UNIVERSIDADE DE BRASÍLIA

FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA

SANITIZAÇÃO DE OVOS INCUBÁVEIS COM ÓLEO ESSENCIAL DE CRAVO-DA-ÍNDIA

GABRIEL DA SILVA OLIVEIRA

DISSERTAÇÃO DE MESTRADO EM CIÊNCIAS ANIMAIS

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ABRIL DE 2021

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Dissertação de mestrado submetida ao programa de Pós-Graduação em Ciências Animais da Universidade de Brasília, como parte dos requisitos necessários para obtenção do grau de mestre em Ciências Animais.

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1. Carga microbiana 2. Eclodibilidade 3. Incubação artificial 4. Óleo essencial de cravo-da-índia 5. Sanitizantes

Dedico este trabalho a minha mãe **Divonê** e meu pai **Demerval**, as minhas irmãs **Brenda** e **Silvana** e ao meu irmão **Romário**, por serem meus alicerces e os maiores incentivadores dos meus objetivos e sonhos.

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A **todos** aqueles que não foram citados, mas que de alguma maneira foram importantes ao longo dessa jornada.

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RESUMO

SANITIZAÇÃO DE OVOS INCUBÁVEIS COM ÓLEO ESSENCIAL DE CRAVO-DA-ÍNDIA

Gabriel da Silva Oliveira[1], Sheila Tavares Nascimento[1], Vinícius Machado dos Santos[2]

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O objetivo deste estudo foi avaliar o efeito da sanitização de ovos incubáveis com óleo essencial de cravo-da-índia como alternativa ao paraformaldeído sobre a redução da contagem microbiana da casca, rendimento da incubação e qualidade de pintos neonatos. Um total de 1.460 ovos incubáveis marrons com peso médio de 58,64 ± 0,49 g (de matrizes da linhagem CPK [Pesadão Vermelho] de 37 semanas) foram coletados sob condições assépticas e distribuídos aleatoriamente em 4 tratamentos (não sanitizados [controle negativo], sanitizados com álcool de cereais, óleo essencial de cravo-da-índia e paraformaldeído) antes da incubação. A contagem de bactérias mesófilas aeróbias totais da casca foi significativamente menor após a pulverização com óleo essencial de cravo-da-índia (2,30 ± 0,24 $\log_{10}$ UFC/mL) do que em ovos não sanitizados (3,49 ± 0,34 $\log_{10}$ UFC/mL) ou em ovos pulverizados com álcool de cereais (3,09 ± 0,14 $\log_{10}$ UFC/mL), mas não diferiu significativamente da contagem no grupo paraformaldeído (2,23 ± 0,29 $\log_{10}$ UFC/mL). O valor médio de eclodibilidade para os ovos tratados com óleo essencial de cravo-da-índia (84,69 ± 1,65%) foi estatisticamente semelhante ao paraformaldeído (81,87 ± 3,92%), mas significativamente superior ao controle negativo (74,03 ± 3,58%) e álcool de cereais (73,59 ± 2,87%). Na avaliação do escore Pasgar© foi determinado que o óleo essencial de cravo-da-índia (9,21 ± 0,89) teve um efeito superior na qualidade física dos pintos em comparação com os efeitos dos outros tratamentos. Portanto, o óleo essencial de cravo-da-índia é eficaz e seguro para ovos destinados à incubação e recomenda-se a sua utilização como alternativa ao paraformaldeído na sanitização desses ovos.

Palavras-chave: Contagem bacteriana, eclodibilidade, óleo essencial, ovos incubáveis, sanitizantes.

