu, K.; Archer, G.S.; Tucker, Z.S.; Coufal, C.D. Effectiveness of Duck Hatching Egg Sanitization with the Combination of Hydrogen Peroxide and Ultraviolet Light. *J. Appl. Poult. Res.* **2019**, *28*, 301–306. [CrossRef]
69. Oliveira, G.S.; Nascimento, S.T.; Dos Santos, V.M.; Dallago, B.S.L. Spraying Hatching Eggs with Clove Essential Oil Does Not Compromise the Quality of Embryos and One-Day-Old Chicks or Broiler Performance. *Animals* **2021**, *11*, 2045. [CrossRef] [PubMed]
70. Soares, A.C.B.; Brito, D.A.P.; Soares, S.C.P.; Gomes, K.S.; Saldanha, G.K.M.S.; Soares, V.S. Maintenance of quality of eggs submitted to treatment with propolis extract and sanitizers. *Acta Sci. Anim. Sci.* **2022**, *44*, e53584. [CrossRef]
71. Hrnčár, C.; Prachárová, S.; Bujko, J. The Effect of Disinfection of Hatching Eggs on Hatchability of Oravka Chickens. *Sci. Pap. Anim. Sci. Biotechnol.* **2012**, *45*, 411–414.
72. Zeweil, H.S.; Rizk, R.E.; Bekhet, G.M.; Ahmed, M.R. Comparing the Effectiveness of Egg Disinfectants against Bacteria and Mitotic Indices of Developing Chick Embryos. *J. Basic Appl. Zool.* **2015**, *70*, 1–15. [CrossRef]
73. Wlazlo, L.; Drabik, K.; Al-Shammari, K.I.A.; Batkowska, J.; Nowakowicz-Debek, B.; Gryzińska, M. Use of Reactive Oxygen Species (Ozone, Hydrogen Peroxide) for Disinfection of Hatching Eggs. *Poult. Sci.* **2020**, *99*, 2478–2484. [CrossRef]
74. Oliveira, G.S.; Santos, V.M.; Nascimento, S.T.; Rodrigues, J.C. Alternative sanitizers to paraformaldehyde for incubation of fertile eggs. *Poult. Sci.* **2020**, *99*, 2001–2006. [CrossRef]
75. Yildirim, I.; Ozsan, M.; Yetisir, R. The Use of Oregano (*Origanum vulgare* L.) Essential Oil as Alternative Hatching Egg Disinfectant versus Formaldehyde Fumigation in Quails (*Coturnix coturnix japonica*) Eggs. *Rev. Med. Vet.* **2003**, *154*, 367–370.
76. Poonkodi, K. Chemical composition of essential oil of *Ocimum basilicum* L. (Basil) and its biological activities-an overview. *J. Crit. Rev.* **2016**, *3*, 56–62.
77. Jain, S.; Arora, P.; Popli, H. A comprehensive review on *Citrus aurantifolia* essential oil: Its phytochemistry and pharmacological aspects. *Braz. J. Nat. Sci.* **2020**, *3*, 354. [CrossRef]
78. Ezeorba, T.P.C.; Chukwudozie, K.I.; Ezema, C.A.; Anaduaka, E.G.; Nweze, E.J.; Okeke, E.S. Potentials for health and therapeutic benefits of garlic essential oils: Recent findings and future prospects. *Pharmacol. Res. Mod. Chin. Med.* **2022**, *3*, 100075. [CrossRef]
79. Oliveira, G.d.S.; McManus, C.; dos Santos, V.M. Garlic as Active Principle of Sanitiser for Hatching Eggs. *Worlds Poult. Sci. J.* **2022**, *78*, 1037–1052. [CrossRef]
80. Mustafa, A.A.; Mirza, R.A.; Aziz, H.I. Lavender Essential Oil in Sanitation on Fertile Egg. *Passer J. Basic Appl. Sci.* **2023**, *5*, 377–381. [CrossRef]
81. Nogueira, W.C.L.; Pena, A.C.S.; de Souza, C.N.; Azevedo, I.L.; Fariafilho, D.E.; Almeida, A.C. Disinfection of Fertile Eggs of Free-Range Poultry with Essential Oils. *Rev. Bras. Saude Prod. Anim.* **2019**, *20*, e0822019. [CrossRef]
82. Bekhet, G.; Khalifa, A.Y.Z. Essential Oil Sanitizers to Sanitize Hatching Eggs. *J. Appl. Anim. Res.* **2022**, *50*, 695–701. [CrossRef]
83. Tebrün, W.; Motola, G.; Hafez, M.H.; Bachmeier, J.; Schmidt, V.; Renfert, K.; Reichelt, C.; Brüggemann-Schwarze, S.; Pees, M. Preliminary Study: Health and Performance Assessment in Broiler Chicks Following Application of Six Different Hatching Egg Disinfection Protocols. *PLoS ONE* **2020**, *15*, e0232825. [CrossRef]
84. Oliveira, G.D.S.; McManus, C.; dos Santos, V.M. Essential oils and propolis as additives in egg coatings. *World's Poult. Sci. J.* **2022**, *78*, 1053–1066. [CrossRef]
85. Zeweil, H.; Rizk, R.; Bekhet, G.; Ahmed, R. Effect of Egg Disinfection on Hatching Performance for Bandarah Chicken Strain. *Egypt. Poult. Sci. J.* **2013**, *33*, 289–307.
86. Oliveira, G.d.S.; McManus, C.; dos Santos, V.M. Control of *Escherichia coli* in Poultry Using the In Ovo Injection Technique. *Antibiotics* **2024**, *13*, 205. [CrossRef]
87. Oliveira, G.d.S.; McManus, C.; Salgado, C.B.; dos Santos, V.M. Bibliographical Mapping of Research into the Relationship between In Ovo Injection Practice and Hatchability in Poultry. *Vet. Sci.* **2023**, *10*, 296. [CrossRef]

Paper five

Hatching Egg Sanitizers Based on Essential Oils: Microbiological Parameters, Hatchability, and Poultry Health

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Article

Hatching Egg Sanitizers Based on Essential Oils: Microbiological Parameters, Hatchability, and Poultry Health

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Abstract: Background: Eggshell contamination threatens the viability of hatching eggs. This contamination can be caused by harmless, opportunistic, or pathogenic bacteria. Although necessary, the use of synthetic antibiotics to treat eggshells can present several significant problems: They can be toxic and damage the shell, and, most worryingly, they can lead to bacterial resistance. Faced with these challenges, the objective of this research was to create and test a sanitizing plan for hatching eggs using essential oils derived from *Citrus aurantifolia* (CAEO), *Ocimum basilicum* (OBEO), or *Allium sativum* (ASEO). **Methods:** Sanitizing solutions containing specific concentrations of these essential oils were prepared, and their antimicrobial properties and contributions to poultry safety and hatching parameters were investigated. **Results:** The bacterial load was reduced in eggshells sanitized with essential oils, and the degree of bacterial inhibition, along with their safety profile, may be directly related to optimal hatchability rates, lower incidences of contaminated dead embryos, and the hatching of healthy chicks. **Conclusions:** Together, these results reinforce the importance of essential oils in the development of effective and safe treatments for managing hatching eggs.

Keywords: *Allium sativum*; *Citrus aurantifolia*; egg incubation; eggshell; egg microbiology; essential oils; hatching eggs; natural products; *Ocimum basilicum*; scanning microscopy

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1. Introduction

Embryonic development is a complex physiological process characterized by intricate communication between the internal and external structures of eggs. This sophisticated dialog between structures is essential for the proper development of the embryo. However, the interactions between the eggshell and the embryo present a significant duality. Although these interactions can promote embryonic development, they can also be harmful. The eggshell, as it is an interface with the external environment, is subject to the presence of various contaminants, including pathogenic microorganisms [1,2]. Microbiological tests confirmed the presence of microbial contamination on the eggshell post collection (Figure 1). This contamination can compromise the integrity of the embryo, negatively impacting its development [3]. Sanitary practices are needed to mitigate the risk of bacterial contamination in hatcheries, feed and input storage areas, and facilities supporting poultry activities, such as administrative areas, bathrooms, changing rooms, laboratories, and warehouses. The same attention should be given to production facilities, sanitization rooms, egg storage areas, egg transport vehicles, and personal hygiene practices, with particular emphasis on sanitizing hatching eggs.

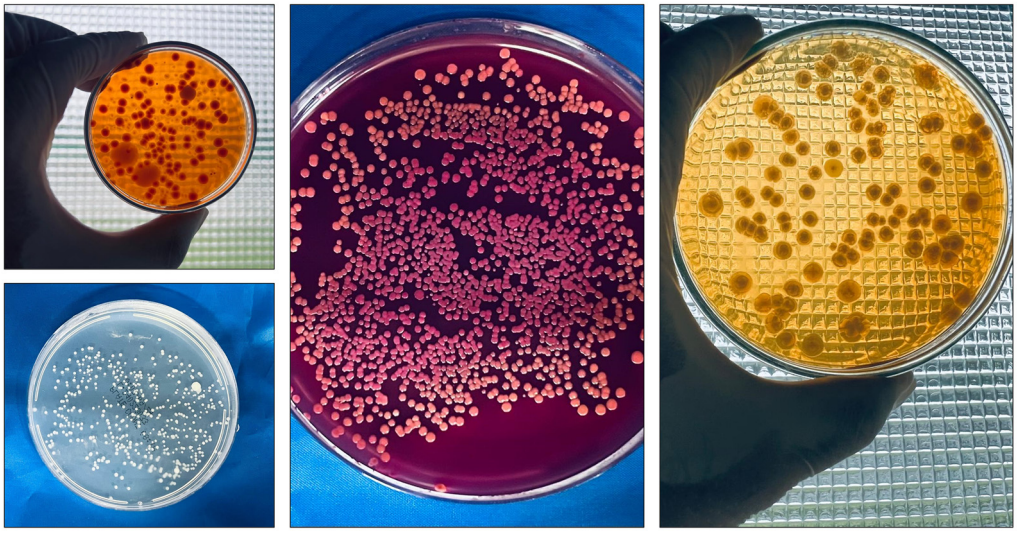


Figure 1. Bacterial colonies grown on different culture media from samples collected from eggshells (immediately after collection).

Sanitizers used in poultry farming are normally formulated with a synthetic chemical base (e.g., formaldehyde, glutaraldehyde, or quaternary ammonium) and have been proven to be efficient at reducing the level of contamination by pathogenic bacteria that cause fatal infections [4–7]. On the other hand, these sanitizers can also be associated with health problems ranging from allergic skin reactions to the development of malignant cancer [8–11]. This problem worsens when companies do not warn users of their sanitizers that they must handle these with personal protective equipment. Poultry researchers, teachers, and professionals play vital roles in the theoretical and practical education of poultry producers and managers about advanced sanitization techniques, adherence to healthy sanitizers, modification of harmful protocols, and immediate recognition of complications and irregularities in practice.

The selection of hatching egg sanitizers that contain compounds derived from plants, such as essential oils, can be a beneficial decision, considering the results of reviews, published in renowned scientific journals, on the benefits of essential oils for the sanitization of hatching eggs and the protection of human health [12–18]. Oliveira et al. [12] suggested the use of essential oils as sanitizing agents for hatching eggs, emphasizing their safety and effectiveness in reducing the microbial load on eggshells, which can significantly increase hatchability rates. Lesgards et al. [18] favored the advancement of essential oils in medical research, highlighting that these compounds may exhibit advanced anticancer properties by inducing cell death in cancer cells without affecting normal cells.

The plants *Citrus aurantifolia* (Tahiti lemon), *Ocimum basilicum* (basil), and *Allium sativum* (garlic) (Figure 2) are among the best-known sources of essential oils worldwide. *Citrus aurantifolia* is a plant that can reach an average height of 5.10 m and a canopy area of approximately 17.50 m^2, with an estimated volume of 105.16 m^3. The leaf area can reach 16.53 cm^2. Each tree can produce an average of 405 fruits, with a fruit set rate of 30.95%, yielding approximately 18.64 kg/tree. The number of flowers/clusters can reach 7.00, and the number of fruits/clusters can reach 2.17. The fruits can have a juice content of approximately 39.89%, with total soluble solids measuring 7.64 °Brix. The acidity can reach 7.94%. Each fruit may contain an average of 24.49 mg of ascorbic acid/100 mL of juice. The polar diameter of the fruits is 3.31 cm, the equatorial diameter is 3.17 cm, and the average volume is 42.36 mL. The average peel thickness can reach 2.33 mm [19]. *Ocimum basilicum* is a plant that can reach an average height of 53.4 cm, with approximately 16.0 branches/plant. The plant diameter can reach approximately 41.0 cm. The leaf blade has an average length of 6.4 cm and a width of 3.4 cm. The time to flowering can reach 72 days, with approximately 32.2 inflorescences per plant, each averaging 15.6 cm in length,

and approximately 11.1 clusters/main stem. In terms of the yield, the average plant weight can reach 153.4 g. The yield of fresh herbs can reach 16.6 kg/100 m^2, whereas the yield of air-dried herbs can reach 3.5 kg/100 m^2. The plant can also contain an essential oil content of 1.03% and has an essential oil efficiency of 0.31 g per plant [20]. *Allium sativum* can reach an average height of 67.40 cm and produces approximately 7.80 leaves per plant, with an average leaf length of 34.20 cm and a leaf width of 3.01 cm. The bulb can have a neck thickness of 1.45 cm and a diameter of 3.70 cm. The bulb yield per plant can average 25.33 g, with approximately 20.55 cloves per bulb. Each clove weighed an average of 2.62 g, with a length of 1.01 cm and a diameter of 1.04 cm. The total soluble solids can reach approximately 36.01% [21].

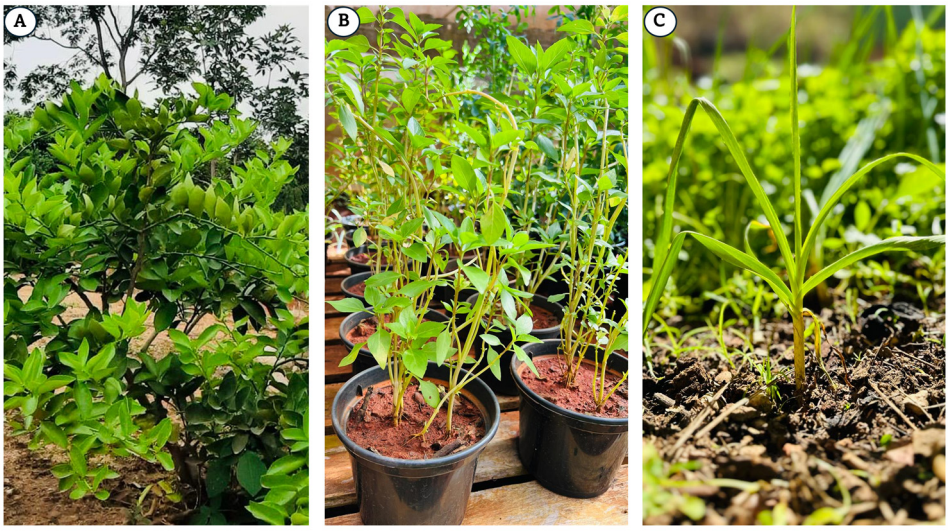


Figure 2. *Citrus aurantifolia* (**A**), *Ocimum basilicum* (**B**), and *Allium sativum* (**C**) plants.

The essential oil extracted from the *Citrus aurantifolia* (CAEO) plant is an aromatic natural compound that is predominantly composed of hydrocarbons in the form of monoterpenes and sesquiterpenes, with D-limonene as the main component [22]. This essential oil was effective in inhibiting in vitro bacterial strains, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus subtilis*, in addition to the fungus *Candida albicans* [22]. The essential oil extracted from *Ocimum basilicum* (OBEO) is a light yellowish and volatile substance, which composition is dominated by monoterpenes, sesquiterpenes, and phenylpropanoids, with estragole as the main constituent [23]. Exhibiting potent antimicrobial activity in vitro, this oil is effective against methicillin-resistant *Staphylococcus aureus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Salmonella* spp., *Klebsiella pneumoniae*, and *Candida albicans* [23]. The essential oil extracted from *Allium sativum* (ASEO) is a bioapplicable natural chemical solution that stores several bioactive compounds. This essential oil has demonstrated significant in vitro antibacterial activities against several bacterial strains, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Listeria innocua* [24]. Remarkable findings were obtained from in vivo tests, where CAEO, OBEO, and ASEO showed robust abilities to substantially decrease the bacterial loads on the shells of hatching eggs [14]. This reduction was particularly evident with mesophilic and enterobacteria [14].

The antimicrobial properties of CAEO, OBEO, and ASEO are not inferior to those of commercial sanitizers [14]. This not only promotes a substantial reduction in microbial contamination but also can encourage the production of next-generation antimicrobials based on CAEO, OBEO, and ASEO, with the capacity to promote green poultry farming [25]. As we continue to explore and better understand these essential oils within the poultry industry, new findings have emerged to strengthen the development of advanced natural antimicrobial solutions capable of meeting the needs of the poultry industry, which is

increasingly aware of the importance of sustainability. Therefore, this study aimed to provide a thorough evaluation of the effects of CAEO, OBEO, and ASEO on hatching eggs. This assessment ranges from evaluating the microbiology of eggshells after the application of these oils to analyzing newly hatched chicks. This study seeks to fill gaps in the understanding of the potential benefits of these essential oils in the face of the microbial challenges faced by poultry farms in the management of hatching eggs.

2. Results and Discussion

The main options for controlling the bacterial contamination of eggshells on commercial poultry farms are based on conventional therapies, such as fumigation with formaldehyde (FA) [6]. However, this sanitization model can lead to adverse effects, as both poultry and humans cannot tolerate constant exposure, especially at high concentrations, because of potential health risks [26–29]. Sanitizing eggshells is essential for bacterial control, but the sanitizers used in this process raise significant concerns regarding health and safety. In our previous study [14], we reported that CAEO, OBEO, and ASEO effectively reduce the bacterial loads on eggshells. In this study, we expand this investigation and demonstrate how approaches based on CAEO, OBEO, and ASEO can influence the hatching and health parameters of poultry.

We began our study with a series of analyses to characterize the hatching eggs. This step was crucial to ensure that the eggs were suitable for subsequent evaluations and to minimize potential biases that could affect the study's primary objective. The qualities and microbiological characteristics of the eggs are detailed in Table 1. After confirming that the eggs met the expected normal parameters, we proceeded with the subsequent stages of the study.

Table 1. Internal evaluation of eggs during pre-incubation.

Analysis	Result
Egg weight (g) *	58.83 ± 4.08
Albumen height (mm) *	6.70 ± 1.02
Haugh unit *	80.24 ± 6.34
Yolk index *	0.40 ± 0.03
Albumen pH *	8.14 ± 0.25
Yolk pH *	6.07 ± 0.15
Mesophilic bacteria (log_{10} CFU/mL) **	2.22 ± 0.38
Enterobacteriaceae (log_{10} CFU/mL) **	1.89 ± 0.15

The results are presented as the means ± standard deviations of 30 * or 15 ** eggs.