ABSTRACT

SANITIZATION OF HATCHING EGGS WITH CLOVE ESSENTIAL OIL

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The aim of this study was to evaluate the efficacy of sanitizing hatching eggs with clove essential oil as an alternative to paraformaldehyde; effects on the reduction in eggshell microbial count, incubation yield, and neonatal chick quality were measured. A total of 1,460 brown hatching eggs with a mean weight of 58.64 ± 0.49 g (from 37-wk-old CPK [Pesadão Vermelho] breeder hens) were collected under aseptic conditions and randomly distributed into 4 treatments (nonsanitized [negative control] and sanitized with grain alcohol, clove essential oil, and paraformaldehyde) before incubation. The count of total aerobic mesophilic bacteria was significantly lower after spraying with clove essential oil (2.30 ± 0.24 $\log_{10}$ CFU/mL) than on nonsanitized eggs (3.49 ± 0.34 $\log_{10}$ CFU/mL) or on eggs sprayed with grain alcohol (3.09 ± 0.14 $\log_{10}$ CFU/mL) but did not differ significantly from the count in the paraformaldehyde group (2.23 ± 0.29 $\log_{10}$ CFU/mL). The mean value hatchability of fertile eggs for the eggs treated with clove essential oil (84.69 ± 1.65%) was statistically similar to paraformaldehyde (81.87 ± 3.92%), but significantly higher than the negative control (74.03 ± 3.58%) and grain alcohol (73.59 ± 2.87%). In the Pasgar© score assessment, it was determined that the clove essential oil (9.21 ± 0.89) had a superior effect on the physical quality of the chicks compared with the effects of the other treatments. Clove essential oil is effective and safe for eggs intended for incubation. Its use as an alternative to paraformaldehyde in the sanitation of fertile eggs is recommended.

Key words: Bacterial enumeration, essential oil, hatching eggs, hatchability, sanitizers.

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ESTRUTURA DA DISSERTAÇÃO

A **Figura I** fornece a representação esquemática da estrutura da dissertação. O capítulo 1 compreendeu a introdução geral para compreensão da pesquisa, objetivos da pesquisa e finalizou-se com a revisão de literatura, em que se discorreu brevemente sobre ovos e questões associadas a eles, como a contaminação microbiana e a sanitização.

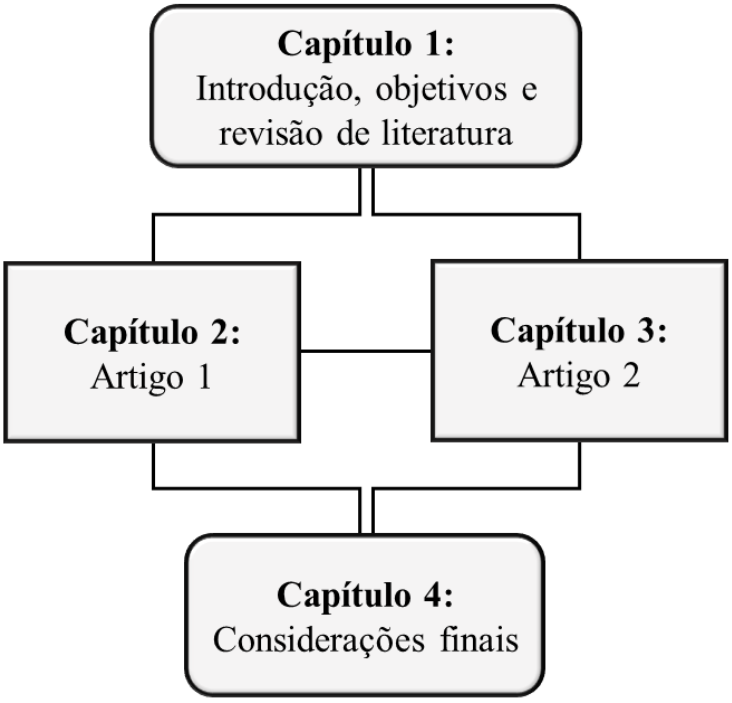

Figura I. Fluxograma da dissertação.

Os capítulos subsequentes (2 e 3), considerados a parte do desenvolvimento do trabalho, foram estruturados na forma de artigos científicos, conforme descrito a seguir:

Capítulo 2 (Artigo 1): Nesse capítulo foram apresentados os resultados da primeira parte da pesquisa, na qual foi avaliado o efeito da sanitização de ovos férteis com óleo essencial de cravo-da-índia como alternativa ao paraformaldeído sobre a redução da contagem microbiana da casca, rendimento da incubação e qualidade de pintos neonatos.

Capítulo 3 (Artigo 2): Nesse capítulo foram apresentados os resultados da segunda parte da pesquisa, na qual foi verificado se a sanitização de ovos incubáveis com óleo essencial de cravo afeta o desempenho de frangos de corte. Além disso, foi investigado se esse óleo afeta a janela de nascimento e a qualidade de embriões e pintos de um dia de idade.

Capítulo 4: Considerações finais.

CAPÍTULO 1 – INTRODUÇÃO E REVISÃO DE LITERATURA[1]

[1]Parte da revisão foi submetida a ***World's Poultry Science Journal***.

1. INTRODUÇÃO

Durante a produção e manejo de ovos incubáveis na avicultura, a contaminação microbiana da casca do ovo é uma preocupação. Os microrganismos colonizadores da casca do ovo podem potencialmente penetra-lá através dos poros ou deficiências estruturais e contaminar as estruturas internas dos ovos. Isso pode resultar na infecção do embrião em desenvolvimento, o que pode resultar em morte (Reid et al., 1961; Padron, 1990; Deeming, 1995; Berrang et al., 1999). Portanto, reduzir os microrganismos da casca de ovos incubáveis o máximo possível pode potencialmente prevenir a redução nos índices produtivos da avicultura, como os percentuais de eclodibilidade (Kuo et al., 1996; Copur et al., 2011; Shahein & Sedeek, 2014).

O número de microrganismos da casca do ovo pode ser reduzido pela ação antimicrobiana de diferentes sanitizantes (Brake & Sheldon, 1990; Scott et al., 1993; Zeweil et al., 2015; Melo et al., 2019). Na avicultura, os sanitizantes geralmente são aplicados nos ovos incubáveis após a coleta e o gás formaldeído tem sido o sanitizante quase sempre utilizado (Williams, 1970; Jabbar et al., 2019). No entanto, por ser reconhecido como inseguro devido à sua toxicidade (Cadirci, 2009; Zeweil et al., 2015), pesquisadores têm sido incentivados a estudar compostos sanitizantes alternativos, incluindo compostos biocidas naturais, como óleos essenciais (Yildirim et al., 2003; Baylan et al., 2015; Oliveira et al., 2020a).

O óleo essencial de cravo-da-índia, com nome científico *Syzygium aromaticum,* é um composto aromático, volátil e um dos antimicrobianos naturais mais potentes devido à presença de eugenol e outros compostos fenólicos (Craveiro & Queiroz, 1993; Chaieb et al., 2007). O uso desse óleo essencial como sanitizante para ovos incubáveis foi testado por Oliveira et al. (2020a). Os autores observaram que o óleo essencial de cravo-da-índia propiciou eclodibilidade (92,37 ± 3,25%) semelhante ao do paraformaldeído (94,44 ± 4,54%). Contudo, o efeito da sanitização de ovos incubáveis com esse óleo essencial na redução da contagem microbiana da casca, qualidade de embriões e pintos de um dia e no desempenho de frangos de corte não foram avaliados. Portanto, torna-se fundamental a realização de estudos adicionais que avaliem o potencial da sanitização de ovos incubáveis com óleo essencial de

cravo-da-índia, considerando ainda que o uso de produtos antimicrobianos menos tóxicos pela avicultura é indispensável.

2. OBJETIVOS

2.1. Objetivo geral

Avaliar a eficácia da sanitização de ovos incubáveis com óleo essencial de cravo-da-índia na fase de pré-incubação.