To elucidate the antibacterial effects of the essential oils on the eggshells, we counted mesophilic bacteria and Enterobacteriaceae at three distinct time points: once before incubation and twice during the incubation process. Overall, we found that compared with the other treatments, the essential oils were notably more effective ($p < 0.05$) at reducing eggshell contamination (Table 2). In addition to significantly lowering the initial bacterial counts, the essential oils demonstrated superior control of contamination recurrence, keeping the bacterial loads stable until the 18th day of incubation. The contamination levels observed on eggshells sanitized with the essential oils during incubation were lower than that in the reference treatment (FA), with the essential oils showing significantly reduced loads of mesophilic bacteria and Enterobacteriaceae on the 18th day of incubation. Until the ninth day of incubation, the eggshells treated with CAEO and ASEO were free of Enterobacteriaceae. On the ninth day, the Enterobacteriaceae loads were minimal on eggs sanitized with OBEO but did not significantly differ from those in the groups treated with the other essential oils. On the other hand, eggshells sanitized with FA, the standard model from the supplier farm, were less effective at preventing recontamination, as they presented significant increases in mesophilic bacteria and Enterobacteriaceae loads during incubation. The ASEO was the most effective at reducing the bacterial contamination of the eggshells.

Table 2. Bacterial counts of eggshells during pre-incubation and incubation.

Sanitizer	Mesophilic Bacteria		
	Day 0	Day 9	Day 18
NE	3.71 ± 0.67^{aB}	4.40 ± 0.44^{aAB}	5.74 ± 0.28^{aA}
GA	3.01 ± 0.72^{abA}	3.48 ± 0.18^{aA}	4.20 ± 1.09^{abA}
FA	1.78 ± 0.56^{bB}	2.90 ± 0.54^{abAB}	4.03 ± 0.26^{bA}
CAEO	1.12 ± 0.42^{bA}	1.76 ± 0.18^{bcA}	2.31 ± 0.46^{cA}
OBEO	1.15 ± 0.53^{bA}	1.51 ± 0.12^{bcA}	2.10 ± 0.48^{cA}
ASEO	1.01 ± 0.05^{bA}	1.19 ± 0.34^{cA}	1.98 ± 0.23^{cA}
p value	<0.0001	<0.0001	<0.0001
Sanitizer	**Enterobacteriaceae**		
	Day 0	Day 9	Day 18
NE	1.59 ± 0.39^{aB}	2.56 ± 0.37^{aAB}	2.96 ± 0.21^{aA}
GA	1.41 ± 0.37^{aA}	1.99 ± 0.19^{aA}	2.03 ± 0.57^{abA}
FA	0.00 ± 0.00 * ^{bC}	1.00 ± 0.45^{bB}	2.42 ± 0.49^{aA}
CAEO	0.00 ± 0.00^{bA}	0.00 ± 0.00^{cA}	0.92 ± 0.20^{cA}
OBEO	0.00 ± 0.00^{bB}	0.54 ± 0.47^{bcAB}	1.08 ± 0.68^{bcA}
ASEO	0.00 ± 0.00^{bA}	0.00 ± 0.00^{cA}	0.35 ± 0.60^{cA}
p value	<0.0001	<0.0001	<0.0001

$^{A–C; a–c}$ Different uppercase (row) or lowercase (column) letters indicate significant differences among means ($p < 0.05$). Non-sanitized eggs—NE; grain alcohol—GA; formaldehyde—FA; *Citrus aurantifolia* essential oil—CAEO; *Ocimum basilicum* essential oil—OBEO; *Allium sativum* essential oil—ASEO. * Indicates a count below detection limits of <10 CFU/mL. The results are presented as the means ($\log_{10}$ CFU/mL) ± standard deviations of triplicate measurements.

More specifically, the essential oils reduced, on average, 70.53% of the mesophilic bacterial loads, and no Enterobacteriaceae were detected during the pre-incubation period. On the ninth day, the average reductions were 66.21% for mesophiles and 92.97% for Enterobacteriaceae. On the 18th day, the reductions were 62.89% for mesophiles and 73.54% for Enterobacteriaceae. These data are consistent with the findings presented in our previous review [12], where we demonstrated that on the basis of various studies, essential oils can reduce the number of mesophilic bacteria by up to 80.77%. These results are also supported by previous analyses of hatching egg sanitizers based on *Lavandula angustifolia* [30], *Origanum vulgare* [31], and *Cuminum cyminum* [32]. The reasons why essential oils kill bacteria are well documented. The main reasons for this include the inhibition of biofilm formation; the induction of apoptosis mediated by oxidative stress; the disruption of DNA synthesis; the leakage of intracellular proteins, ATP, and nucleic acids; and changes in the metabolic profile, which encompasses amino acid restriction and disturbances in energy metabolism [33,34].

On the basis of the ultrastructural analysis of the eggshells after sanitization, we observed that non-sanitized eggshells maintained remarkably preserved structural integrity, with few visible flaws and a consistently smooth surface texture (Figure 3). The cracks observed on the eggshell surface were minimal, small in extent, and scattered, with no significant interconnectivity. The surface appeared uniform, with a smooth texture and few irregularities or aggregates. There were almost no visible particles or contaminants, suggesting a clean and intact surface with minimal signs of wear. On the other hand, eggshells sanitized with essential oils exhibited cracks ranging from few and superficial to numerous and deep, with from minimal to significant interconnectivity. The surface homogeneity also varied, ranging from good uniformity to a marked lack of uniformity. The presence of particles on the eggshell surface varied from a few to a substantial number. The wear on the shells ranged from mild to severe. Specifically, eggshells sanitized with OBEO showed no signs of advanced degradation, whereas those treated with CAEO and ASEO presented considerable stress and surface deterioration. We suggest that a significant portion of the effects observed on eggs sanitized with essential oils was associated with the

GA used as a solvent. Eggshells sanitized solely with GA presented deep, interconnected cracks covering a large part of the surface, characterized by poor homogeneity, a highly irregular texture, and a marked presence of defects. The significant number of particles indicated degradation and contamination, with clear signs of structural failure on the surface. Eggshells sanitized with FA showed significant patterns of cracks that increased in complexity as the magnification of the analysis increased. The cracks were deep, interconnected, and present throughout the surface. This surface was the least homogeneous among all the treated surfaces, with an extremely irregular texture and signs of failure in multiple areas. The presence of particles was widespread and intense, indicating a high likelihood of contamination and/or internal degradation. The wear of the eggshells in this treatment group was extreme, with severe degradation, and the structure appeared to be on the brink of complete failure. Therefore, as expected, the non-sanitized eggshells presented the best scenario in terms of the integrity, whereas the eggshells sanitized with FA were the most compromised, severely affecting the structural integrity.

Overall, the incubation process was concluded at 516 h and 12 min, but the first hatch occurred at 469 h and 49 min, whereas the last hatch occurred at 501 h and 27 min, covering a period of 31 h and 38 min. This hatch window is within the expected range for commercial setters [35]. Previously, an incubation cycle involving non-sanitized eggs and those sanitized with ethyl alcohol, essential oils, or FA lasted an average of 31 h and 45 min [36].

We quantified the eggs' weight loss based on the eggs' initial weights and observed that the eggs sanitized with the essential oils did not show weight losses different from those of the non-sanitized eggs (Table 3). In contrast, the eggs sanitized with FA showed weight losses that exceeded the ideal limit required to ensure an adequate air cell by the end of embryonic development [37]. Losses of this magnitude have a significant impact on embryonic development; for example, excessive internal water loss can cause severe dehydration in the embryo [37]. The elevated weight losses of the eggs sanitized with FA corroborate the observation that it was the sanitizing agent that most compromised the integrity of the eggshell (Figure 3). In contrast, the damage caused by the essential oils to the eggshells did not result in weight losses outside the normal range observed for eggs treated with these oils (Figure 3). One possible explanation for this phenomenon is that despite the combination of alcohol and oil causing damage to the shells, the oily and residual characteristics of the essential oils, even at low concentrations, may have minimized the significant weight loss. Importantly, in recent years, essential oils have been used in egg coatings to improve their structures and reduce weight loss in eggs stored for more than 20 days [38–40].

To gain insights into hatching outcomes, we analyzed the hatchability rate of fertile eggs in response to the sanitizers applied to the eggshells (Table 3). First, we observed that under the same incubation conditions, the conventional FA-based sanitizer significantly reduced ($p < 0.05$) the hatchability rate, whereas the essential oils promoted the highest hatchability rates. Our hatchability results are in close alignment with previous findings on the sanitization of eggs using essential oils of *Syzygium aromaticum* at a concentration of 0.39% [41] and *Cuminum cyminum* and *Origanum vulgare*, each at 0.5% [32]. Compared with non-sanitized eggs, the application of these essential oils led to significant increases in hatch rates: for every 320 incubated eggs, 34 additional eggs successfully hatched. Similarly, for *C. cyminum* and *O. vulgare*, for every 50 incubated eggs, 6 more eggs hatched than in the untreated control group. Oliveira et al. [13] conducted a review that contrasted the effects on hatchability of eggs sanitized with FA and essential oils. The review revealed that on average, essential oils do not produce inferior results compared with FA in terms of hatchability.

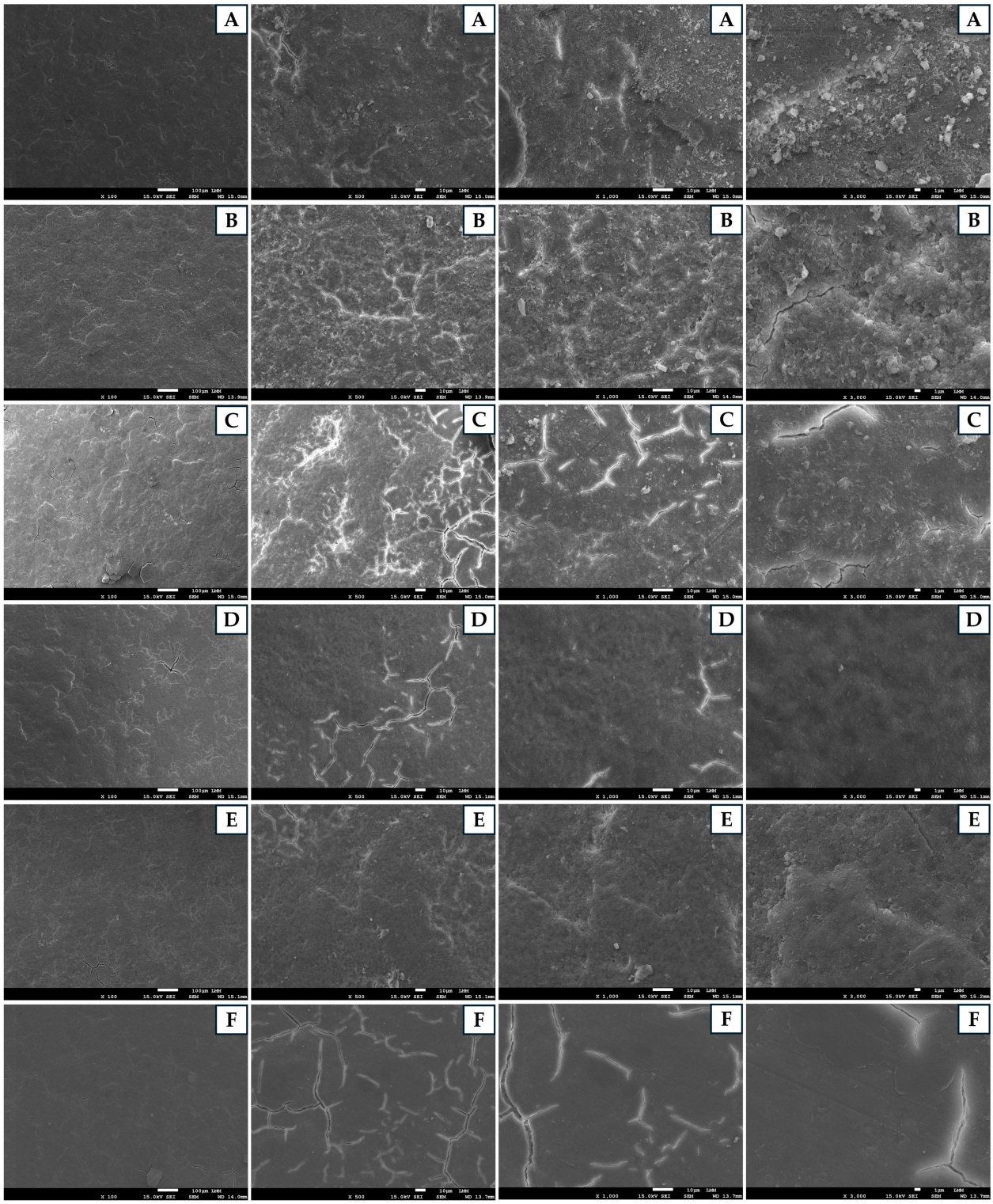


Figure 3. Scanning electron microscopy photographs of eggshells after different sanitization processes. ((**A**) non-sanitized eggs—NE): high degree of integrity and minimal degradation. ((**B**) grain alcohol—GA): low degree of integrity, severe degradation, and evident structural failure. ((**C**) formaldehyde—FA): critical degree of integrity, severe degradation, and risk of structural failure. ((**D**) *Citrus aurantifolia* essential oil—CAEO): reduced degree of integrity, significant cracks, and irregular texture. ((**E**) *Ocimum basilicum* essential oil—OBEO): moderate degree of integrity and some cracks and particles. ((**F**) *Allium sativum* essential oil—ASEO): highly reduced degree of integrity, many cracks, and many particles.

Table 3. Analysis of eggs' weight losses, hatchability of fertile eggs, embryonic mortality, and percentage of contaminated eggs throughout the incubation cycle.

Sanitizer	Egg Weight Loss	Hatchability	Dead			
			Early	Mid	Late	Contaminated
NE	12.88 ± 1.41 ^{b}	81.30 ± 2.88 ^{ab}	3.90 ± 1.43 ^{b}	0.86 ± 1.49 ^{a}	7.03 ± 6.58 ^{a}	6.91 ± 3.18 ^{a}
GA	13.65 ± 1.15 ^{ab}	82.56 ± 3.21 ^{ab}	7.70 ± 3.21 ^{ab}	1.70 ± 1.20 ^{a}	6.35 ± 3.15 ^{a}	1.68 ± 1.20 ^{b}
FA	15.11 ± 1.46 ^{a}	75.34 ± 5.87 ^{b}	15.74 ± 6.70 ^{a}	1.27 ± 1.41 ^{a}	5.95 ± 2.54 ^{a}	1.70 ± 2.08 ^{b}
CAEO	13.71 ± 0.99 ^{ab}	89.58 ± 2.62 ^{a}	2.59 ± 1.91 ^{b}	1.28 ± 1.41 ^{a}	6.12 ± 3.68 ^{a}	0.43 ± 0.75 ^{b}
OBEO	12.91 ± 1.17 ^{b}	88.31 ± 4.61 ^{a}	4.33 ± 1.99 ^{b}	0.86 ± 0.86 ^{a}	6.50 ± 2.65 ^{a}	0.00 ± 0.00 ^{b}
ASEO	13.80 ± 1.18 ^{ab}	87.83 ± 5.49 ^{a}	3.47 ± 2.16 ^{b}	2.19 ± 2.28 ^{a}	6.51 ± 2.32 ^{a}	0.00 ±0.00 ^{b}
p value	0.0221	0.0058	0.0015	0.8737	0.9995	0.0006

^{a,b} Different lowercase (column) letters indicate significant differences among means ($p < 0.05$). Non-sanitized eggs—NE; grain alcohol—GA; formaldehyde—FA; *Citrus aurantifolia* essential oil—CAEO; *Ocimum basilicum* essential oil—OBEO; *Allium sativum* essential oil—ASEO. The results are presented as the means ± standard deviations of 240 eggs.

Embryonic mortality can be divided into three phases (Figure 4). We compared the mortality rates among the treatments. FA increased ($p < 0.05$) the rate of early embryonic mortality compared with both non-sanitized eggs and those treated with GA and essential oils (Table 3). Considering the average rate of early mortality associated with essential oils, we demonstrated that compared with FA, these oils reduced the mortality rate during this period by approximately 75%. The high early mortality rate observed in eggs sanitized with FA can possibly be attributed to the residual presence of this compound on the eggshell during the first days of development or because it can fix itself to the internal contents of the eggs. Similarly, Bekhet [42] reported an increase in the early embryonic mortality rate for eggs sanitized with FA. No differences were found for intermediate or late mortality rates among the treatments. The highest number of contaminated embryos was expected for the non-sanitized eggs, as these eggs are exposed to a more significant bacterial challenge than are the sanitized eggs.

Figure 4. Dead chick embryos at different stages of development. The stages are categorized as follows: (Early) 0–7 days of incubation, (Mid) 8–18 days of incubation, and (Late) 19–21 days of incubation.

To the best of our knowledge, our study is pioneering in linking the sanitization of hatching eggs with essential oils and the level of bacterial contamination in the yolk sac. Our findings indicate that interventions with CAEO, OBEO, and ASEO reduced ($p < 0.05$) the mesophilic bacterial load by 55.36% and the Enterobacteriaceae load by 56.98% in the yolk sacs of 18-day-old embryos (Table 4). Our hypothesis is that the use of essential oils helps to maintain low bacterial levels on the eggshell, thereby impeding the penetration of contaminating bacteria into the embryo. In this way, the embryo benefits from the antibacterial barrier created by the essential oils on the eggshell. Li et al. [43] corroborated our findings. They did not isolate nalidixic-acid-resistant *E. coli* in any of the yolk sac samples from chicks hatched from eggs artificially contaminated with bacteria on the shell and subsequently sanitized with 1.5% lysozyme. In contrast, when sanitization was performed with distilled water instead of lysozyme, 40% of the yolk sac samples contained *E. coli*. Although partial, our recent findings also provide important support for our previous hypothesis that the sanitization of hatching eggs with essential oils contributes to significant reductions in the microbial loads of newly hatched chicks [44]. We also noticed that a significant number of dead embryos due to contamination were identified in the non-sanitized eggs, whereas no mortality was recorded among embryos from the eggs sanitized with OBEO or ASEO (Table 3). Thus, we demonstrated the relationships among the practice of sanitizing eggshells, the reduction in bacterial counts on the eggshells, and the survival rates and bacterial qualities of the embryos. FA did not have the potential to reduce the bacterial loads in the yolk sacs of the embryos.

Table 4. Bacterial counts in the yolk sacs of the embryos at 18 days of development.

Sanitizer	Mesophilic Bacteria	Enterobacteriaceae
NE	3.21 ± 0.30^{a}	2.65 ± 0.18^{a}
GA	2.94 ± 0.52^{ab}	2.14 ± 0.15^{ab}
FA	2.93 ± 1.00^{ab}	2.36 ± 0.29^{a}
CAEO	1.52 ± 0.39^{bc}	1.24 ± 0.29^{bc}
OBEO	1.66 ± 0.47^{bc}	1.11 ± 0.61^{c}
ASEO	1.10 ± 0.20^{c}	1.07 ± 0.27^{c}
p value	0.0014	0.0002

[a–c] Different lowercase (column) letters indicate significant differences among means ($p < 0.05$). Non-sanitized eggs—NE; grain alcohol—GA; formaldehyde—FA; *Citrus aurantifolia* essential oil—CAEO; *Ocimum basilicum* essential oil—OBEO; *Allium sativum* essential oil—ASEO. The results are presented as the means ($\log_{10}$ CFU/mL) ± standard deviations of seven yolk sacs.