2.2. Objetivos específicos

- Avaliar o efeito do óleo essencial de cravo-da-índia na redução da contagem microbiana da casca;
- Avaliar o efeito do óleo essencial de cravo-da-índia na perda de peso, eclosão, eclodibilidade, mortalidade embrionária e rendimento de pintos;
- Avaliar o efeito do óleo essencial de cravo-da-índia na qualidade de embriões e pintos de um dia;
- Avaliar o efeito do óleo essencial de cravo-da-índia no desempenho de frangos de corte.

3. REVISÃO DE LITERATURA

3.1. Fisiologia da formação do ovo em galinhas

O ovário e o oviduto da galinha (*Gallus gallus domesticus*) (Figura 1.1) são responsáveis pela formação do ovo. No ovário ocorre a formação das células germinativas que, posteriormente, são transformadas em oócitos (óvulos) (Speake et al., 1998). Esses são incorporados por sais minerais, proteínas e lipídios oriundos do metabolismo hepático para a formação dos folículos (Speake et al., 1998). De 10 a 12 dias depois que a ave atinge a maturidade sexual, o folículo completa seu crescimento, é ovulado e continua se desenvolvendo à medida que percorre os compartimentos do oviduto, por um tempo médio de 26 horas, em que os constituintes do ovo são secretados e depositados (Lajusticia, 2002; Mine & Kovacs-Nolan, 2004). Esse ciclo ovulatório é contínuo e ocorre em dias sucessivos, ocorrendo uma ou duas horas mais tarde a cada dia. Quando o atraso total excede oito horas, o ciclo termina e não ocorre ovulação por um intervalo de um ou mais dias, para então iniciar um novo ciclo (Johnston & Gous, 2003).

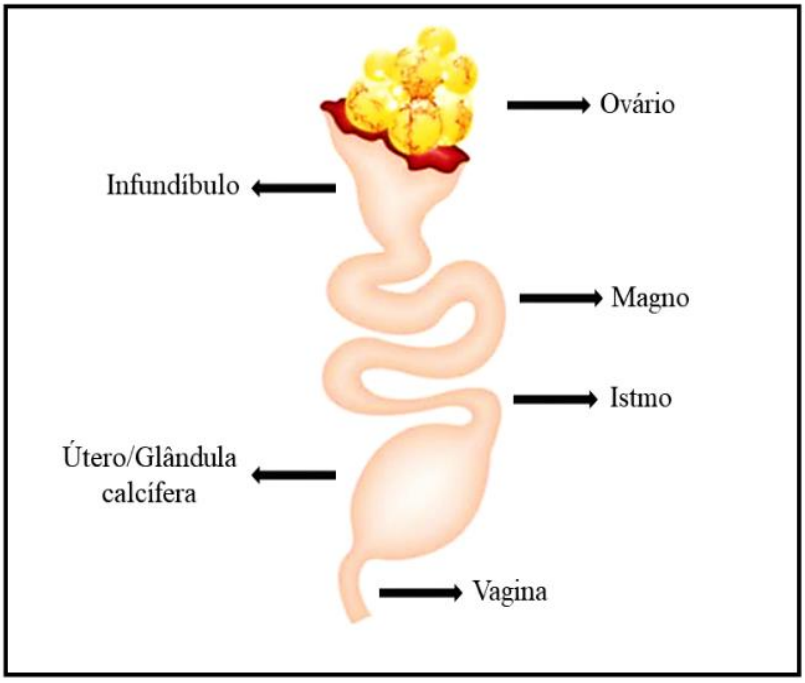

Figura 1.1. Desenho esquemático do sistema reprodutivo da galinha. **Fonte**: Adaptado de Barbosa (2011).

O oviduto da galinha (Figura 1.1) é uma estrutura tubular, tortuosa e longa, que se estende do ovário à cloaca e é composto pelo infundíbulo (local da fertilização), magno (deposição das três camadas do albúmen e a chalaza), istmo (formação da membrana interna e externa da casca), glândula calcífera ou útero (deposição de água e sais minerais, formação e pigmentação da casca e a deposição da cutícula) e a vagina (armazenamento de espermatozoides e oviposição) (Lajusticia, 2002; Mine & Kovacs-Nolan, 2004; Liu et al., 2010; Wilson et al., 2017). No momento da oviposição, as membranas interna e externa formam a câmara de ar ao se separarem na fração mais larga do ovo devido ao gradiente de temperatura interna do ovo e do ambiente (Kutchai & Steen, 1971; Vieira, 2007).

3.2. Estrutura, composição e defesa antimicrobiana do ovo

O ovo é uma fonte encapsulada de macro e micronutrientes, com um complexo sistema bioquímico, que fornece suporte nutricional e energético para o embrião até a eclosão (Réhault-Godbert et al., 2019) e também proteção natural contra a contaminação microbiana (Gantois et al., 2009; Guyot et al., 2016); consiste em três frações principais: a gema (27,5% do peso do total do ovo), o albúmen (63%) e a casca (9,5%) (Cotterill & Geiger, 1977).

3.2.1. Gema

A gema é constituída por agregados proteicos não solúveis (grânulos) suspensos em um líquido amarelo claro (plasma) (Anton, 2013). Contém aproximadamente 48% de água, 32,6% de lipídios,

16% de proteínas (incluindo proteínas antimicrobianas, como a ovotransferrina e a ovomucóide), carboidratos, minerais e vitaminas (Mine & Kovacs-Nolan, 2004; Mann & Mann, 2008; Kusum et al., 2018). A gema está fortemente associada à membrana vitelínica (espessura = 10 µm) – uma estrutura formada por uma camada mesodérmica externa de células achatadas e uma camada endodérmica interna de células epiteliais colunares, que fornece proteção antimicrobiana física e química e dá forma à gema do ovo, além de evitar o vazamento da gema em direção ao albúmen e por ser um dos meios de transferência do conteúdo da gema para o embrião (Bellairs et al., 1963; Back et al., 1982; Noble & Cocchi, 1990; Speake et al., 1998).

Em ovos férteis, a gema é a fonte quase exclusiva de nutrientes para o embrião (Bauer et al., 2013). Sabe-se, por exemplo, que 90% da energia total produzida pelo embrião se originam da β-oxidação de ácidos graxos dos lipídios da gema (Noble & Cocchi, 1990; Speake et al., 1998) e é utilizada para sua manutenção e desenvolvimento (Speake et al., 1998). Somente 10% de energia são derivados do metabolismo de proteínas e carboidratos (Fiske & Boyden, 1926).

3.2.2. Albúmen

O albúmen é um fluido depositado em torno da membrana vitelínica pelo magno. É estruturado em quatro camadas distintas: uma camada fina externa (fluida), uma camada espessa (gelatinosa), uma camada fina interna (fluida) e a chalaza (gelatinosa), que equivalem a 23,2, 57,3, 16,8 e 2,7% do total do albúmen, respectivamente (Nys & Guyot, 2011). É menos denso que a gema, pois é composto basicamente de água (88%) e proteínas (9,7-11%) (Mine, 2002). A estrutura viscosa, o pH alcalino e algumas proteínas do albúmen, como a ovotransferrina (12%) e lisozima (3,4%) exercem papéis fundamentais no impedimento ao crescimento ou sobrevivência de microrganismos no albúmen (Kang et al., 2006; Guyot et al., 2016).

Em ovos férteis, o albúmen serve como uma fonte importante de água para o embrião em desenvolvimento e o seu teor de proteínas é utilizado como base para a síntese de tecido embrionário (Willems et al., 2014). Antes de ser absorvido pelo embrião durante a embriogênese, o albúmen flui para a gema. Nesse sentido, uma das possíveis rotas de transferência do conteúdo do albúmen para o embrião é o sistema de saco de albúmen - cavidade amniótica - lúmen intestinal – gema (Carinci & Manzoli-Guidotti, 1968; Shbailat & Abuassaf, 2018).