Although the essential oils did not influence the chick weights, FA significantly reduced ($p < 0.05$) the chick weight compared with those of chicks from the non-sanitized eggs. This reduction is probably because of the degree of embryo dehydration caused by excessive egg weight loss during incubation because of eggshell damage. According to Aviagen [45], the low weights of chicks compared with what is expected on the basis of the weight of the eggs, indicating poor yield, is associated with chick dehydration, reduced yolk reserves, and excessive levels of activity and noise. Compared with the non-sanitized eggs, the hatching eggs sanitized with the essential oils tended to produce healthier chicks, with a significant difference observed only for OBEO (Table 5). To identify the main cause of the decline in chick quality from the non-sanitized eggs, we conducted a detailed analysis of individual factors (reflex, navel, legs, and beak). The analysis revealed that poor navel healing was the primary factor responsible for the reduced quality of the chicks hatched from the non-sanitized eggs (Figure 5). These findings highlight the importance for sanitizing hatching eggs. Among the treatments, OBEO produced the healthiest chicks.

Table 5. Monitoring of chick hatch weights and quality scores.

Sanitizer	Chick Weight (g) [1]	Pasgar Score [2]
NE	39.66 ± 0.83 [a]	8.56 ± 1.06 [b]
GA	38.85 ± 1.14 [ab]	8.81 ± 0.73 [ab]
FA	38.46 ± 0.81 [b]	9.06 ± 0.83 [ab]
CAEO	39.11 ± 0.65 [ab]	9.38 ± 1.05 [ab]
OBEO	39.42 ± 0.87 [ab]	9.63 ± 0.70 [a]
ASEO	39.06 ± 0.94 [ab]	9.44 ± 0.79 [ab]
p value	0.0458	0.0093

[a,b] Different lowercase (column) letters indicate significant differences among means ($p < 0.05$). Non-sanitized eggs—NE; grain alcohol—GA; formaldehyde—FA; *Citrus aurantifolia* essential oil—CAEO; *Ocimum basilicum* essential oil—OBEO; *Allium sativum* essential oil—ASEO. [1] Results are the means ± standard deviations of all the chicks that hatched; [2] Results are the means ± standard deviations of 40 chicks.

Figure 5. Navels of newly hatched chicks with poor healing.

FA caused morphological changes, ranging from mild to moderate, in the tracheae and lungs of the embryos in all the embryo samples (Table 6; Figures 6 and 7). Our findings corroborate the results of histological analyses carried out by Turkish researchers, who, approximately 16 years ago, confirmed that sanitizing hatching eggs with FA has adverse effects on the embryos' tracheae [46]. Given the ability of FA to penetrate eggshells after exposure [27], especially owing to the damage caused to the shells' integrity, we suggest that there may be an accumulation of toxic chemical compounds within the eggs' internal compartments, with implications that extend throughout embryonic development. Although we previously suggested that proper sanitization of hatching eggs with FA could prevent adverse effects on embryonic development [44], our recent results highlight the need for further investigation to understand the factors that favor the toxic effects of FA on embryos, even when applied under appropriate conditions. To advance this field, we propose a multifactorial study on the sanitization of hatching eggs with FA that considers the characteristics of eggshells from different genetic strains; the purity, formulation, and quality of the FA; and the genetic variation of the embryos.

Table 6. Evaluation of trachea and lung tissue samples from different treatments [1].

Sanitizer	Tracheal Lesion			Lung Lesion	
	Epithelial Cell Necrosis	Goblet Cell Hyperplasia	Lymphocytic Inflammation	Congestion	Bronchial Epithelial Necrosis
NE	–	–	–	–	–
GA	–	–	–	–	–
FA	–	++	+	++	–
CAEO	–	–	–	–	–
OBEO	–	–	–	–	–
ASEO	–	–	–	–	–

[1] The data are presented in the following intensity categories: absent (–), mild (+), and moderate (++). Non-sanitized eggs—NE; grain alcohol—GA; formaldehyde—FA; *Citrus aurantifolia* essential oil—CAEO; *Ocimum basilicum* essential oil—OBEO; *Allium sativum* essential oil—ASEO. The results are the means obtained by measuring samples of six embryos.

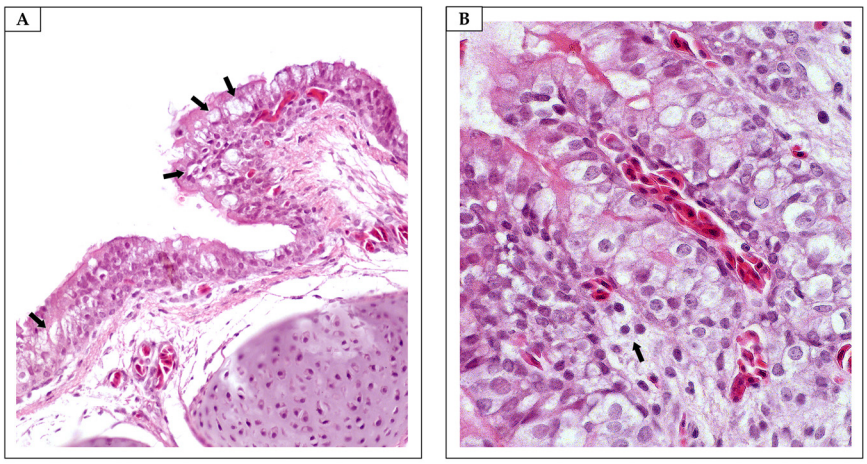


Figure 6. Tracheae. (**A**) Formaldehyde (FA) group. Multifocal goblet cell hyperplasia (arrows) (H&E, objective 40×). (**B**) FA group. Mononuclear inflammatory infiltration (arrows) (H&E, objective 40×).

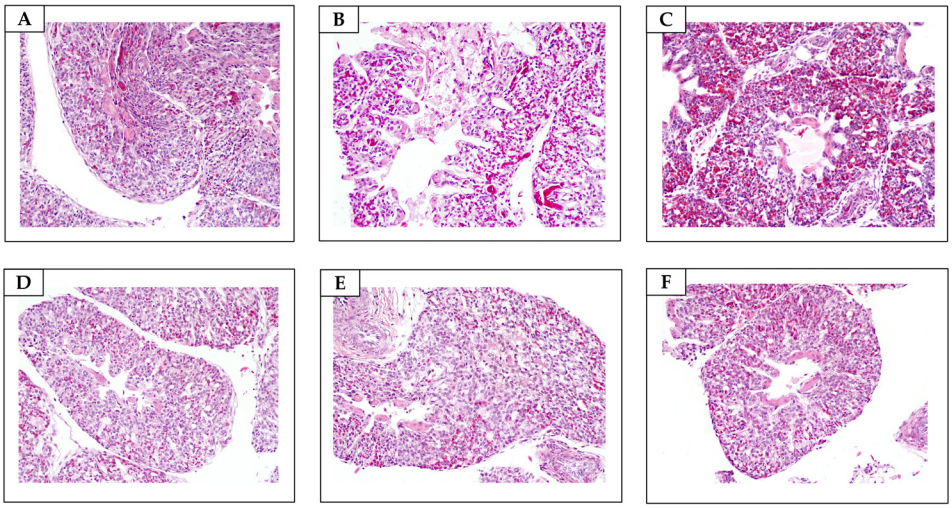


Figure 7. Lungs. (**A**) Non-sanitized eggs (NE). Unremarkable microscopic changes (H&E, objective 20×). (**B**) Grain alcohol (GA). Unremarkable microscopic changes (H&E, objective 20×). (**C**) Formaldehyde (FA). Severe pulmonary congestion (H&E, objective 20×). (**D**) *Citrus aurantifolia* essential oil (CAEO). (**E**) *Ocimum basilicum* essential oil (OBEO). (**F**) *Allium sativum* essential oil (ASEO). Unremarkable microscopic changes (H&E, objective 20×).

Unlike those treated with FA, trachea and lung samples from embryos in the essential oil group presented no detectable morphological changes (Table 6, Figures 7 and 8). These findings suggest that the potential penetration and accumulation of essential oils in the embryonic environment do not reach levels capable of inducing morphological alterations in the poultry respiratory system. Oliveira et al. [13] also demonstrated that chicks hatched from eggs sanitized with *Syzygium aromaticum* essential oil did not exhibit histopathological changes in the trachea. This allows us to speculate that although poultry have tissues with varying degrees of sensitivity, the embryonic mortalities observed in the group exposed to the essential oils were not attributable to toxicity associated with the egg sanitization process using these natural compounds. This conclusion is supported by previous studies that reported the absence of histopathological alterations in the digestive, cardiovascular, and central nervous systems and muscular tissues of one-day-old chicks hatched from eggs sanitized with essential oils [13,32].

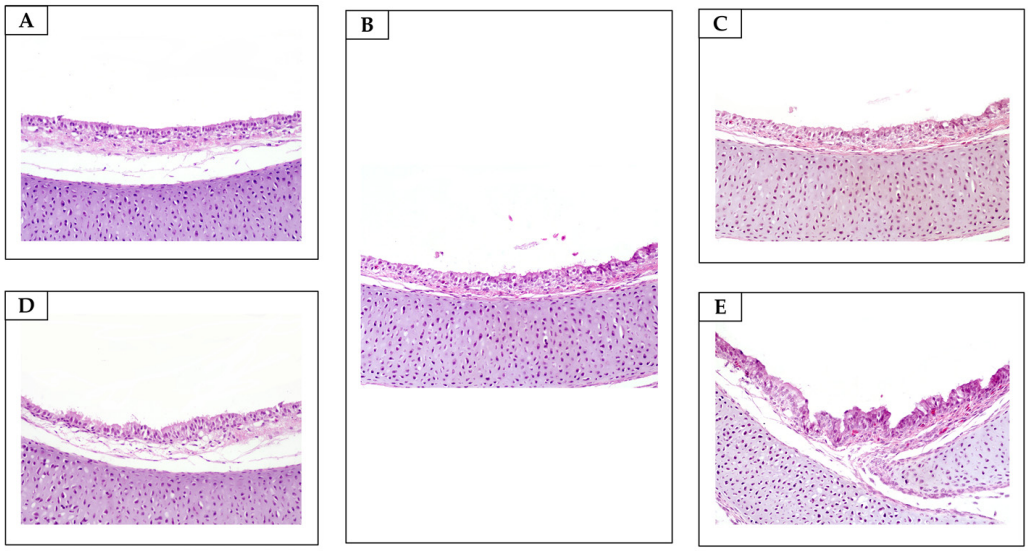
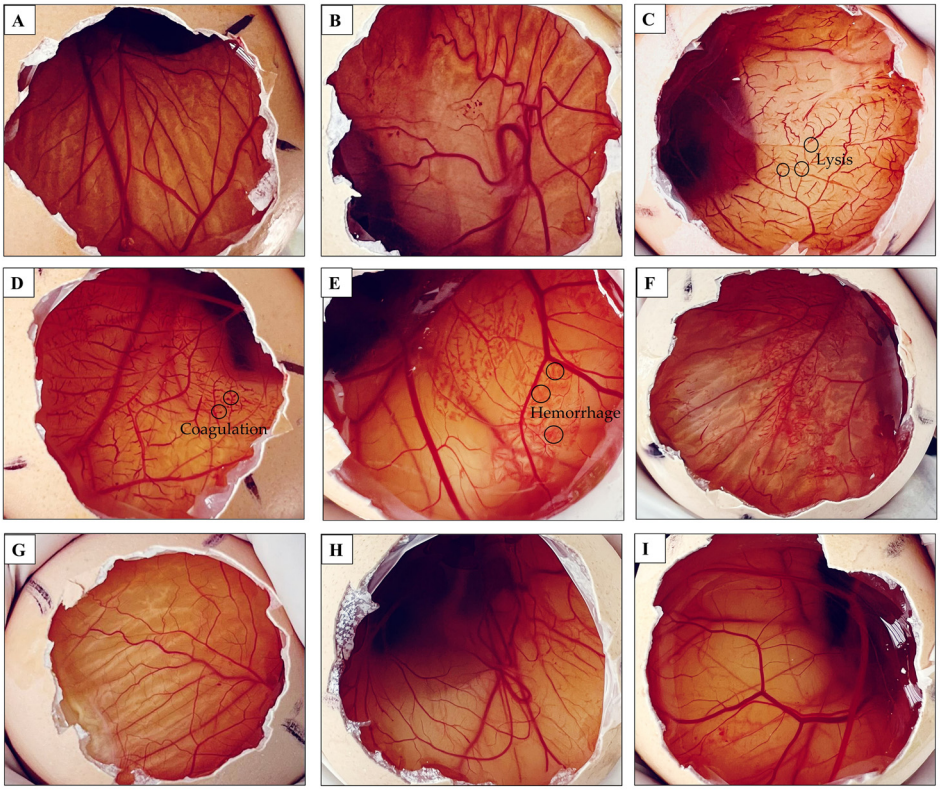


Figure 8. Tracheae. Unremarkable microscopic changes (H&E, objective 20×). (**A**) Non-sanitized eggs (NE). (**B**) Grain alcohol (GA). (**C**) *Citrus aurantifolia* essential oil (CAEO). (**D**) *Ocimum basilicum* essential oil (OBEO). (**E**) *Allium sativum* essential oil (ASEO).

Changes in the chorioallantoic membrane (CAM) became visible after the application of the sanitizing solutions (Figure 9). GA caused lysis in the CAMs of all eight embryos tested within the first 30 s post application, as exemplified in Figure 9. To determine whether the severe toxicity observed when the essential oils were diluted in GA was, indeed, attributable to the solvent, we evaluated the membranes exposed to the pure essential oils. We found that although the pure essential oils were highly concentrated compared with the tested dilutions, they exhibited low toxicities. None of the eight CAMs evaluated showed lysis, hemorrhage, or coagulation within the first 2 min of exposure, which contrasts with the results observed in the other treatments. We provide a robust scientific foundation for the topical use of essential-oil-based sanitizers on eggshells, reaffirming that this management approach is safe and, in cases of embryonic contact, minimal and non-toxic. Exposure to GA, which is used as a diluent, did not significantly affect the embryonic viability; if excessive or prolonged penetration occurred, we would have observed elevated embryo mortality associated with massive hemorrhages, coagulation, and lysis of blood vessels. These findings underscore that sanitizing with essential oils diluted in GA is a safe practice. However, under no circumstances should GA be used as a diluent for injectable applications because of its potential risk of embryonic damage. For injecting essential oils into eggs, we strongly recommend using alternative and proven safe diluents. For example, the medium-chain triglycerides used as a diluent for essential oils in

hen's egg test chorioallantoic membrane (HET-CAM) assays did not induce irritation to the CAM, as evidenced by the absence of hemorrhage, coagulation, and vasoconstriction [47].



Sanitizers	Concentrations	Irritation score	Toxicity
NE	-	-	-
FA	1.5%	7.71 ± 3.40	Moderate
GA	93.8%	16.75 ± 2.49	Severe
CAEO	9.38 mg/mL	12.63 ± 4.41	Severe
OBEO	4.69 mg/mL	12.13 ± 4.09	Severe
ASEO	1.17 mg/mL	13.11 ± 3.92	Severe
CAEO	Pure	1.75 ± 3.41	Weak
OBEO	Pure	2.13 ± 3.94	Weak
ASEO	Pure	1.67 ± 3.56	Weak

Figure 9. Photographic sequences of the hen's egg test chorioallantoic membrane (HET-CAM) assay illustrating the CAM without the application of any sanitizing product (NE) (**A**) and the CAMs after contact with different sanitizers: formaldehyde (FA) (**B**), grain alcohol (GA) (**C**), *Citrus aurantifolia* essential oil (CAEO) (**D**), *Ocimum basilicum* essential oil (OBEO) (**E**), and *Allium sativum* essential oil (ASEO) (**F**). Additionally, the figure shows the membranes exposed to pure CAEO (**G**), OBEO (**H**), and ASEO (**I**). The irritation score is presented as the mean ± standard deviation of eight eggs, highlighting the toxic/irritant effects of each tested substance.

3. Materials and Methods

The CAEO (extracted from the fruit peel by pressing) (Phytoterápica®, Rio de Janeiro, Brazil), OBEO (extracted from the leaves and flowers by steam distillation) (BioEssência®, São Paulo, Brazil), and ASEO (extracted from the rhizomes by steam distillation) (Laszlo®, Minas Gerais, Brazil) were purchased commercially. The chemical composition of each essential oil was identified via gas chromatography coupled with mass spectrometry. The three main compounds in the CAEO were D-limonene (18.32%), p-cymene (18.04%), and limonene 1,2-diol (7.10%). In OBEO, the main constituents were estragole (69.57%), linalool (20.70%), and (E)-α-bisabolene (2.22%) (BioEssência®, São Paulo, Brazil). In the ASEO, the highlights were di-2-propenyl-trisulfide (30.72%), diallyl disulfide (18.95%), and methyl and allyl trisulfide (11.84%) [48].

For this antibacterial assay [49], *S. aureus* and *E. coli* (100 µL; optical density: 0.5 McFarland; American Type Culture Collection, Manassas, VA, USA) were inoculated separately on Mueller–Hinton agar (Difco, BD, Sparks, MD, USA). Sterile disks (6 mm in diameter; Laborclin, Pinhais, Paraná, Brazil) were supplemented with 10 µL of CAEO, OBEO, or ASEO. These disks, including the antibiotic (azithromycin at 15 µg; Laborclin, Pinhais, Paraná, Brazil) and sterilized distilled water disks, were transferred to the surface of the already inoculated Mueller–Hinton agar (Difco, BD, Sparks, MD, USA) and incubated at 36 °C for 24 h. Inhibition zones were measured to confirm the antibacterial activities. A procedure similar to that described above was also used to evaluate the minimum inhibitory concentrations (MICs) of the essential oils. In a Petri dish containing 100 µL of the bacterial suspension seeded on Muller–Hinton agar (Difco, BD, Sparks MD, USA), sterile disks soaked with 10 µL of CAEO, OBEO, or ASEO diluted in 0.5% Tween 80 at twenty concentrations ranging from 300 to 0.0003 mg/mL were fixed. Tween 80, at a concentration of 0.5%, served as a negative control, and azithromycin at 15 µg (Laborclin, Pinhais, Paraná, Brazil) served as a positive control. The plates were incubated for 24 h at 36 °C.

The three lowest effective concentrations of each essential oil, determined via the disk diffusion method, were tested in triplicate via the broth dilution method to confirm the effectiveness of these concentrations [50]. In this test, each essential oil was diluted until it reached the three desired concentrations in test tubes containing Mueller–Hinton broth (Difco, BD, Sparks, MD, USA), followed by the addition of the bacterial strains. In one of the tubes, the essential oil was not diluted, serving as a negative control. After the tubes were incubated at 36 °C for 24 h, the efficiency of each oil was visually measured in the absence of bacterial growth. The MIC value was recorded as the lowest concentration of each essential oil that inhibited bacterial growth.

The essential oils inhibited both bacteria (Figure 10), depending on the concentration tested. CAEO demonstrated inhibitory activity against *E. coli* at concentrations ranging from 300 to 9.38 mg/mL, with inhibition zones between 28.33 and 9.33 mm. Similarly, it was effective against *S. aureus* at concentrations ranging from 300 to 1.17 mg/mL, with inhibition zones ranging from 30.33 to 7.67 mm. OBEO demonstrated antimicrobial activity against *E. coli* at concentrations ranging from 300 to 1.17 mg/mL, with inhibition zones ranging from 16.67 to 7.00 mm. For *S. aureus*, the concentrations ranged from 300 to 4.69 mg/mL, with inhibition zones between 12.67 and 7.67 mm. ASEO oil exhibited a potent inhibitory effect on *E. coli* at concentrations ranging from 300 to 0.29 mg/mL, with inhibition halos ranging from 36.33 to 7.67 mm. The inhibition of *S. aureus* occurred at concentrations ranging from 300 to 1.17 mg/mL, with halos ranging from 32.00 to 8.00 mm. As a reference, the positive control showed average inhibition zones of 23.67 mm for *E. coli* and 22.00 mm for *S. aureus*. Although higher concentrations may be more effective at reducing eggshell contamination [51], they can also result in increased operating costs, potential damage to the integrity of the shell's cuticular layer [52], and the blockage of pores [53] and a consequent reduction in the hatchability rate [12,53]. The lowest concentration of each essential oil tested, which demonstrated effectiveness in the disk diffusion method, also proved to be effective in the broth dilution method. Therefore, to prepare each essential-oil-based sanitizer, the lowest concentration of each oil that was effective against both bacteria was selected.