3.2.3. Casca

A casca de ovo é um invólucro poroso e complexo, que protege o embrião do ambiente físico e microbiano. É formada por uma rede de fibras proteicas associadas a cristais de carbonato de cálcio (94% do peso total da casca), fosfato de cálcio (1%), carbonato de magnésio (1%) e substâncias

orgânicas (4%) (Murakami et al., 2007). Apresenta uma estrutura multicamada, que compreende uma camada mais interna, composta pelas membranas interna (espessura = 20 μm) e externa (50 μm), camada mamilar (100-110 μm), camada paliçada (200 μm), camada de cristal vertical (5-8 μm) e a cutícula (10 μm), estendendo-se da cutícula às membranas da casca os poros (15-65 μm: superfície e 6-23 μm: níveis internos da casca) (Tyler, 1956; Hamilton, 1986). Sua espessura pode, portanto, variar entre 300 e 400 μm (Hincke et al., 2012).

As membranas da casca de ovo (interna e externa) são malhas fibrosas extracelulares altamente reticuladas (Hincke et al., 2012), que funcionam como uma barreira microbiana (Gantois et al., 2009). Entretanto, a membrana interna oferece maior resistência para a entrada de microrganismos, pelo fato de estar coberta por uma camada de material eletrodenso, chamado membrana limitante (Gantois et al., 2009). Ambas possuem capacidade hidrofóbica, o que reduz a disponibilidade de água para os microrganismos e, assim, criam um ambiente adverso para sua multiplicação (Moro, 2002). Algumas proteínas, como a lisozima, a ovotransferrina e a ovocalixina-36 são detectadas a partir das membranas da casca do ovo e estão envolvidas na defesa antimicrobiana (Hincke et al., 2000; Gautron et al., 2006). Alguns pesquisadores sugerem que as duas membranas formam a barreira microbiana mais eficaz (Mayes & Takeballi, 1983).

Em ovos férteis, a casca apresenta uma série de funções que contribuem para o desenvolvimento do embrião, como permitir as trocas gasosas (O_2 e CO_2) e a perda de água necessária pelos poros microscópicos (Balkan et al., 2006), bem como a capacidade de transferir parte do cálcio por intermédio da membrana corioalantoide (Torres & Korver, 2018), que é utilizado para a mineralização do esqueleto embrionário (Johnston & Comar, 1995). Essa dissolução parcial da casca facilita a eclosão/bicada do pinto (Athanasiadou et al., 2018).

3.3. Mecanismos de contaminação microbiana em ovos

A contaminação microbiana em ovos pode ocorrer por duas rotas principais: transmissão vertical e transmissão horizontal (Barrow & Lovell, 1991). A transmissão vertical caracteriza-se pela contaminação do conteúdo interno do ovo durante sua formação no oviduto, devido à infecção do trato reprodutivo da galinha (Barrow & Lovell, 1991). A contaminação horizontal ocorre quando, após a postura, os ovos são expostos a superfícies contaminadas e os microrganismos colonizam e penetram a casca através de trincas microscópicas, rachaduras ou pelos poros da casca (Barrow & Lovell, 1991). Portanto, no ovo fértil, tanto a contaminação por transmissão vertical quanto a horizontal podem ameaçar o desenvolvimento embrionário. No entanto, é mais comum que a contaminação ocorra por transmissão horizontal, uma vez que se presume que 90% dos ovos estejam estéreis na oviposição (Mayes & Takeballi, 1983; Barrow & Lovell, 1991).