A flock of 58,138 breeder hens from a commercial farm located in Planaltina, the Federal District, Brazil, was subjected to rigorous and comprehensive testing. The results confirmed that the flock was completely free of *Salmonella* spp., *Mycoplasma gallisepticum*, and *Mycoplasma synoviae*, ensuring the high quality and safety of the collected eggs. This conclusion is based on extensive bacteriological and serological analyses of samples, including serum, egg, meconium, and cloacal swabs. These tests provide a reliable foundation for the subsequent experimental procedures [54,55].

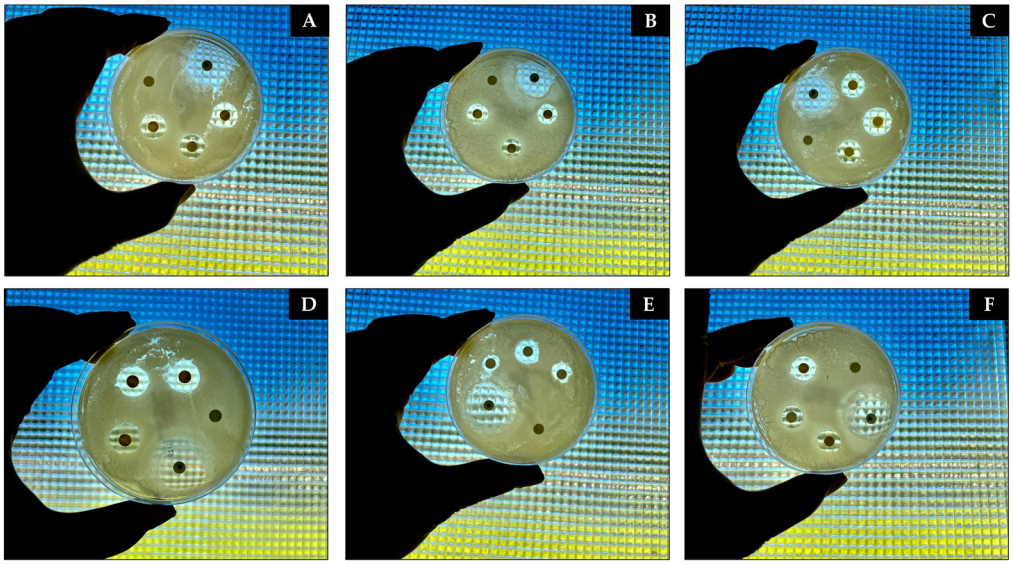


Figure 10. Photographic images of the disk diffusion assay results illustrating the initial antibacterial effects of CAEO, OBEO, and ASEO after 24 h of incubation at 36 °C. (**A**) Inhibition zone produced by ASEO against *E. coli*. (**B**) Inhibition zone observed for OBEO against *S. aureus*. (**C**) Inhibition zone generated by CAEO against *E. coli*. (**D**) Inhibition zone resulting from ASEO against *S. aureus*. (**E**) Inhibition zone of OBEO against *E. coli*. (**F**) Inhibition zone formed by CAEO against *S. aureus*.

A total of 1695 hatching eggs from broiler breeders (flock 27) aged 57 weeks, from the Pescoço Pelado Vermelho (PSÇ) lineage, were donated by this commercial farm. The accumulation of fecal matter and feed residue on the surface of hatching eggs can inactivate the antimicrobial properties of sanitizers or impair the interactions between them and microorganisms [27,56–58]. When collecting, the professionals at the donor farm discarded all the eggs that were cracked, broken, and/or had excess organic matter. Therefore, all the donated eggs were clean and intact and were subjected to sanitization using GA, FA, CAEO, OBEO, or ASEO. A control group was not subjected to any sanitization with the mentioned products. A total of 275 hatching eggs were used and appropriately identified for each treatment.

Before sanitization, the internal quality of the hatching eggs was initially tracked to assess the viability of the embryonic microenvironment and ensure that these eggs were as healthy as possible. For this analysis, 30 eggs were identified, weighed, and then carefully opened using scissors. The contents were gently dispersed on a glass surface. Initially, the height of the albumen was measured using a digital caliper. The yolk was subsequently separated from the albumen with a yolk separator, and its diameter and height were measured with a digital caliper. To complete the analysis, the pH values of the albumen and yolk of all the eggs were measured using a calibrated pH meter. The final results were obtained by applying the collected data to the formulae for the Haugh unit (HU = 100 log (albumen height + 7.57 − 1.7 egg weight$^{0.37}$) [59] and the yolk index (YI = yolk height/yolk diameter) [60].

The eggs intended for the microbiological analysis were immediately placed in sterile bags (Labplas, Sainte-Julie, QC, Canada) after collection and stored under refrigeration (approximately 5 °C) until the analysis, which was carried out 24 h later. The microbiological quality characteristics of the contents of 15 hatching eggs were evaluated using a method similar to that described by Figueiredo et al. [61], with some modifications. For the analysis, 15 mL of the egg content was collected. These samples were placed in labeled sterile plastic bags (Labplas, Sainte-Julie, QC, Canada) to which 135 mL of 0.1% peptone water (Laborclin, Pinhais, Paraná, Brazil) was added. The plastic bags containing the solutions were then homogenized for two minutes. Serial dilutions of the homogenized solutions were carried out in 0.1% peptone water (Laborclin, Pinhais, Paraná, Brazil). The bacterial load was then

quantified by plating 100 µL of the dilutions on plate count agar and red violet bile glucose agar (Ionlab, Araucária, Paraná, Brazil). These culture media were selected for counting the total aerobic mesophilic bacteria and Enterobacteriaceae, respectively. The plates were incubated at 36 °C for a period of 48 h. After this interval, the numbers of developed colonies were recorded and expressed as decimal logarithms (log_{10}).

To prepare the sanitizers, each essential oil was carefully diluted in GA until it reached the following final concentrations: CAEO, 9.38 mg/mL; OBEO, 4.69 mg/mL; and ASEO, 1.17 mg/mL. The concentration of the GA used as a vehicle for the essential oils, was 93.8%. FA, at a concentration of 5 g/m^3, was applied to the eggs through fumigation in a closed environment for 15 min, following the routine standards established at the farm. The liquid sanitizing solutions were applied to the eggs (~2.5–3 mL/egg) via hand sprayers. The eggs were left to dry naturally at room temperature for approximately 30 min. The non-sanitized eggs were kept under similar conditions. From preparing the solutions to their application, the professionals involved were properly equipped with masks, gloves, protective glasses, lab coats, and caps. According to Franco et al. [62], eggs must be sanitized within 30 min after collection, as delays in cleaning can favor a reduction in chicken viability because of contamination. All the eggs were sanitized simultaneously 20 min after collection and then sent to an experimental laboratory for artificial incubation.

We evaluated the bacterial counts on the eggshells, following a methodology similar to that used in this study for analyzing the microbiological content of the eggs, with adaptations from the protocol described by Shahein and Sedeek [36] (Figure 11). We subjected hatching eggs (nine eggs/treatment) to washing for two minutes in 0.1% peptone saline solution (Laborclin, Pinhais, Paraná, Brazil) and placed them in sterile transparent bags (Labplas, Sainte-Julie, QC, Canada). Each bag was filled with 165 mL of the solution along with three eggs/treatment. Serial dilutions of the washing solutions were carried out in 0.1% peptone water (Laborclin, Pinhais, Paraná, Brazil). We conducted this analysis in triplicate per treatment immediately after sanitization on the ninth and 18th days of incubation.

We prepared eight eggshells from each treatment for scanning electron microscopy (SEM) analysis following the protocol established by Mahato et al. [63]. After the internal contents were removed, the eggshells were carefully washed with distilled water. The eggshell membrane was then removed using tweezers. A small equatorial section of the shell, approximately 5 mm × 5 mm in size, was subsequently extracted and boiled in a 2% sodium hydroxide solution for 10 min to remove any residual membrane. The samples were rinsed again with distilled water and left to air-dry at room temperature for 12 h. The dried samples were then metalized and examined under a JEOL JSM-7001F scanning electron microscope (Jeol Ltd., Akishima, Tokyo, Japan) at standard magnifications of ×100, ×500, ×1000, and ×3000.

Before the hatching eggs arrived at the experimental laboratory, the egg storage chamber and setters were rigorously cleaned and sanitized. The storage chamber and setters were initially washed with water and detergent. Then, the sanitizer Lysoform (2.5%; SC Johnson, Racine, WI, USA) was applied and left to act for at least 10 min, according to the manufacturer's recommendation. The eggs were stored for 24 h in a storage chamber with a controlled temperature between 19 and 21 °C and a relative humidity between 50 and 60%. After the storage period, the incubation and hatching methodology adopted by Oliveira et al. [64] was used. Briefly, the eggs were weighed after storage and incubated in single-stage setters (Chocmaster, Curitiba, Paraná, Brazil), as illustrated in Figure 12. A total of four setters were used. In each incubation tray, the eggs from each treatment group were distributed randomly. During incubation, the mean temperature was maintained at 37.7 °C, and the mean relative humidity was 60%. Egg turning occurred automatically every hour, as recommended by Oliveira et al. [65]. Candling was performed individually on each egg on the 10th day of incubation, allowing for the removal of infertile eggs and those with embryonic mortality. During the birth period, the mean temperature was adjusted to 36.6 °C, and the mean relative humidity was adjusted to 65%. The climatic variables of the incubation room were monitored every five minutes via a HOBO data logger (Onset,

Bourne, MA, USA) during the 21 days of incubation, ensuring the optimal functioning of the setters.

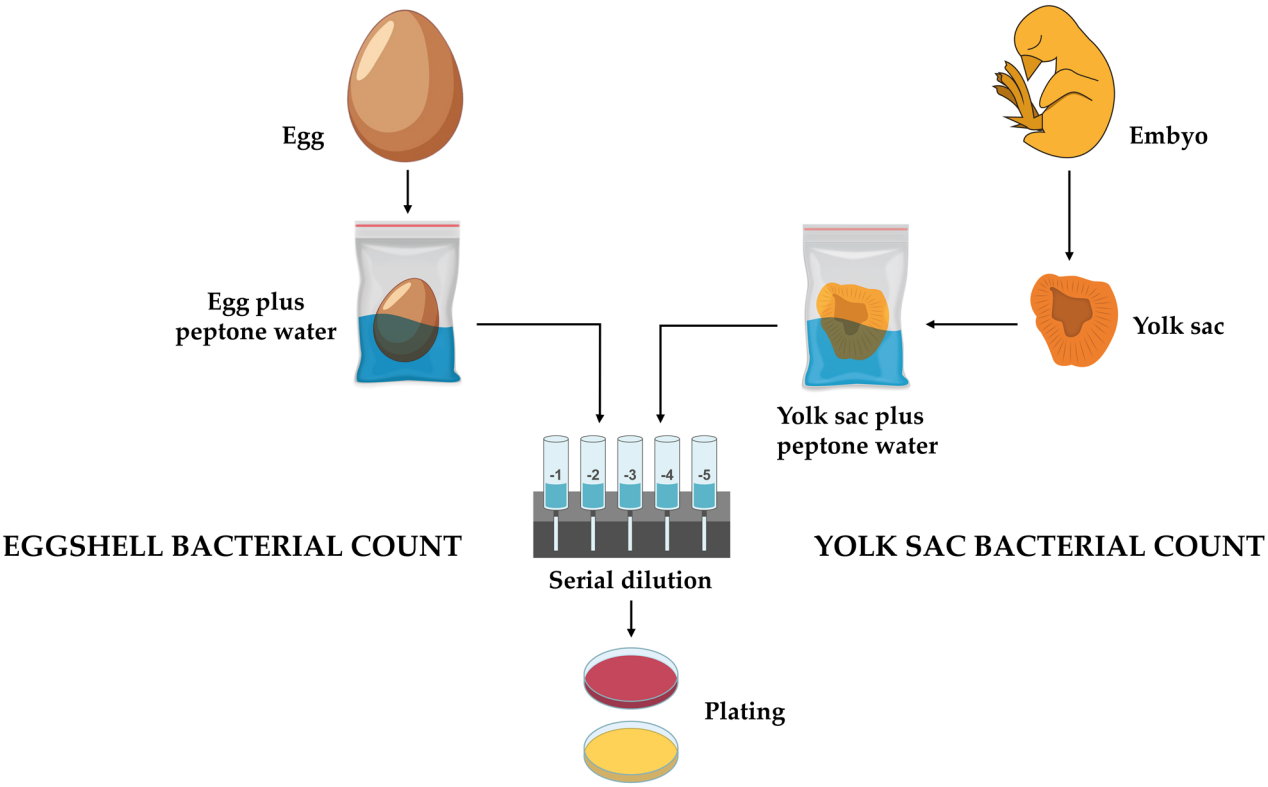


Figure 11. Detailed schematic of the bacterial count protocol for eggshells and yolk sacs. This diagram illustrates the process from sample extraction (egg or embryo/yolk sac) to the plating of homogenized solutions, ensuring the precise quantification of the bacterial presence. Yellow plate: mesophilic bacteria count; red plate: Enterobacteriaceae count.

The parameters used to evaluate the feasibility of the egg sanitization during incubation and hatching were as follows: egg weight loss (%), hatchability of fertile eggs (%), early dead (%), mid dead (%), late dead (%), and contaminated eggs (%), according to the methodology described by Baylan et al. [66], with very few changes. To evaluate the quality of the hatched chicks, we used the Pasgar score, which considers the reflexes, navel, legs, and beak, according to the methodology described by Boerjan [67]. Next, the bodyweights of the same chicks were measured using a high-precision analytical scale (Gehaka, São Paulo, São Paulo, Brazil).

In accordance with the methodology of Upadhyaya et al. [68], the bacterial load of the yolk sac contents was determined. Seven chick embryos from each treatment were removed from the setter at 18 days of development and euthanized by cervical dislocation by a trained researcher, with the aim of collecting the contents of the yolk sac. To ensure the sterility and integrity of the procedure, the bags containing the yolk sac contents were transferred to sterile transparent bags (Labplas, Sainte-Julie, QC, Canada), each containing 150 mL of 0.1% peptone saline solution (Laborclin, Pinhais, Paraná, Brazil). The contents were then homogenized for two minutes. The homogenized solutions were subjected to serial dilutions following a careful and standardized protocol. This procedure employed the same microbiological counting methods as those used previously (Figure 11).

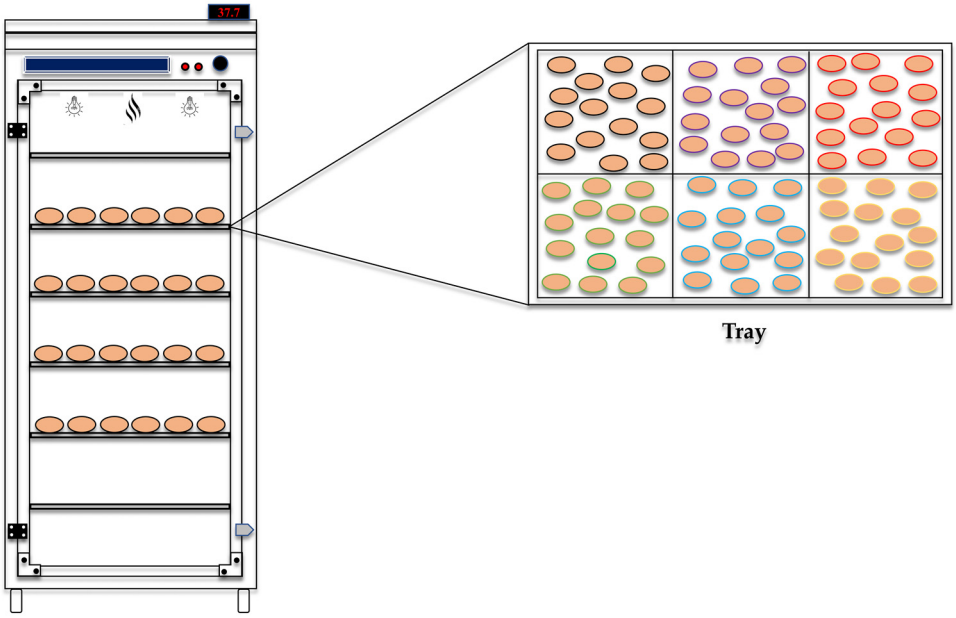


Figure 12. Schematic representation of egg incubation under different experimental conditions in the setter. In this figure, we present a detailed illustration of the arrangement of the hatching eggs inside the setter, highlighting each treatment's organization and specific environment, which may vary across trays on the basis of randomness. The colored outlines clearly identify the six different treatments applied during this study, facilitating the distinction of and comparison between the experimental conditions.

In this investigation, six embryos from each treatment group at 18 days of development were euthanized by cervical dislocation to assess the effects of the sanitizing solutions on the poultry respiratory system. In line with ethical and methodological standards, necropsies were conducted, and tissue samples from the tracheae and lungs were collected. These samples were fixed in 10% buffered formalin (pH 7.0), embedded in paraffin, and stained with hematoxylin and eosin (H&E). A semi-quantitative analysis of the pathological changes in all the tissues was performed, and the results were categorized as absent (−), mild (+), moderate (++), or severe (+++). Epithelial cell degeneration/necrosis, goblet cell hyperplasia, and lymphocytic inflammation were the main lesions evaluated in the tracheae (adapted from Hayretdağ and Kolankaya [46]). Bronchial epithelial degeneration/necrosis and congestion were histologically assessed in the lungs.

We measured the toxicity profiles of the sanitizing solutions on embryos via HET-CAM [69]. A total of 120 non-sanitized hatching eggs (mean weight: 56.33 ± 3.64 g) from 51-week-old PSÇ-lineage broiler breeders (batch 21) were incubated until the 9th day of embryonic development, under the same conditions described in the "incubation and hatching" section, in a single-stage setter (Premium Ecologica, Belo Horizonte, Minas Gerais, Brazil). After this period, all the eggs were subjected to candling to confirm embryonic development. In embryonated eggs, an opening was made in the shell above the air chamber with the help of surgical scissors. After being moistened with a 0.9% saline solution, the eggshell membranes were carefully removed to expose the CAMs. Then, 200 µL of each sanitizing solution, at the same concentration used to sanitize the eggshells, was pipetted onto the membrane. Each sanitizing solution was applied to eight eggs, except for the treatments with the essential oils, which were applied to 16 eggs: eight with GA as the carrier and eight without GA as the carrier. The harmful effects of the sanitizing solutions were evaluated based on the degrees of hemorrhage, coagulation, and lysis of the blood vessels of the CAMs, as described in detail in the study by Derouiche and Abdennour [70]. Observations, after the sanitizing solutions were applied, were carried out with the aid of a magnifying glass, allowing for detailed monitoring of their effects on the CAMs.

We conducted the experiment in accordance with a completely randomized design, which included six treatments and four repetitions in each treatment group, with 60 eggs per repetition. We performed the bacterial counts of the eggshells in triplicate, and the bacterial analysis of the yolk sac was conducted in seven repetitions, with each embryo considered as an independent experimental unit. Analysis of variance was performed via PROC GLM. Tukey's test was used to compare the treatment means ($p < 0.05$). We used SAS Studio University Edition software (SAS Inst. Inc., Cary, NC, USA) for all the statistical analyses.

4. Conclusions

The natural and safe sanitization plan using CAEO, OBEO, and ASEO, as tested and described in this study, provides effective bacterial control for hatching eggs, increasing hatching rates, and resulting in healthier poultry without morphological alterations. This work reaffirms the promise of essential oils as a feasible solution for the sanitary management of hatching eggs. The early use of essential oils during the pre-incubation phase for the bacterial treatment of the eggshells was driven by the pursuit of safer and more effective interventions in the poultry sector. The high efficiency of the essential oils at low concentrations, as reflected in the reduced amounts needed for formulating sanitizers, can help to reduce the higher costs associated with these products compared to some synthetic alternatives, which could hinder their use. Another point to consider is that the combined application of essential oils with practical, economical, and efficient sanitization methods can eliminate the need to increase the concentrations of essential oils in egg-hatching sanitization practices, thereby minimizing the undesirable economic effects associated with the use of higher concentrations. Another important aspect for making them economically feasible is the selection of oils extracted from plants cultivated in various parts of the world or those that are easily accessible, such as CAEO, OBEO, and ASEO. Our findings expand the understanding of the topical application of essential oils to hatching eggs. According to the results of this study, we suggest a detailed protocol for sanitizing hatching eggs with essential oils (Table 7). Considering concerns regarding bacterial resistance, we recommend the cautious use of essential oils and the adoption of proper application methodologies and a management plan that promotes the periodic rotation of at least two different essential oils, along with frequent monitoring of the microbiota present on the eggshells.

Table 7. Step-by-step instructions for sanitizing hatching eggs with essential oils.

Step	Activity
1	Choose an essential oil with a proven effectiveness.
2	Add the essential oil to the correct amount of grain alcohol—the lower the concentration required for the microbial control of the eggshell the better.
3	Mix the solution carefully to ensure the proper dispersion of the essential oil.
4	After dilution, the sanitizer will be ready for use.
5	Store the sanitizer in a clean, labeled spray bottle in a cool, dark place.
6	Be sure to wear personal protective equipment and wash your hands before handling the sanitizer.
7	Apply the sanitizer as soon as possible after egg collection.
8	Choose eggs with the cleanest possible shells, avoiding those that are excessively dirty.
9	Do not wash the eggs before applying the sanitizer.
10	Position an egg so that the surface is accessible for sanitizer application.
11	Spray the sanitizer over the entire surface of the egg, ensuring uniform coverage.
12	Avoid applying excess sanitizer (use an average of 3 mL/egg).
13	Allow the eggs to dry naturally at room temperature.
14	After complete drying, store the eggs in a sanitized place until incubation.
15	When sanitization is complete, dispose of the sanitizer in a suitable manner.

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References

1. Amer, M.M.; Elbayoumi, K.M.; Amin Girh, Z.M.S.; Mekky, H.M.; Rabie, N.S. A Study on Bacterial Contamination of Dead in Shell Chicken Embryos and Culled One Day Old Chicks. *Int. J. Pharm. Clin. Res.* **2017**, *7*, 5–11.
2. Addo, M.G.; Raissa, M.A.; Badu, K.; Agordzo, S. Bacterial Contamination of Chicken Eggs from Poultry Farms and Retail Markets in The New Juaben Municipality, Ghana. *Microbiol. Res. J. Int.* **2020**, *30*, 58–66. [CrossRef]
3. Rezaee, M.S.; Liebhart, D.; Hess, C.; Hess, M.; Paudel, S. Bacterial Infection in Chicken Embryos and Consequences of Yolk Sac Constitution for Embryo Survival. *Vet. Pathol.* **2021**, *58*, 71–79. [CrossRef] [PubMed]
4. Gehan, Z.M.; Anwer, W.; Amer, H.M.; EL-Sabagh, I.M.; Rezk, A.; Badawy, E.M. In Vitro Efficacy Comparisons of Disinfectants Used in the Commercial Poultry Farms. *Int. J. Poult. Sci.* **2009**, *8*, 237–241.
5. Rui, B.R.; Angrimani DD, S.R.; Cruz, L.V.; Machado, T.L.; Lopes, H.C. Principais Métodos de Desinfecção e Desinfectantes Utilizados na Avicultura: Revisão de Literatura. *Rev. Científica Eletrônica De Med. Veterinária Garça* **2011**, *9*, 1–14.
6. Amoah, I.B.; Asiedu, P.; Arthur, C.T.; Aboagye, I.F. Effect of Formaldehyde Treatment on Bacteria-Infected Hatching Eggs of *Gallus gallus domesticus* Linnaeus, 1758. *Ghana J. Sci.* **2020**, *61*, 82–90. [CrossRef]
7. Aidaros, H.; Hafez, E.; El Bahgy, H. In Vitro Efficacy Testing of Some Commercial Disinfectants Against Pathogenic Bacteria Isolated from Different Poultry Farms. *Adv. Anim. Vet. Sci.* **2022**, *10*, 1–8. [CrossRef]
8. Takigawa, T.; Endo, Y. Effects of Glutaraldehyde Exposure on Human Health. *J. Occup. Health* **2006**, *48*, 75–87. [CrossRef]
9. Kim, K.-H.; Jahan, S.A.; Lee, J.-T. Exposure to Formaldehyde and Its Potential Human Health Hazards. *J. Environ. Sci. Health Part C* **2011**, *29*, 277–299. [CrossRef]
10. Luz, A.; DeLeo, P.; Pechacek, N.; Freemantle, M. Human Health Hazard Assessment of Quaternary Ammonium Compounds: Didecyl Dimethyl Ammonium Chloride and Alkyl (C12–C16) Dimethyl Benzyl Ammonium Chloride. *Regul. Toxicol. Pharmacol.* **2020**, *116*, 104717. [CrossRef]
11. Wijaya, R.I.; Ilyas, M. Allergic Contact Dermatitis Among Healthcare Workers Exposed to Glutaraldehyde. *Indones. J. Community Occup. Med.* **2021**, *1*, 94–100. [CrossRef]
12. Oliveira, G.d.S.; dos Santos, V.M.; Nascimento, S.T. Essential Oils as Sanitisers for Hatching Eggs. *Worlds Poult. Sci. J.* **2021**, *77*, 605–617.
13. Oliveira, G.D.S.; McManus, C.; de Araújo, M.V.; de Sousa, D.E.R.; de Macêdo, I.L.; de Castro, M.B.; dos Santos, V.M. Sanitizing Hatching Eggs with Essential Oils: Avian and Microbiological Safety. *Microorganisms* **2023**, *11*, 1890. [CrossRef]
14. Oliveira, G.d.S.; McManus, C.; Vale, I.R.R.; Dos Santos, V.M. Obtaining Microbiologically Safe Hatching Eggs from Hatcheries: Using Essential Oils for Integrated Sanitization Strategies in Hatching Eggs, Poultry Houses and Poultry. *Pathogens* **2024**, *13*, 260. [CrossRef]
15. Lizarraga-Valderrama, L.R. Effects of Essential Oils on Central Nervous System: Focus on Mental Health. *Phyther. Res.* **2021**, *35*, 657–679. [CrossRef]
16. Mota, K.S.D.; Garcia, A.C.M.; Santos, M.D.; Costa, I.C.P. Effect of Topical Use of Essential Oils on Wound Healing in Humans: Systematic Review Protocol. *Research, Soc. Dev.* **2022**, *11*, e165111436096. [CrossRef]

17. Alves-Silva, J.M.; Zuzarte, M.; Girão, H.; Salgueiro, L. The Role of Essential Oils and Their Main Compounds in the Management of Cardiovascular Disease Risk Factors. *Molecules* **2021**, *26*, 3506. [CrossRef]
18. Lesgards, J.-F.; Baldovini, N.; Vidal, N.; Pietri, S. Anticancer Activities of Essential Oils Constituents and Synergy with Conventional Therapies: A Review. *Phyther. Res.* **2014**, *28*, 1423–1446. [CrossRef]
19. Kamatyanatti, M.; Nagre, P.K.; Ramteke, V.; Baghe, M.M. Evaluation of Acid Lime (*Citrus aurantifolia* swingle) Genotypes During Hasth Bahar for Growth, Yield and Quality Attributes. *Ecoscan* **2016**, *9*, 277–283.
20. Nurzyńska-Wierdak, R. Comparing the Growth and Flowering of Selected Basil (*Ocimum basilicum* L.) Varieties. *Acta Agrobot.* **2007**, *60*, 127–131. [CrossRef]
21. Raja, H.; Ram, C.N.; Bhargav, K.K.; Pandey, M.; Jain, A. Genetic Variability Assessment in Garlic (*Allium sativum* L.) Genotypes. *J. Pharmacogn. Phytochem.* **2017**, *6*, 1781–1786.
22. Mohammed, I.O.; Alrasheid, A.A.; Hussein Ayoub, S.M. GC-MS Analysis and Study of the Antimicrobial Activity of *Citrus paradisi*, *Citrus aurantifolia*, and *Citrus sinensis* Peel Essential Oils as Hand Sanitizer. *Int. J. Microbiol.* **2024**, 4957712. [CrossRef] [PubMed]
23. Hamid, S.; Oukil, N.F.; Moussa, H.; Djihad, N.; Mróz, M.; Kusznierewicz, B.; Attia, A.; Djenadi, K.; Mahdjoub, M.M.; Bouhenna, M.M.; et al. Chemical and Biological Characterization of *Ocimum basilicum* L. Phenolic Extract and Essential Oil Derived Through Ultrasound and Microwave-Assisted Extraction Techniques. *Food Biosci.* **2024**, *60*, 104359. [CrossRef]
24. Yasin, G.; Jasim, S.A.; Mahmudiono, T.; Al-Shawi, S.G.; Shichiyakh, R.A.; Shoukat, S.; Kadhim, A.J.; Iswanto, A.H.; Saleh, M.M.; Fenjan, M. Investigating the Effect of Garlic (*Allium sativum*) Essential Oil on Foodborne Pathogenic Microorganisms. *Food Sci. Technol.* **2022**, *42*, e03822. [CrossRef]
25. Oliveira, G.D.S.; McManus, C.; Sousa, H.A.D.F.; Santos, P.H.G.D.S.; dos Santos, V.M. A Mini-Review of the Main Effects of Essential Oils from *Citrus aurantifolia*, *Ocimum basilicum*, and *Allium sativum* as Safe Antimicrobial Activity in Poultry. *Animals* **2024**, *14*, 382. [CrossRef]
26. Freitas AG, D. *Efeito da Fumigação de Nascedouros com Formaldeído Sobre o Trato Respiratório E Desempenho de Frangos de Corte*; Graduation Completion Work; Federal University of Uberlândia: Minas Gerais, Brazil, 2007.
27. Cadirci, S. Disinfection of Hatching Eggs by Formaldehyde Fumigation-a Review. *Eur. Poult. Sci.* **2009**, *73*, 116–123.
28. Nielsen, G.D.; Wolkoff, P. Cancer Effects of Formaldehyde: A Proposal for an Indoor Air Guideline Value. *Arch. Toxicol.* **2010**, *84*, 423–446. [CrossRef]
29. Instituto Nacional de Câncer (Brasil). *Formaldeído, Câncer e os Riscos para a Saúde/Instituto Nacional de Câncer*; INCA: Rio de Janeiro, Brazil, 2024; 22p.
30. Mustafa, A.A.; Mirza, R.A.; Aziz, H.I. Lavender Essential Oil in Sanitation on Fertile Egg. *Passer. J. Basic. Appl. Sci.* **2023**, *5*, 377–381. [CrossRef]
31. Yildirim, I.; Ozsan, M.; Yetisir, R. The Use of Oregano (*Origanum vulgare* L.) Essential Oil as Alternative Hatching Egg Disinfectant versus Formaldehyde Fumigation in Quails (*Coturnix coturnix* japonica). *Eggs. Rev. Med. Vet.* **2003**, *154*, 367–370.
32. Bekhet, G.; Khalifa, A.Y.Z. Essential Oil Sanitizers to Sanitize Hatching Eggs. *J. Appl. Anim. Res.* **2022**, *50*, 695–701. [CrossRef]
33. Bai, J.; Li, J.; Chen, Z.; Bai, X.; Yang, Z.; Wang, Z.; Yang, Y. Antibacterial Activity and Mechanism of Clove Essential Oil Against Foodborne Pathogens. *LWT* **2022**, *173*, 114249. [CrossRef]
34. Yuan, Y.; Liu, L.; Guo, L.; Wang, L.; Liu, Y. Antibacterial Mechanism of Rose Essential Oil Against *Pseudomonas Putida* Isolated from White *Hypsizygus Marmoreus* at Cellular And Metabolic Levels. *Ind. Crops Prod.* **2023**, *196*, 116523. [CrossRef]
35. Decuypere, E.; Tona, K.; Bruggeman, V.; Bamelis, F. The Day-Old Chick, A Crucial Hinge Between Breeders and Broilers. *World's Poult. Sci. J.* **2001**, *57*, 127–138. [CrossRef]
36. Shahein, E.H.A.; Sedeek, E.K. Role of Spraying Hatching Eggs with Natural Disinfectants on Hatching Characteristics and Eggshell Bacterial Counts. *Egypt. Poult. Sci. J.* **2014**, *34*, 213–230. [CrossRef]
37. Molenaar, R.; Reijrink, I.A.M.; Meijerhof, R.; Van den Brand, H. Meeting Embryonic Requirements of Broilers Throughout Incubation: A review. *Rev. Bras. Cienc. Avic.* **2010**, *12*, 137–148. [CrossRef]
38. Pires, P.G.S.; Leuven, A.F.R.; Franceschi, C.H.; Machado, G.S.; Pires, P.D.S.; Moraes, P.O.; Kindlein, L.; Andretta, I. Effects of Rice Protein Coating Enriched with Essential Oils on Internal Quality and Shelf Life of Eggs During Room Temperature Storage. *Poult. Sci.* **2020**, *99*, 604–611. [CrossRef]
39. Sun, R.; Song, G.; Zhang, H.; Zhang, H.; Chi, Y.; Ma, Y.; Li, H.; Bai, S.; Zhang, X. Effect of Basil Essential Oil and Beeswax Incorporation on The Physical, Structural, and Antibacterial Properties of Chitosan Emulsion Based Coating for Eggs Preservation. *LWT* **2021**, *150*, 112020. [CrossRef]
40. Oliveira, G.D.S.; McManus, C.; Pires, P.G.D.S.; dos Santos, V.M. Combination of Cassava Starch Biopolymer and Essential Oils for Coating Table Eggs. *Front. Sustain. Food Syst.* **2022**, *6*, 957229. [CrossRef]
41. Oliveira, G.S.; Nascimento, S.T.; dos Santos, V.M.; Silva, M.G. Clove Essential Oil in the Sanitation of Fertile Eggs. *Poult. Sci.* **2020**, *99*, 5509–5516. [CrossRef]
42. Bekhet, G.M. Impact of Hatching Egg Disinfection on Hatching Characteristics and Chick Embryos. *Indian. J. Anim. Res.* **2021**, *55*, 353–358. [CrossRef]
43. Li, X.; Anderson, D.; Rathgeber, B.; McLean, N.; MacIsaac, J. Fumigating Broiler Hatching Eggs with Lysozyme Product (Inovapure) to Reduce Eggshell Microbial Load. *Poult. Sci.* **2018**, *97*, 4252–4261. [CrossRef] [PubMed]