3.4. Microbiota contaminante na casca e no conteúdo interno de ovos

Conforme descrito anteriormente, os ovos normalmente recebem sua primeira contaminação microbiana após a oviposição, e esses microrganismos podem penetrar a casca dos ovos (Berrang et al., 1999). Entre os gêneros bacterianos presentes na superfície da casca e no conteúdo interno de ovos que foram descritos, estão a *Arthrobacter, Bacillus, Micrococcus, Proteus, Streptococcus, Alcaligenes, Klebsiella, Enterobacter, Escherichia, Pseudomonas, Salmonella, Citrobacter, Serratia, Acinetobacter, Flavobacterium, Cytophaga e Aeromonas* (Mayes & Takeballi, 1983, Board & Tranter, 1995; Cook et al., 2003). Entre os gêneros fúngicos presentes na microbiota da casca e no conteúdo interno dos ovos estão *Alternaria , Penicillium, Chaetomium, Trichothecium, Scopulariopsis, Botryotrichum, Engyodontium, Fusarium, Purpureocillium, Aspergillus, Penicillium, Fusarium, Mucor*, *Rhizopus*, *Cladosporium*, *Candida* e *Trichoderma* (Cook et al., 2003; Neamatallah et al., 2009; Tomczyk et al., 2018).

No entanto, os fungos do gênero *Aspergillus* e as bactérias do gênero *Pseudomonas* são contaminantes, que presentes na casca de ovos férteis podem aumentar as chances de infecção dos embriões em desenvolvimento e dos pintos, pois ambos podem danificar a camada cuticular e, assim, facilitar sua própria entrada e a de outras bactérias no interior do ovo (Board et al., 1979). A penetração transcasca, por exemplo, de bactérias gram-negativas pertencentes à família Enterobacteriaceae, como *Escherichia coli,* pode representar um risco para o desenvolvimento de embriões e pintos, uma vez que essa bactéria é a principal responsável pela infecção do saco vitelino (Harry, 1957; Cortés et al., 2004). Essa infecção reduzirá a qualidade dos embriões e pintos e causará mortalidade desses animais (Harry, 1957; Cortés et al., 2004). Logo, diminuir a microbiota contaminante da casca do ovo pode aumentar a qualidade e a capacidade de sobrevivência de embriões e pintos.

3.5. Sanitização de ovos incubáveis

3.5.1. Relevância e problemática

A sanitização de ovos incubáveis é uma prática adotada pela avicultura que visa à segurança microbiológica dos ovos e os melhores resultados de eclodibilidade. Geralmente é realizada na forma seca (fumigação) (Samberg & Meroz, 1995; Whistler & Sheldon, 1989; Jabbar et al., 2019), em que o sanitizante é volatilizado em um ambiente totalmente fechado com circulação e expulsão dos gases de exaustão, ou na forma úmida (pulverização ou imersão) (Scott et al., 1993; Buhr et al., 1994a, Buhr et al., 1994b; Cony et al., 2008), que consiste em aplicar o sanitizante por meio de pulverização na casca do ovo ou por imersão do ovo no sanitizante. Além disso, os ovos também podem ser sanitizados por radiação ultravioleta (Chavez et al., 2002, Coufal et al., 2003; Melo et al., 2019). Recomenda-se que a

sanitização seja realizada imediatamente ou no máximo 30 minutos após a coleta dos ovos para minimizar as chances de penetração de microrganismos pelos poros da casca (Samberg & Meroz, 1995; Araújo & Albino, 2011) e, assim, reduzir a incidência de infecções microbianas em embriões e pintos.