44. Oliveira, G.S.; Nascimento, S.T.; dos Santos, V.M.; Dallago, B.S.L. Spraying Hatching Eggs with Clove Essential Oil Does Not Compromise the Quality of Embryos and One-Day-Old Chicks or Broiler Performance. *Animals* **2021**, *11*, 2045. [CrossRef] [PubMed]
45. Aviagen How to Measure Chick Yield. 2011. Available online: http://en.aviagen.com/assets/Tech_Center/BB_Resources_Tools/Hatchery_How_Tos/02HowTo2MeasureChickYield.pdf (accessed on 16 July 2024).
46. Hayretdağ, S.; Kolankaya, D. Investigation of the Effects of Pre-Incubation Formaldehyde Fumigation on the Tracheal Epithelium of Chicken Embryos and Chicks. *Turk. J. Vet. Anim. Sci.* **2008**, *32*, 263–267.
47. Corrêa, A.N.R.; Weimer, P.; Rossi, R.C.; Hoffmann, J.F.; Koester, L.S.; Suyenaga, E.S.; Ferreira, C.D. Lime and Orange Essential Oils and D-Limonene as A Potential COVID-19 Inhibitor: Computational, in Chemico, and Cytotoxicity Analysis. *Food Biosci.* **2023**, *51*, 102348. [CrossRef]
48. Vale, I.R.R.; Oliveira, G.d.S.; McManus, C.; de Araújo, M.V.; Salgado, C.B.; Pires, P.G.d.S.; de Campos, T.A.; Gonçalves, L.F.; Almeida, A.P.C.; Martins, G.D.S.; et al. Whey Protein Isolate and Garlic Essential Oil as an Antimicrobial Coating to Preserve the Internal Quality of Quail Eggs. *Coatings* **2023**, *13*, 1369. [CrossRef]
49. Bauer, A.W.; Kirby, W.M.M.; Sherris, J.C.; Turck, M. Antibiotic Susceptibility Testing by a Standardized Single Disk Method. *Am. J. Clin. Pathol.* **1966**, *45*, 493–496. [CrossRef]
50. Wiegand, I.; Hilpert, K.; Hancock, R.E. Agar and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances. *Nat. Protoc.* **2008**, *3*, 163–175. [CrossRef]
51. Chung, H.; Kim, H.; Myeong, D.; Kim, S.; Choe, N.H. Effect of Chlorine Dioxide Gas Application to Egg Surface: Microbial Reduction Effect, Quality of Eggs, and Hatchability. *Korean J. Food Sci. Anim. Resour.* **2018**, *38*, 487–497.
52. He, Z.; Chen, X.; Shi, X.; Li, X.; Li, C.; Li, J.; Xu, G.; Yang, N.; Zheng, J. Acetic Acid, Vinegar, and Citric Acid as Washing Materials for Cuticle Removal to Improve Hatching Performance of Quail Eggs. *Poult. Sci.* **2020**, *99*, 3865–3876. [CrossRef]
53. Hasyim, A.R.; Lase, J.A.; Alwiyah, A.; Suroto, S.; Khairiyah, K.; Hutagalung, M.; Harahap, S.M.; el Ramija, K.; Lestari, D.; Ardiarini, N.; et al. The Effectiveness of Cherry Leaf Extract (*Muntingia calabura* L.) as an Antibacterial Against Hatchability of Kub Chicken Eggs in Artificial Hatchery. *Bul. Peternak.* **2021**, *45*, 214. [CrossRef]
54. Ministério da Agricultura, Pecuária e Abastecimento. Portaria n° 208, de 20 de Dezembro de 1994. Aprova Normas de Credenciamento e Monitoramento de Laboratórios de Diagnóstico das Micoplasmoses Aviárias. Diário Oficial União. 26 dez 1994. Seção 1, p. 20510. Available online: https://www.defesa.agricultura.sp.gov.br/legislacoes/portaria-sda-208-de-20-12-1994,373.html (accessed on 20 May 2024).
55. Ministério da Agricultura, Pecuária e Abastecimento. Portaria n° 126, de 03 de Novembro de 1995. Aprova Normas Para Credeciamento e Monitoramento de Laboratórios de Diagnóstico das Salmonellas Aviárias (S. Enteritidis, S. Gallinarum, S. Pullorum e S. Typhimurium). Diário Oficial União. 6 nov 1995. Seção 1, p. 17694. Available online: https://www.defesa.agricultura.sp.gov.br/legislacoes/portaria-sda-126-de-03-11-1995,372.html (accessed on 20 May 2024).
56. Brake, J.; Sheldon, B.W. Effect of A Quaternary Ammonium Sanitizer for Hatching Eggs on Their Contamination, Permeability, Water Loss and Hatchability. *Poult. Sci.* **1990**, *69*, 517–525. [CrossRef] [PubMed]
57. Bailey, J.S.; Cox, N.A.; Berrang, M.E. Bactericidal Treatment of Hatching Eggs III: Effect of Organic Contaminants on Efficacy of Egg Sanitizers. *J. Appl. Poult. Res.* **2001**, *10*, 117–120. [CrossRef]
58. Nogueira, W.C.L.; Pena, A.C.S.; de Souza, C.N.; Azevedo, I.L.; Fariafilho, D.E.; Almeida, A.C. Disinfection of Fertile Eggs of Free-Range Poultry with Essential Oils. *Rev. Bras. Saude Prod. Anim.* **2019**, *20*, e0822019. [CrossRef]
59. Haugh, R.R. A New Method for Determining the Quality of an Egg. *US Egg Poult.* **1937**, *39*, 27–49.
60. Funk, E.M. The relation of the Yolk Index Determined in Natural Position to the Yolk Index as Determined after Separating the Yolk from the Albumen. *Poult. Sci.* **1948**, *27*, 367. [CrossRef]
61. Figueiredo, T.C.; Assis, D.C.S.; Menezes, L.D.M.; Oliveira, D.D.; Lima, A.L.; Souza, M.R.; Heneine, L.G.D.; Cançado, S.V. Effects of Packaging, Mineral Oil Coating, and Storage Time on Biogenic Amine Levels and Internal Quality of Eggs. *Poult. Sci.* **2014**, *93*, 3171–3178. [CrossRef]
62. Franco, C.M.; Araújo, I.C.; Lopes, T.S.; Gonçalves, T.F.; Costa, B.T.; Sousa, L.S.; Araújo, I.M.; Souza, M.R.; Lara, L.J. Sanitization at Different Times After Floor Egg Collection for Hatchability, Microbiological Quality, and Performance of Broilers. *Poult. Sci.* **2023**, *102*, 103156. [CrossRef]
63. Mahato, P.L.; Weatherby, T.; Ewell, K.; Jha, R.; Mishra, B. Scanning Electron Microscope-Based Evaluation of Eggshell Quality. *Poult. Sci.* **2024**, *103*, 103428. [CrossRef] [PubMed]
64. Oliveira, G.S.; dos Santos, V.M.; Nascimento, S.T.; Rodrigues, J.C. Alternative Sanitizers to Paraformaldehyde for Incubation of Fertile Eggs. *Poult. Sci.* **2020**, *99*, 2001–2006. [CrossRef]
65. Oliveira, G.D.S.; dos Santos, V.M.; Rodrigues, J.C.; Nascimento, S.T. Effects of Different Egg Turning Frequencies on Incubation Efficiency Parameters. *Poult. Sci.* **2020**, *99*, 4417–4420. [CrossRef]
66. Baylan, M.; Akpınar, G.C.; Canogullari, S.D.; Ayasan, T. The Effects of Using Garlic Extract for Quail Hatching Egg Disinfection on Hatching Results and Performance. *Rev. Bras. Cienc. Avic.* **2018**, *20*, 343–350. [CrossRef]
67. Boerjan, M. Chick Vitality and Uniformity. *Int. Hatch. Pract.* **2006**, *20*, 7–8.
68. Upadhyaya, I.; Yin, H.B.; Nair, M.S.; Chen, C.H.; Upadhyay, A.; Darre, M.J.; Venkitanarayanan, K. Efficacy of Fumigation with Trans-Cinnamaldehyde and Eugenol in Reducing *Salmonella enterica* serovar Enteritidis on Embryonated Egg Shells. *Poult. Sci.* **2015**, *94*, 1685–1690. [CrossRef] [PubMed]

69. Luepke, N. Hen's Egg Chorioallantoic Membrane Test for Irritation Potential. *Food Chem. Toxicol.* **1985**, *23*, 287–291. [CrossRef]
70. Derouiche, M.T.T.; Abdennour, S. HET-CAM test. Application to Shampoos in Developing Countries. *Toxicol. Vitr.* **2017**, *45*, 393–396. [CrossRef]

Paper six

Multivariate Analysis of Microbiological and Incubation Parameters in Hatching Eggs Sanitized with or Without Essential Oils

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Article

Multivariate Analysis of Microbiological and Incubation Parameters in Hatching Eggs Sanitized with or Without Essential Oils

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Simple Summary

In the poultry chain, the beneficial interaction between health and productivity must be constant. These elements need to work together to ensure efficient and safe results. For example, the implementation of hatching egg sanitization practices has proven to be an effective strategy for achieving good productivity rates and obtaining healthy chicks. It is essential to understand how these factors correlate to ensure both bird health and maximum productivity. Therefore, we conducted a multivariate analysis of microbiological and incubation parameters to evaluate whether bacterial contamination of the eggshell and yolk sac negatively affects hatchability (HI) and to compare the effectiveness of different sanitization protocols in reducing bacterial contamination in these regions.

Abstract

Aspects related to the contamination of hatching eggs, sanitary management during pre-incubation, and the performance of the incubation process can compromise productive efficiency in poultry farming. When these factors negatively influence poultry farming, they can destabilize the generation and distribution of financial resources throughout the production chain, as well as limit public access to poultry-derived proteins. Understanding how these aspects are interrelated is essential for making decisions that benefit poultry health and productivity. Therefore, we conducted a multivariate analysis of microbiological and incubation parameters to evaluate whether bacterial contamination of the eggshell and yolk sac negatively affects HI and to compare the effectiveness of different sanitization protocols in reducing bacterial contamination in these regions. To achieve this, we utilized the raw data from our previous research on the sanitization of hatching eggs and conducted a detailed statistical analysis to evaluate the relationships between the studied variables. The correlation analysis revealed that eggshell mesophilic bacterial contamination (EGM) was strongly associated with yolk sac mesophilic bacterial contamination (YSM) (r = 0.76) and yolk sac contamination by Enterobacteriaceae (YSE) (r = 0.73). The principal component analysis indicated a negative association between HI performance and eggshell and yolk sac contamination. Results indicated beneficial associations between the reduction of contamination in hatching eggs and increased hatchability rates when using essential oils. The bacterial load of hatching eggs contributes to reduced productivity, reaffirming the need for proper egg sanitization, especially using essential oils.

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Keywords: eggshell contamination; egg sanitization; hatchability; poultry production; sanitizers; yolk sac contamination

1. Introduction

Egg contamination is of interest due to its occurrence in non-sterile environments [1], exposure to unsanitary conditions after laying [2], and, most importantly, the effects of this contamination on poultry health. Egg contamination has been found to be associated with yolk sac infection [3]. This contamination has been previously described through two routes: vertical, which occurs during the formation of the egg, meaning bacteria are already present in the egg's content when it is formed; and horizontal, when the already-formed egg is exposed to bacteria that penetrate the shell and migrate into the contents [3]. Each route of contamination requires a strategic control plan aligned with poultry health.

When it comes to sanitizing hatching eggshells, horizontal bacterial contamination is more relevant than vertical contamination, both from a productive and economic standpoint, as it is the only type that can be directly minimized through the topical application of sanitizer on the eggshell [4]. Reducing surface contamination is critical for four main reasons: (1) reducing the presence of pathogenic microbiota; (2) minimizing complications arising from this microbiota, including infections and embryonic mortality; (3) preventing cross-microbial transmission in the poultry environment; and (4) ensuring that poultry professionals are exposed to minimal microbiological risks in the workplace.

Proper sanitization is a viable option to reduce contamination of eggshells [5]. This process involves the application of synthetic or natural chemical compounds to surfaces, whether they are inanimate or not, in effective doses, following the manufacturer's recommendations or based on scientific evidence, and according to the specific needs of each stage of poultry production. This procedure is followed by the conservation and monitoring of the sanitized surface. The frequency of sanitization may vary from minutes to months, depending on the production stage. For instance, after egg collection, the sanitation of hatching eggs typically occurs at intervals of minutes, with the application being carried out based on the frequency of collection, the source batch, and the amount that the sanitization environment can accommodate.

The effectiveness of hatching egg sanitization in reducing contamination can vary significantly. In their review, Oliveira et al. [6] compared formaldehyde, the primary compound used for egg sanitization, with essential oils. They demonstrated that both substances exhibit significant antibacterial activity in reducing eggshell bacteria. This is due to the specific antibacterial mechanisms of each. As reviewed by Ricke et al. [7], formaldehyde targets the spore cores and cell walls of bacteria. On the other hand, Li et al. [8] reported that essential oils inhibit bacteria by interacting with the cell membrane, impairing its function and physiological activity through direct interaction with the hydrocarbon chains of the phospholipid tails. However, the antibacterial effects of formaldehyde and essential oils in reducing eggshell contamination are not always similar, and one may be more effective than the other, due to the influence of factors such as the intrinsic characteristics of each (see review by Oliveira et al. [6]).

Sanitizers for hatching eggs can be validated in both laboratory and industrial settings not only based on microbial levels on the eggs, but also on incubation outcomes, or a combination of both [9–13]. The incubation results encompass various parameters, such as egg weight loss, chick characteristics, mortality rates, and, most importantly, hatchability (HI) [14,15]. The latter parameter serves as the primary indicator for assessing the efficiency of the incubation process. In parallel, the microbiological parameters include the microbial

count present on the eggshell and in the embryos. Previous studies have indicated that various pre-incubation and incubation factors can influence HI rates [16,17]. However, these studies have not delved into the specific aspects of egg sanitization that truly influence these variations.

This study aims to fill this gap by applying multivariate analysis of microbiological and incubation parameters, exploring the relationship between contamination of the eggshell and yolk sac (mesophilic bacteria and Enterobacteriaceae) with HI in hatching eggs sanitized or not with essential oils. Multivariate analysis is a methodology that allows the simultaneous evaluation of multiple factors, providing a more precise understanding of the relationships between bacterial contamination, sanitization, and HI.

2. Materials and Methods

In this study, we used the data matrix generated in our previous research on hatching egg sanitization [18], with approval from the Ethics Committee on Animal Use at the Federal Institute of Brasília (IFB Document Number 6739150424). Briefly, the research involved analyzing the application of different essential oils and control treatments on hatching eggs after collection, namely: (1) Non-sanitized eggs (NE); (2) Spraying with grain alcohol (GA) at a concentration of 93.8%; (3) Fumigation with formaldehyde (FA) at a concentration of 5 g/m^3; (4) Spraying with *Citrus aurantifolia* essential oil (CAEO) at a concentration of 9.38 mg/mL; (5) Spraying with *Ocimum basilicum* essential oil (OBEO) at a concentration of 4.69 mg/mL; and (6) Spraying with *Allium sativum* essential oil (ASEO) at a concentration of 1.17 mg/mL.

This study used hatching eggs with an average egg weight per replicate ranging from 56 to 60 g from a 57-week-old broiler breeder from the Pescoço Pelado Vermelho (PSÇ) lineage. Except for the control treatment, all eggs were sanitized simultaneously 20 min after collection, then naturally air-dried at room temperature for approximately 30 min. Subsequently, they were stored for 24 h at a temperature between 19 and 21 °C and relative humidity between 50% and 60%. Liquid sanitizers were applied using hand sprayers, whereas the gaseous sanitizing agent was used in a closed environment according to the farm's standard protocols. For liquid sanitizers, a dose of approximately 2.5–3 mL per egg was applied. After the storage period, eggs were incubated in single-stage setters (Chocmaster, Curitiba, Paraná, Brazil) at an average temperature of 37.7 °C and relative humidity of 60%, with hourly turning throughout the incubation period. During hatching, eggs were maintained at 36.6 °C with an average relative humidity of 65% without turning.

Throughout the experiment, various parameters were performed, including hatchability of fertile eggs (HI, %), which represents the relationship between the number of hatched chicks and the number of fertile eggs; number of contaminated eggs (CE,%) throughout the incubation process; early mortality (EM, %); intermediate mortality (IM, %); late mortality (LM,%); egg weight (EW, g) after storage; egg weight loss (EWL, %) calculated as the relationship between egg weight on day 0 and day 18 of incubation; chick weight (CW, g) after hatching; yolk sac bacterial count ($\log_{10}$ CFU/mL) on the 18th day of embryonic development; and eggshell bacterial count ($\log_{10}$ CFU/mL) after sanitization.

The raw data from the previous study was used to investigate the relationships between microbiological and incubation parameters, particularly to explore, for the first time in the field of hatching egg sanitization, the correlation between egg contamination and HI, comparing the effectiveness of different sanitization protocols through various statistical correlation analyses. The application of this methodological approach in this area of research aims to provide quantitative evidence to either support or refute previously established assumptions regarding this relationship. Through these analyses, this study seeks to measure the strength and direction of this correlation objectively. The results

obtained will enable a more precise understanding of the actual impact of contamination on HI, contributing to developing more effective strategies to mitigate this issue. Additionally, this will enhance the comprehension of the effectiveness of sanitization protocols in reducing contamination.

For this study, the analysis was conducted based on six treatments and four repetitions (each repetition comprising 60 eggs; totaling 1440 eggs) for the following parameters: hatchability of fertile eggs (HI), number of contaminated eggs (CE), early mortality (EM), intermediate mortality (IM), late mortality (LM), egg weight (EW), egg weight loss (EWL), and chick weight (CW). Additionally, the parameters of bacterial count in the yolk sac (yolk sac mesophiles (YSM) and yolk sac Enterobacteriaceae (YSE)) were evaluated based on six treatments with seven repetitions, and bacterial count on the eggshell (eggshell mesophiles (EGM) and eggshell Enterobacteriaceae (EGE)) were evaluated based on six treatments in triplicate.

The data were analyzed through multivariate analyses using SAS v.9.4 software (SAS Institute Inc., Cary, NC, USA). The analyses included correlations between incubation and microbiological variables, as well as principal component (PROC PRINCOMP), path, and cluster analyses. The paths (PROC CALIS) were defined based on the movement of effects of the internal and external factors affecting contamination and HI. Hierarchical clusters (PROC CLUSTER) were formed using the treatments, and then discrimination analyses (PROC STEPDISC and PROC DISCRIM) were used to define the factors that separated the clusters.

3. Results

The correlation analysis (Table 1) was conducted to elucidate the factors that influenced the HI rates. A significant negative correlation was identified between HI and EM (r = −0.79). Furthermore, it was found that EGM was strongly associated with YSM (r = 0.76) and YSE (r = 0.73), similar to EGE, which was also strongly correlated with YSM (r = 0.62) and YSE (r = 0.64). YSE was the main factor positively correlated with EM (r = 0.35), although this correlation was not statistically significant.

Table 1. Correlation between the analyzed variables.

	CE	EM	IM	LM	EW	EWL	CW	YSM	YSE	EGM	EGE
HI	−0.35 ns	−0.79 ****	−0.10 ns	−0.25 ns	0.03 ns	−0.18 ns	0.08 ns	−0.30 ns	−0.47 ns	−0.37 ns	−0.17 ns
CE		0.11 ns	−0.08 ns	−0.31 ns	−0.03 ns	−0.25 ns	0.30 ns	0.49 *	0.71 ***	0.70 **	0.61 **
EM			−0.04 ns	−0.19 ns	−0.15 ns	0.24 ns	−0.35 ns	0.18 ns	0.35 ns	0.04 ns	0.04 ns
IM				−0.12 ns	0.15 ns	0.09 ns	0.23 ns	−0.23 ns	−0.22 ns	−0.17 ns	−0.11 ns
LM					0.14 ns	0.11 ns	0.06 ns	−0.11 ns	−0.31 ns	0.03 ns	−0.29 ns
EW						0.18 ns	0.63 ***	−0.48 *	−0.42 ns	−0.35 ns	−0.38 ns
EWL							−0.37 ns	0.30 ns	0.20 ns	0.05 ns	−0.35 ns
CW								−0.37 ns	−0.16 ns	−0.13 ns	0.08 ns
YSM									0.82 ****	0.76 ***	0.62 **
YSE										0.73 ***	0.64 **
EGM											0.79 ****

Abbreviations: hatchability of fertile eggs (HI); number of contaminated eggs (CE); early mortality (EM); intermediate mortality (IM); late mortality (LM); egg weight (EW); egg weight loss (EWL); chick weight (CW); yolk sac mesophiles (YSM); yolk sac Enterobacteriaceae (YSE); eggshell mesophiles (EGM); eggshell Enterobacteriaceae (EGE), not significant (ns); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In the principal component analysis, the first principal component accounted for 40.3% of the total variance, while the second principal component explained 17.6% of the variance (Figure 1). This structure revealed that HI was positioned opposite to variables associated with eggshell and yolk sac contamination, indicating a negative association between HI performance and contamination factors. Although this negative correlation was more pronounced between HI and the contamination variables, the spatial configuration also demonstrated that CW was oriented in the opposite direction to the contamination

variables, suggesting a negative correlation. Although this remains a premature finding, it may suggest a possible indication that chicks with greater body weight tend to be associated with lower levels of contamination. CE exhibited a positive correlation with eggshell and yolk sac contamination, reinforcing that both sources of contamination are associated with CE during incubation.

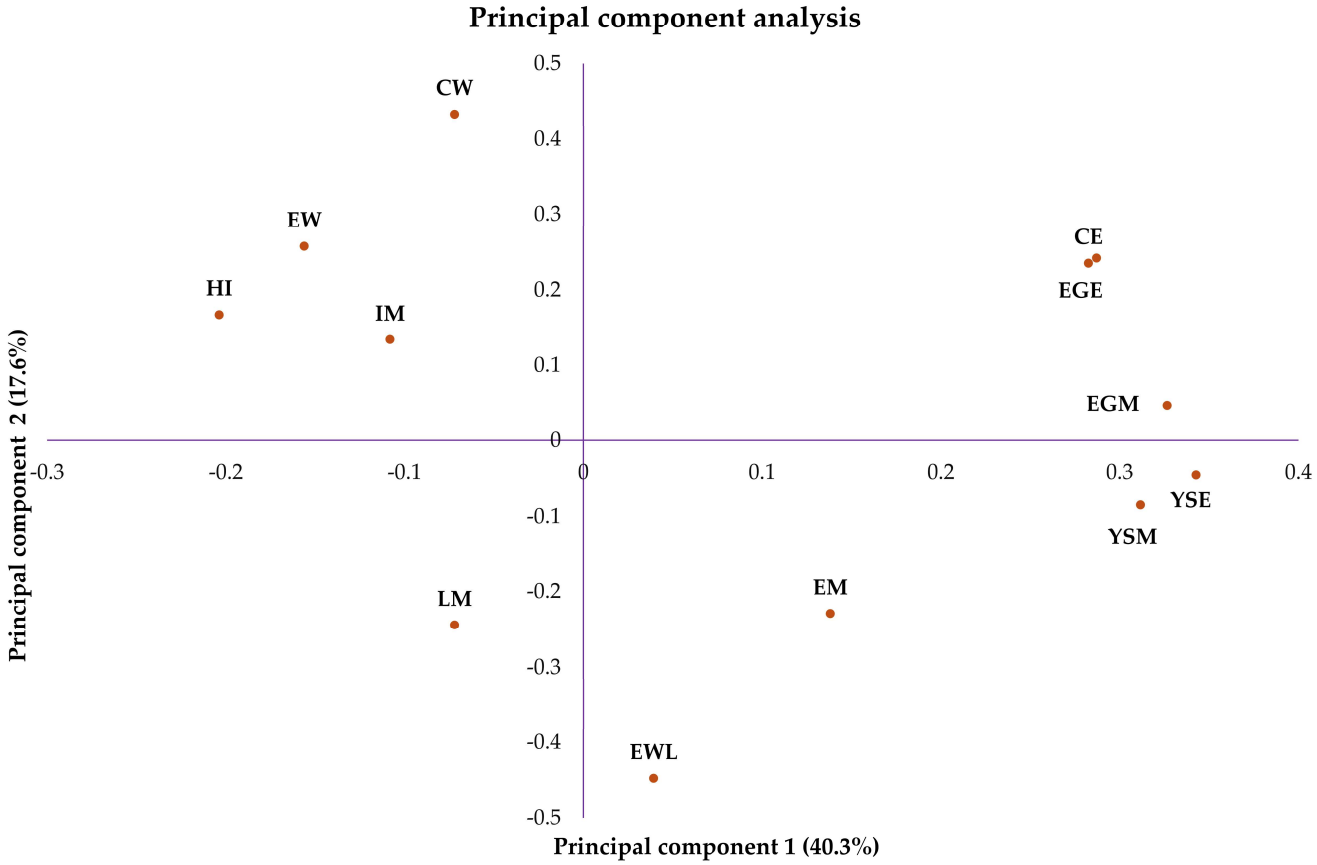
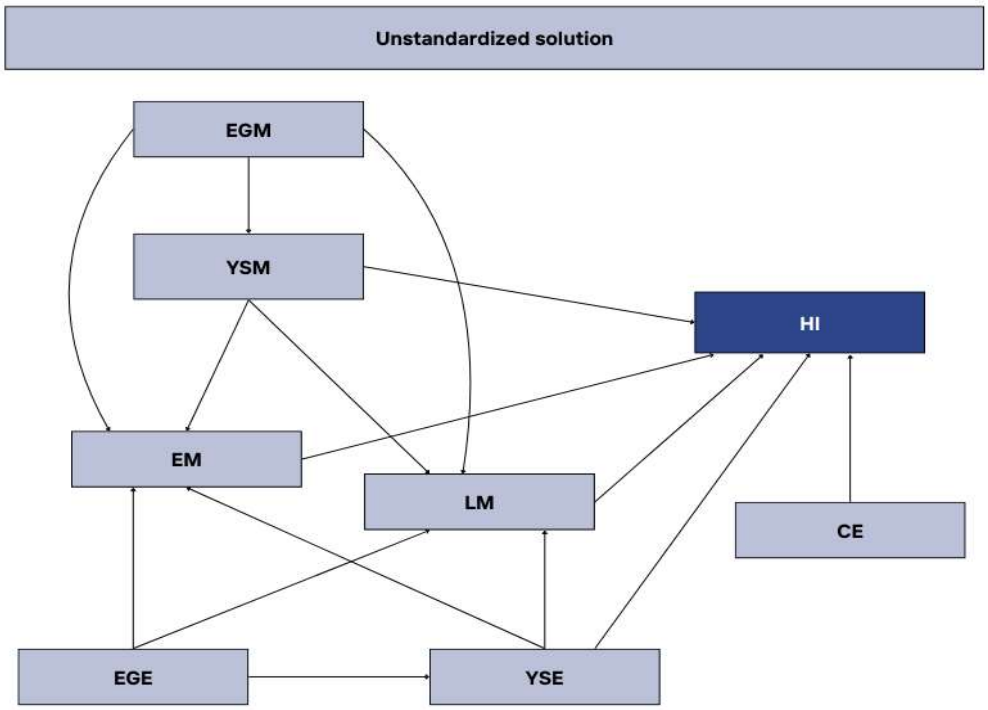


Figure 1. Principal component analysis of the parameters measured during the experimental period. Hatchability of fertile eggs (HI); number of contaminated eggs (CE); early mortality (EM); intermediate mortality (IM); late mortality (LM); egg weight (EW); egg weight loss (EWL); chick weight (CW); yolk sac mesophiles (YSM); yolk sac Enterobacteriaceae (YSE); eggshell mesophiles (EGM); eggshell Enterobacteriaceae (EGE).

In the pathway diagram, we begin with the EGM, which represents the variable responsible for initiating the contamination effects in poultry (Figure 2). According to the proposed hypothetical model, contamination of the eggshell promoted contamination of the yolk sac, which in turn increased embryonic mortality and subsequently affected HI. The change in HI would, therefore, be a response to CE. In the diagram, we excluded pathways that were not statistically significant. This approach ensured a more accurate and clearer representation of significant relationships. The inclusion of statistically similar paths is not justified, as such relationships do not provide sufficient evidence to be considered relevant or discussed within the studied context. The results of the path analysis revealed that contamination of both the eggshell and yolk sac significantly influenced EM and LM. Additionally, the path analysis indicated that EM, LM, CE, YSM, and YSE significantly affected HI.



Standardized results for path list			
Path			*P* value
EGM	=>	YSM	<0.0001
EGM	=>	LM	0.0088
EGE	=>	YSE	<0.0001
EGE	=>	LM	0.0307
YSE	=>	EM	0.0014
YSE	=>	LM	0.0022
EM	=>	HI	<0.0001
LM	=>	HI	<0.0001
CE	=>	HI	<0.0001
YSM	=>	HI	<0.0001
YSE	=>	HI	<0.0001

Figure 2. Path analysis of microbiological and incubation parameters with statistical significance. Hatchability of fertile eggs (HI); number of contaminated eggs (CE); early mortality (EM); late mortality (LM); yolk sac mesophiles (YSM); yolk sac Enterobacteriaceae (YSE); eggshell mesophiles (EGM); eggshell Enterobacteriaceae (EGE).

The hierarchical cluster analysis, based on microbiological and incubation variables (Figure 3), classified the treatments into three distinct clusters. The treatment with FA formed an independent cluster (cluster 2), while the treatments with essential oils were grouped into a single cluster (cluster 3). The remaining treatments comprised Cluster 1. Discriminant analysis identified the variables responsible for separating the clusters, with EGE and EGM emerging as the primary determinants (Table 2). The strength of this separation is evidenced by the high partial R^2 values, with EGE and EGM together accounting for more than 80% of the differentiation between cluster 1 and clusters 2 and 3. Notably, EGM alone contributed nearly 90% to the distinction between clusters 2 and 3. Furthermore, all analyzed variables were statistically significant in differentiating the clusters. These results suggest that the treatments promoted different decontamination and incubation performance profiles (Table 2).

Table 2. Discriminant analysis of the variables responsible for separating the clusters.

	Cluster 1 vs. Clusters 2 and 3			
Variables	R^2 Partial	$p < F$	$p <$ Lambda	$p <$ ASCC
EGE	0.8086	0.0011	<0.0001	<0.0001
EGM	0.8068	0.0024	<0.0001	0.0017
CE	0.7918	0.0067	<0.0001	<0.0001
EM	0.7694	0.0188	<0.0001	0.0002
EWL	0.7513	0.0432	<0.0001	<0.0001
	Cluster 2 vs. cluster 3			
EGM	0.8813	0.0005	0.0005	0.0005

Abbreviations: Eggshell Enterobacteriaceae (EGE); eggshell mesophiles (EGM); number of contaminated eggs (CE); early mortality (EM); egg weight loss (EWL). Coefficient of determination (R^2); Tests for multivariate separation of clusters ($p <$ Lambda and $p <$ ASCC).

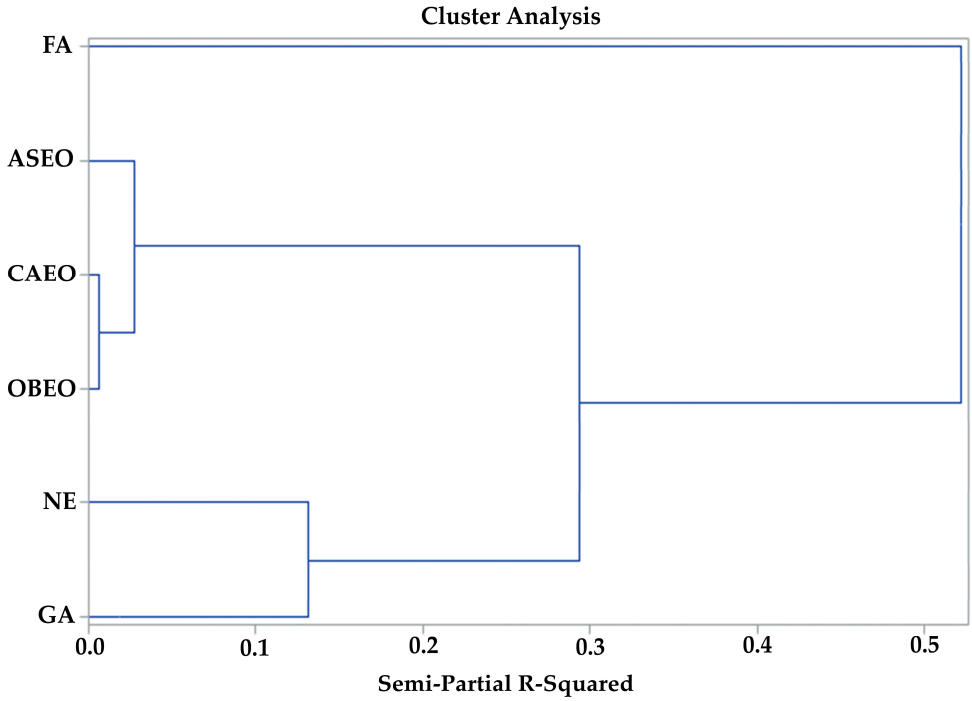


Figure 3. Hierarchical cluster analysis of the parameters measured during the experimental period. non-sanitized eggs (NE); grain alcohol (GA); formaldehyde (FA); *Citrus aurantifolia* essential oil (CAEO); *Ocimum basilicum* essential oil (OBEO); *Allium sativum* essential oil (ASEO).

In addition to the partial R^2, which indicated the individual contribution of each variable to the separation of clusters, the values of $p < F$, $p <$ Lambda, and $p <$ ASCC were also analyzed (Table 2). The $p < F$ showed the differences in means between groups, while $p <$ Lambda and $p <$ ASCC confirmed the multivariate separation of the clusters. All tests showed $p < 0.05$, indicating that the variables significantly discriminate the groups both individually and in combination.

Canonical correlation analysis revealed that the treatments with essential oils (CAEO, OBEO, and ASEO) were positioned close to HI and in the opposite direction of CE, indicating positive associations with HI and negative associations with CE (Figure 4). This configuration suggests that the application of essential oils promoted a reduction in CE and, consequently, an increase in HI. In contrast, the opposite positioning between the treatment with NE and the HI variable reveals a negative correlation, whereas the proximity between NE and CE, both located in the upper right quadrant of the graph, demonstrates a positive correlation. These results indicate that, unlike essential oils, the absence of sanitization results in a higher NE and a lower HI. The FA treatment, in turn, was positioned close to the EM suggesting a positive correlation between these factors, meaning that FA may be associated with increased EM. Furthermore, FA exhibited a negative correlation with both HI and CE, being oriented in the opposite direction to these variables. Although the reduction of CE with FA indicates sanitary efficiency, the lack of a positive association between FA and HI suggests that the bacterial control promoted by FA did not translate into improved HI. This result further supports the hypothesis that the positive correlation observed between FA and EM may have contributed to the decrease in HI, independently of the reduction in NE. This suggests that other contamination sources or factors related to the characteristics of the sanitizer may have played a role in the observed mortality.

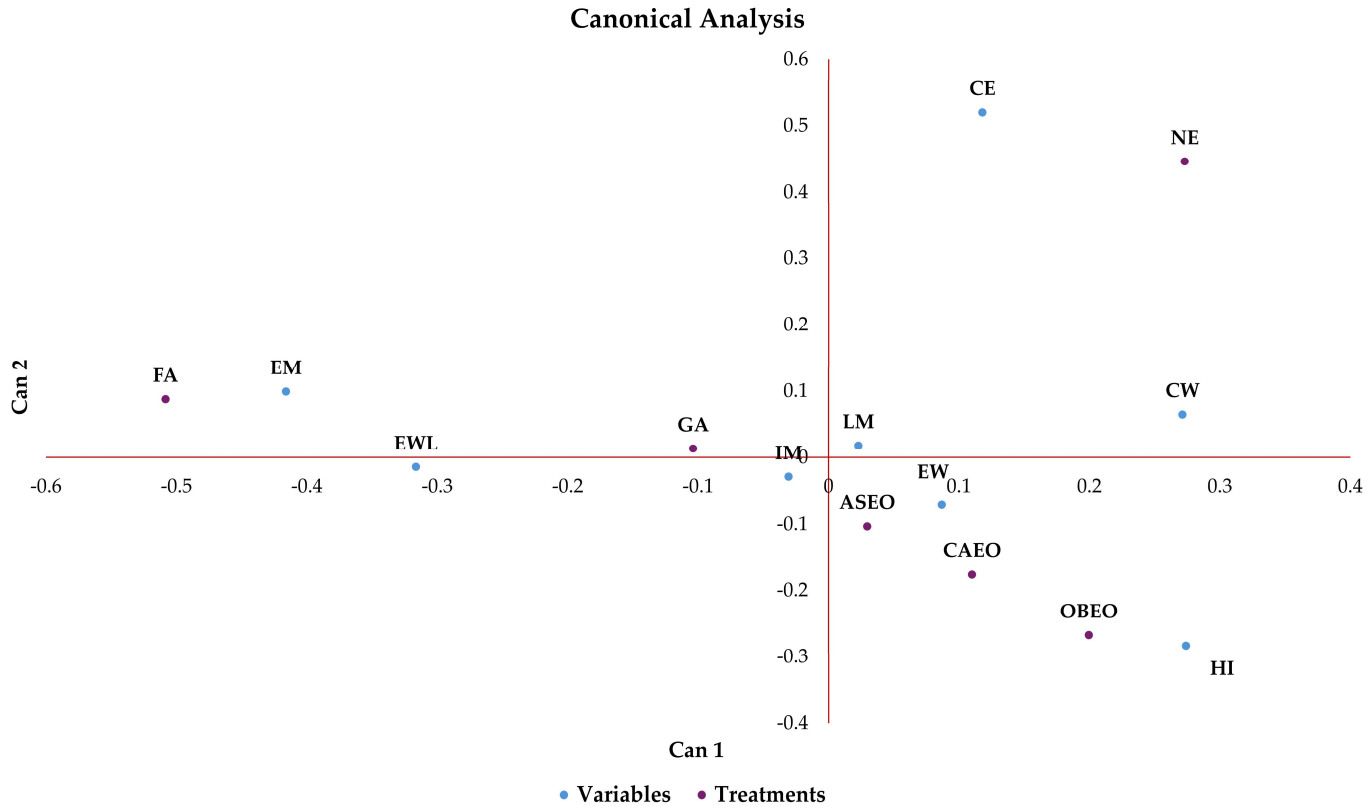


Figure 4. Canonical analysis of the parameters measured during the experimental period. Hatchability of fertile eggs (HI); number of contaminated eggs (CE); early mortality (EM); intermediate mortality (IM); late mortality (LM); egg weight (EW); egg weight loss (EWL); chick weight (CW); non-sanitized eggs (NE); grain alcohol (GA); formaldehyde (FA); *Citrus aurantifolia* essential oil (CAEO); *Ocimum basilicum* essential oil (OBEO); *Allium sativum* essential oil (ASEO).

The canonical structure revealed that, on the first axis (Can1), the separation of treatments was mainly explained by the variables EM (canonical coefficient = −0.416) and HI (canonical coefficient = 0.274) (Figure 4). On the second axis (Can2), CE (canonical coefficient = 0.519) exerted the greatest positive influence, while HI (canonical coefficient = −0.283) showed a negative influence.

Analyzing the principal component figure and treatments, it is observed that the first principal component accounted for 40.9% of the total variance, while the second principal component explained 17.3%. The treatments CAEO, OBEO, and ASEO exhibit a negative relationship with variables associated with contamination (CE, EGM, EGE, YSE, and YSM) (Figure 5). This distribution in the figure indicates that higher levels of these forms of contamination are associated with lower values in these treatments. In other words, ASEO, CAEO, and OBEO are linked to the reduction of the various forms of contamination analyzed, suggesting that these treatments may have a positive effect in decreasing the contaminant load and could be potentially effective within the studied sanitary control context. On the other hand, the FA treatment shows a strong positive correlation with the EM variable, as well as with YSM and YSE, both of which are related to yolk sac contamination and are positively correlated with EM. This indicates that the FA treatment is associated with higher levels of these contamination forms, which can be interpreted as an undesirable effect in the sanitary context. Additionally, FA presents a negative correlation with variables such as EC, EGM, and EGE, suggesting that in these aspects, the treatment may contribute to reducing contamination. Thus, FA tends to be less effective than essential oils in sanitary terms, as measured by YSM and YSE, and

less efficient in productive terms, as measured by HI, since essential oils are positively correlated with HI and FA is negatively correlated with this variable.

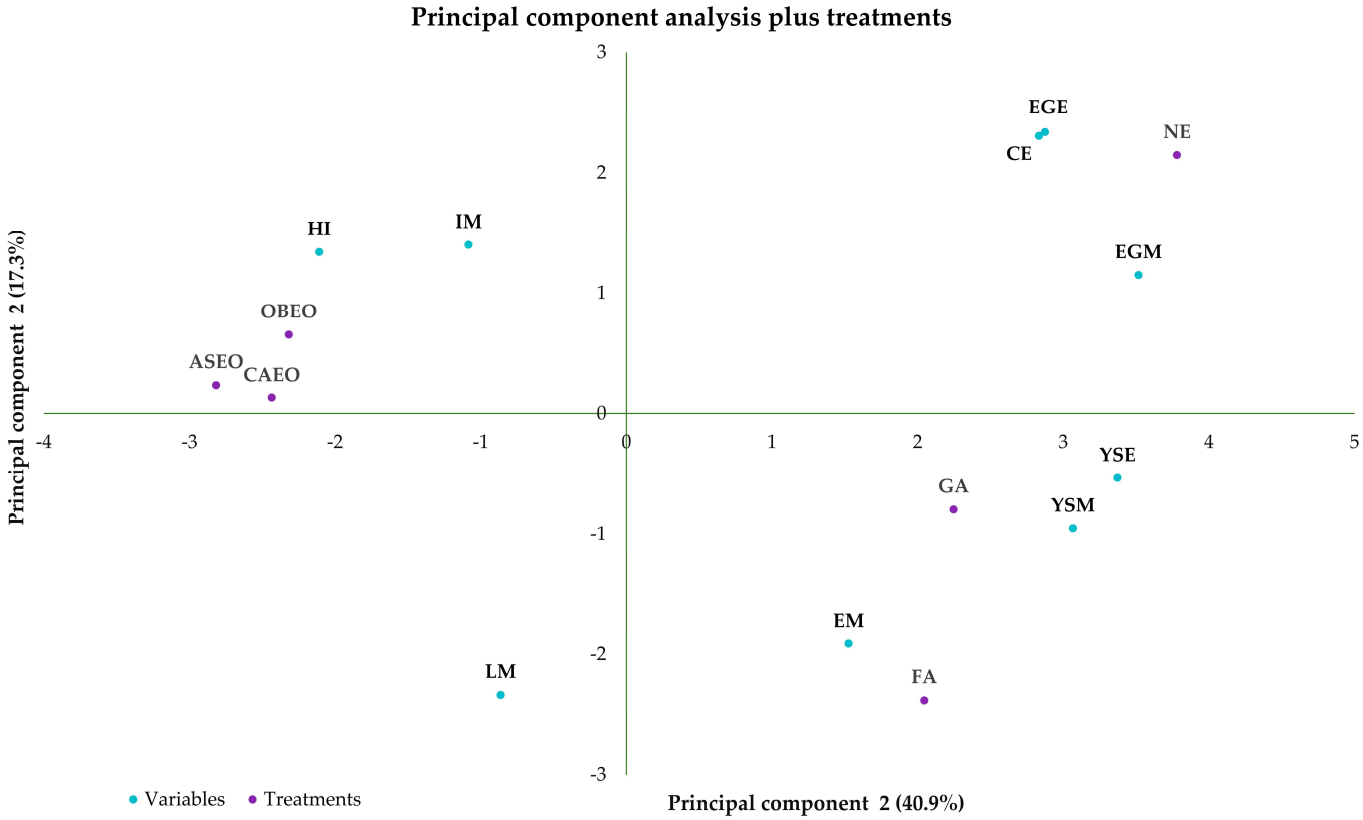


Figure 5. Principal component analysis of the parameters measured during the experimental period plus treatments. Hatchability of fertile eggs (HI); number of contaminated eggs (CE); early mortality (EM); intermediate mortality (IM); late mortality (LM); yolk sac mesophiles (YSM); yolk sac Enterobacteriaceae (YSE); eggshell mesophiles (EGM); eggshell Enterobacteriaceae (EGE); non-sanitized eggs (NE); grain alcohol (GA); formaldehyde (FA); *Citrus aurantifolia* essential oil (CAEO); *Ocimum basilicum* essential oil (OBEO); *Allium sativum* essential oil (ASEO).

4. Discussion

Our correlation analyses allow us to infer that contamination levels, both on the eggshell and in the yolk sac, directly or indirectly influence variation in HI. This variation due to eggshell contamination has been suggested by researchers from Brazil, Turkey, Egypt, the United States, and other countries [10,19–21] and can be explained by the dynamic between sanitized and non-sanitized hatching eggs, with non-sanitized hatching eggs often showing lower HI rates compared to those that are sanitized [15,22–24]. This occurs because embryos from non-sanitized hatching eggs have a significantly higher likelihood of exposure to bacterial contamination and subsequent infection [25]. Oliveira et al. [26] found a significant reduction of over 1.50 $\log_{10}$ in the bacterial load of the yolk sac of embryos from sanitized eggs, compared to embryos from non-sanitized eggs. Thus, the lack of proper egg sanitization, which maintains contamination and may promote an increase in CE during incubation, can represent an additional stress factor in poultry production.

The ASEO, CAEO, and OBEO treatments showed a negative correlation with problematic variables associated with contamination and reduced productivity (Figures 4 and 5), indicating a greater potential to reduce embryonic mortality, mitigate the effects of contamination, and preserve HI. This finding can be supported by various studies, as evidenced in references [6,26–32]. Although eggshell contamination and CE showed a negative correlation with FA (Figures 4 and 5), a positive correlation was ob-

served with contamination-related variables associated with the yolk sac (Figure 5). This suggests a partial antibacterial effect, which may have impacted hatchability, given the observed negative correlation between FA and HI. Ogbu and Oguike [33] reported that reduced HI may be linked to several factors, including EM, egg rots, broken yolks, chicks dead-in-shell, prolonged storage before incubation, poor breeder nutrition, breeder age, and setters and hatchery malfunctions. Among these causes, we highlight EM, which was positively associated with FA sanitization in our study. Therefore, the negative correlation between FA and HI may be related to increased yolk sac contamination, which could have initially triggered embryonic mortality (Figure 4), in addition to the embryo toxicity of FA itself, which may lead to death during the early stages of development. The application of formaldehyde in setters should not be performed under any circumstances during the first four days of development, as it represents a risk to the embryos [34].

5. Conclusions

As global poultry production systems evolve to meet society's demands increasingly safely and sustainably, identifying critical factors that threaten productivity and poultry health has become a priority. When identified early, these factors enable the implementation of proactive measures to prevent adverse impacts on systems managing hundreds of thousands or even millions of eggs and poultry daily. This study confirmed an inverse correlation between microbial load present on the eggshell and in the yolk sac, as well as CE during incubation and HI rates. In practical terms, the higher the level of contamination, the lower the HI. Notably, essential oils reaffirmed their potential as advanced sanitizing agents, proving effective in reducing both external and internal bacterial loads in hatching eggs. In contrast to formaldehyde, which was associated with increased EM and reduced HI, essential oils demonstrated beneficial effects, positioning them as a safer and more efficient solution for microbial control. This research raises a warning flag regarding the toxicity of FA to embryonic survival. Ultimately, the use of essential oil-based sanitizers is recommended for efficiently sanitizing hatching eggs in poultry production.

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References

1. Wen, C.; Li, Q.; Lan, F.; Li, X.; Li, G.; Yan, Y.; Wu, G.; Yang, N.; Sun, C. Microbiota continuum along the chicken oviduct and its association with host genetics and egg formation. *Poult. Sci.* **2021**, *100*, 101104. [CrossRef] [PubMed]
2. Gomes, B.; Pena, P.; Cervantes, R.; Dias, M.; Viegas, C. Microbial contamination of bedding material: One health in poultry production. *Int. J. Environ. Res. Public Health* **2022**, *19*, 16508. [CrossRef] [PubMed]
3. Jahantigh, M.; Rashki, A.; Najimi, M. A study on bacterial flora and antibacterial resistance of yolk sac infection in Japanese quail (*Coturnix japonica*). *Comp. Clin. Pathol.* **2013**, *22*, 645–648. [CrossRef]
4. Eltokhy, E.; Hefny, E.; Koraney, N.; Azam, A. Eggshell contamination with special reference to different methods of disinfection on the isolated bacteria. *Benha Vet. Med. J.* **2021**, *41*, 13–18. [CrossRef]
5. Oliveira, G.D.S.; McManus, C.; Salgado, C.B.; dos Santos, V.M. Effects of sanitizers on microbiological control of hatching eggshells and poultry health during embryogenesis and early stages after hatching in the last decade. *Animals* **2022**, *12*, 2826. [CrossRef]
6. Oliveira, G.D.S.; McManus, C.; de Araújo, M.V.; de Sousa, D.E.R.; de Macêdo, I.L.; de Castro, M.B.; dos Santos, V.M. Sanitizing hatching eggs with essential oils: Avian and microbiological safety. *Microorganisms* **2023**, *11*, 1890. [CrossRef]
7. Ricke, S.C.; Richardson, K.; Dittoe, D.K. Formaldehydes in feed and their potential interaction with the poultry gastrointestinal tract microbial community—A review. *Front. Vet. Sci.* **2019**, *6*, 188. [CrossRef]
8. Li, C.; Zhang, C.; Chen, X.; Cui, H.; Lin, L. The interference mechanism of basil essential oil on the cell membrane barrier and respiratory metabolism of *Listeria monocytogenes*. *Front. Microbiol.* **2022**, *13*, 855905. [CrossRef]
9. Melo, E.F.; Clímaco, W.L.S.; Triginelli, M.V.; Vaz, D.P.; de Souza, M.R.; Baião, N.C.; Pompeu, M.A.; Lara, L.J.C. An evaluation of alternative methods for sanitizing hatching eggs. *Poult. Sci.* **2019**, *98*, 2466–2473. [CrossRef]
10. Oliveira, G.D.S.; Nascimento, S.T.; dos Santos, V.M.; Silva, M.G. Clove essential oil in the sanitation of fertile eggs. *Poult. Sci.* **2020**, *99*, 5509–5516. [CrossRef]
11. Taşdemir, A.N.; Onbaşılar, E.E.; Yalçın, S.; Boyalı, B.; Aygören, H.; Tülek, E.; Sarıçam, S.; Akan, M. Effects of oregano juice on eggshell microbial load, layer embryo development, hatching results, and growth at the first 2 weeks after hatch. *Trop. Anim. Health Prod.* **2021**, *53*, 404. [CrossRef] [PubMed]
12. Mohammadi-Aragh, M.K.; Linhoss, J.E.; Evans, J.D. Effects of various disinfectants on the bacterial load and microbiome of broiler hatching eggs using electrostatic spray. *J. Appl. Poult. Res.* **2022**, *31*, 100278. [CrossRef]
13. Wang, L.; Dong, Q.; Tang, K.; Han, K.; Bai, H.; Yin, Y.; Li, C.; Ma, C.; Teng, L.; Li, J.; et al. Effect of phage spray on hatchability and chick quality of eggs contaminated with *Salmonella typhimurium*. *Viruses* **2024**, *16*, 1338. [CrossRef] [PubMed]
14. Shahein, E.H.A.; Sedeek, E.K. Role of spraying hatching eggs with natural disinfectants on hatching characteristics and eggshell bacterial counts. *Egypt. Poult. Sci. J.* **2014**, *34*, 213–230. [CrossRef]
15. Fouad, W.; Kassab, A.Y.; Abd Elshahed, A.A.; El-damrawy, S.Z. Effect of spraying moringa oil on embryonic development, hatchability, physiological parameters, post-hatch chick growth and bacterial contamination of fertile quail eggs. *Egypt. J. Nutr. Feeds* **2023**, *26*, 203–221. [CrossRef]
16. Mauldin, J.M. Factors affecting hatchability. In *Commercial Chicken Meat and Egg Production*; Springer: Boston, MA, USA, 2022; pp. 727–773.
17. King'ori, A.M. Review of the factors that influence egg fertility and hatchability in poultry. *Int. J. Poult. Sci.* **2011**, *10*, 483–484. [CrossRef]
18. Oliveira, G.D.S.; McManus, C.; Santos, P.H.G.D.S.; de Sousa, D.E.R.; Jivago, J.L.D.P.R.; de Castro, M.B.; dos Santos, V.M. Hatching egg sanitizers based on essential oils: Microbiological parameters, hatchability, and poultry health. *Antibiotics* **2024**, *13*, 1066. [CrossRef]
19. Copur, G.; Arslan, M.; Duru, M.; Baylan, M.; Canogullari, S.; Aksan, E. Use of oregano (*Origanum onites* L.) essential oil as hatching egg disinfectant. *Afr. J. Biotechnol.* **2010**, *9*, 2531–2538.
20. Wells, J.B.; Coufal, C.D.; Parker, H.M.; Kiess, A.S.; Purswell, J.L.; Young, K.M.; McDaniel, C.D. Hatchability of broiler breeder eggs following eggshell sanitization by repeated treatment with a combination of ultraviolet light and hydrogen peroxide. *Int. J. Poult. Sci.* **2011**, *10*, 421–425. [CrossRef]
21. Mousa-Balabel, T.M.; Mohamed, R.A.; Al-Midani, S.A.; El-Samad, M.S.A. Impact of boiler breeders hatching eggs disinfection time on some hatchability parameters. *Int. J. Sci. Basic Appl. Res.* **2016**, *30*, 230–240.
22. Fouad, W.; Abdel-Hafez, M.S.; Abd El-Halim, H.A.H. Influence of spraying garlic oil on embryonic development, hatchability, physiological parameters, post-hatch chick growth and bacterial contamination of fertile quail eggs. *Egypt. Poult. Sci. J.* **2018**, *38*, 877–893. [CrossRef]
23. Badran, A.M.M.; Osman, A.M.R.; Yassein, D.M.M. Comparative study of the effect of some disinfectants on embryonic mortality, hatchability, and some blood components. *Egypt. Poult. Sci. J.* **2018**, *38*, 1069–1081. [CrossRef]
24. Ergun, F.; Taskin, A.; Ergun, D. Effects of spraying cemele pepper (*Capsicum annuum* L.) extracts onto hatching eggs of atak-s layer hens on hatching results, serum glutathione and malondialdehyde levels. *J. Anim. Plant Sci.* **2024**, *34*, 1210–1219. [CrossRef]

25. Upadhyaya, I.; Yin, H.B.; Nair, M.S.; Chen, C.H.; Upadhyay, A.; Darre, M.J.; Venkitanarayanan, K. Efficacy of fumigation with trans-cinnamaldehyde and eugenol in reducing *Salmonella enterica* serovar enteritidis on embryonated eggshells. *Poult. Sci.* **2015**, *94*, 1685–1690. [CrossRef] [PubMed]
26. Oliveira, G.D.S.; McManus, C.; dos Santos, V.M. *Syzygium aromaticum* Essential oil as a safe natural solution to control bacteria in hatching eggs. *Pathogens* **2025**, *14*, 422. [CrossRef]
27. El-Soufi, A.; Al Khatib, A.; Khazaal, S.; El Darra, N.; Raafat, K. Evaluation of essential oils as natural antibacterial agents for eggshell sanitization and quality preservation. *Processes* **2025**, *13*, 224. [CrossRef]
28. Hassan, A.S.I.; Morsy, E.A.; El Moustafa, K.M.; Ibrahim, F.A.; Elmenawey, M.A. Effects of clove essential oil on eggshell bacterial load, antibacterial sensitivity, and hatchability. *Egypt. Pharm. J.* **2023**, *22*, 650–658. [CrossRef]
29. Bekhet, G.M. Impact of hatching egg disinfection on hatching characteristics and chick embryos. *Indian J. Anim. Res.* **2021**, *55*, 353–358. [CrossRef]
30. Bekhet, G.; Khalifa, A.Y.Z. Essential oil sanitizers to sanitize hatching eggs. *J. Appl. Anim. Res.* **2022**, *50*, 695–701. [CrossRef]
31. Mustafa, A.A.; Mirza, R.A.; Aziz, H.I. Lavender essential oil in sanitation on fertile egg. *Passer J. Basic Appl. Sci.* **2023**, *5*, 377–381. [CrossRef]
32. Iraqi, E.E.; El-Sahn, A.A.; El-Barbary, A.M.; Ahmed, M.M.; Elkomy, A.E. Antimicrobial activity of tea tree and lavender essential oils and their effects on hatching performance and eggshell bacterial count of Japanese quail eggs. *BMC Vet. Res.* **2025**, *21*, 176. [CrossRef] [PubMed]
33. Ogbu, O.C.; Oguike, M.A. Hatchability of fertile eggs in poultry industry. *J. Agric. Sustain.* **2019**, *12*, 107–123.
34. Thermote, L. Effective Hygiene within the Hatchery. *Int. Hatch. Pract.* **2006**, *20*, 18–21.

Concluding Remarks

The sanitization of hatching eggs goes beyond a simple management step: it serves as an indispensable bridge to achieving high-quality hatch rates. Although eggs possess a natural antimicrobial barrier, this defense, while complex, is not infallible. The reality is that no poultry management system is entirely sterile. From the farm to the storage and incubation rooms, including equipment, personnel, and hygiene protocols, there are gaps that can facilitate the proliferation of microorganisms. Incubation of eggs, for example, creates an environment that is conducive to bacterial growth and the proliferation of other microbes. This is where the sanitization of hatching eggs becomes not just an option, but a strategic sanitary necessity. By acting as an additional barrier, the sanitizer applied to the eggshell can reduce the risk of embryonic infection due to its antimicrobial properties. Even in cases where external contamination is already present on the eggshell, sanitization can create unfavorable conditions for the survival and proliferation of these bacteria. Therefore, it serves as an additional safeguard for microbiological safety, protecting not only the eggs but also the health of the future poultry. Investing in the sanitization of hatching eggs is not just a preventive measure; it is a responsible and safe commitment to poultry management.

Over the years, contamination in poultry environments has posed significant logistical challenges, leading to losses and high costs associated with prevention and control. Additionally, new formulas for sanitizing poultry environments have emerged, accompanied by concerns about the additional effects of these solutions on animals, the environment, and humans; factors that are increasingly considered when selecting products to be applied in the industry. Behind the global marketing promoting the use of formaldehyde for sanitizing hatching eggs, there are numerous serious reports regarding the health impacts on both animals and humans associated with this compound. These concerns have driven a shift toward the use of new formulations based on natural ingredients. Over 25 years ago, researchers recognized the potential of essential oils as a sustainable and promising technical solution for sanitizing hatching eggs. In this study, we reaffirm the efficacy of the essential oils of *Citrus aurantifolia* (CAEO), *Ocimum basilicum* (OBEO), and *Allium sativum* (ASEO) in this context, highlighting their numerous benefits. This method not only involves the use of plants cultivated worldwide but also combines proven antibacterial properties, safety, ease of application, and broad commercial availability. All other stages of hatching egg management must be carried out with the same biosafety standards required by this sanitization technology; otherwise, the desired results will not be achieved. The acceptance of this green technology for sanitizing hatching eggs is expected to grow as advances in understanding and new findings regarding its applications are achieved. The lack of focus on studies evaluating the economic costs associated with this technology remains a significant gap in the field and needs to be addressed. Similarly, industrial-scale research is scarce but essential to assess its feasibility and applicability under real-world management conditions. These efforts will also promote the familiarization and adoption of this sustainable approach by poultry industry professionals, solidifying its position as a benchmark practice.

Figure 1 illustrates an example of obtaining and applying essential oils to eggs:

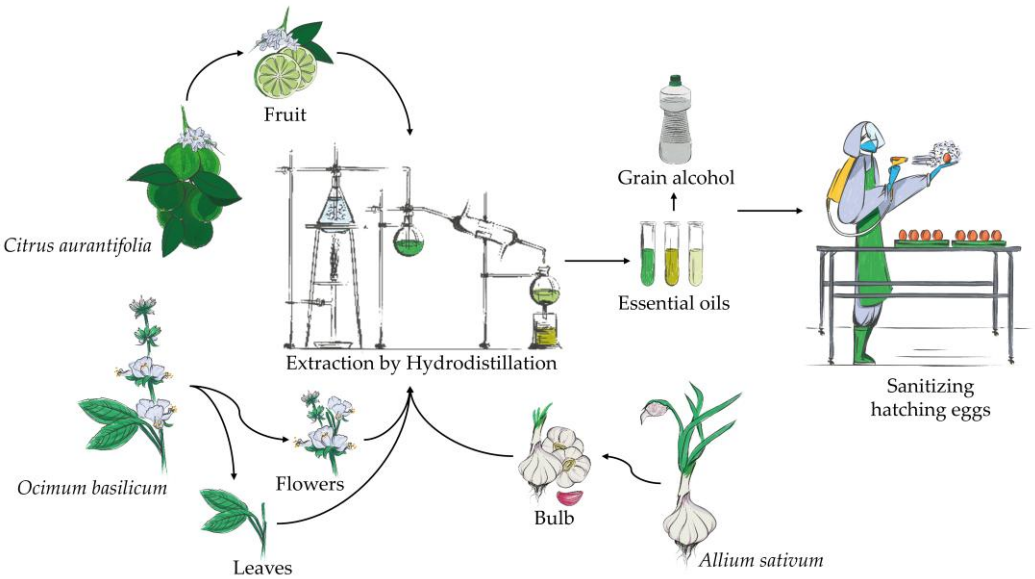

Figure 1. Protocol for obtaining and applying essential oils of CAEO, OBEO and ASEO on hatching eggs.