O paraformaldeído é um pó cristalino branco elaborado a partir da polimerização do formaldeído; é inflamável e incolor, tem um odor pungente e característico e pode emitir gás formaldeído quando aquecido (Taylor et al., 1969). Esse gás também pode ser gerado pela combinação de formalina e permanganato de potássio ($KMnO_4$) na proporção de 2:1 (v/p) (Cadirci, 2009). O formaldeído é o composto sanitizante mais popular para aplicação em ovos incubáveis devido ao seu potencial antimicrobiano (Williams, 1970; Cadirci, 2009), o que foi confirmado por vários estudos. Chung et al. (2018) observaram uma redução significativa no número de bactérias mesófilas aeróbicas totais na casca de ovos incubáveis (de 4,66 ± 0,08 para 2,33 ± 0,10 log_{10} UFC/casca) após fumigação com formaldeído (formalina associada com $KMnO_4$). Pereira et al. (2018) observaram que a fumigação de ovos incubáveis com paraformaldeído reduziu significativamente a contagem de bactérias mesófilas aeróbicas totais na casca de 3,00 ± 0,72 para 2,36 ± 0,87 quando a contagem foi calculada em log_{10} UFC/g e de 4,80 ± 0,72 a 3,92 ± 1,50 quando calculada em log_{10} UFC/ovo. Clímaco et al. (2018) também demonstraram que a fumigação com paraformaldeído (1,10 ± 0,16 log_{10} UFC/mL) reduziu significativamente a contagem de bactérias aeróbicas mesófilas totais na casca de ovos incubáveis em comparação com o tratamento de controle seco (3,14 ± 0,42 log_{10} UFC/mL), controle úmido (3,16 ± 0,44 log_{10} UFC/mL), ácido peracético (2,91 ± 0,67 log_{10} UFC/mL), peróxido de hidrogênio (3,05 ± 0,16 log_{10} UFC/mL), luz ultravioleta (2,20 ± 0,56 log_{10} UFC/mL) e ozônio (2,95 ± 0,41 log_{10} UFC/mL). O formaldeído tem potencial antimicrobiano porque reage irreversivelmente com componentes vitais de nitrogênio dos microrganismos, como proteínas (desnaturação) e ácidos nucléicos (alquilação) (Maris, 1995; Dorman & Deans, 2000).

Apesar de ser um agente antimicrobiano eficiente para ovos incubáveis, o formaldeído é perigoso, pois possui características altamente tóxicas, exigindo cuidados no seu manuseio e aplicação (Cadirci, 2009). A exposição ao formaldeído em embriões de galinha está associada a formas importantes de dano ou perda, como atraso no desenvolvimento, baixo peso, malformações e morte (Cadirci, 2009; Zeweil et al., 2015). Contudo, é fundamental enfatizar que esses efeitos deletérios do formaldeído em embriões de galinha dependem das condições de sua aplicação nos ovos, como tempo de aplicação, quantidade, concentração, tempo de exposição dos ovos, temperatura e umidade relativa durante a aplicação (Samberg & Meroz, 1995; Cadirci, 2009). Os efeitos potenciais da exposição ao formaldeído em humanos incluem irritação dos olhos, pele e mucosas, bronquite, edema pulmonar e câncer (Casteel et al., 1987; Wilbur et al., 1999; Nielsen & Wolkoff, 2010). Portanto, equipamentos de proteção individual, incluindo jalecos, máscaras e luvas, devem ser usados, e exames médicos

periódicos devem ser realizados para garantir a proteção e saúde dos profissionais das granjas e incubatórios (Cadirci, 2009).

Diante do exposto, os pesquisadores têm buscado sanitizantes alternativos eficazes que não representem perigo para o desenvolvimento do embrião de galinha e para a saúde dos profissionais envolvidos no processo de sanitização. Entre as alternativas possíveis que vêm sendo consideradas pela comunidade científica estão os óleos essenciais (Copur et al., 2010; Baylan et al., 2015; Bekhet, 2019).

3.5.2. Óleos essenciais

Os óleos essenciais são produtos naturais aromáticos e voláteis extraídos de plantas usando técnicas de extração, como hidrodestilação e destilação a vapor (Nakatsu et al., 2000; Mohammadhosseini et al., 2013). Eles são compostos por diversas substâncias, das quais dois ou três são componentes principais (20-70%) e o restante são componentes secundários (Bakkali et al., 2008). As concentrações dessas substâncias estão relacionadas às condições ambientais e às características gerais da planta (Barra, 2009; Nurdjannah & Bermawie, 2012). Os componentes predominantes dos óleos essenciais podem ser classificados em terpenóides e fenilpropanóides, ambos contendo compostos fenólicos (Amorati et al., 2013).

Os óleos essenciais têm diversas bioações, incluindo sua importante ação antimicrobiana. Essa ação se deve principalmente à presença de compostos fenólicos, como cinamaldeído, carvacrol, eugenol ou timol (Didry et al.,1994; Helander et al., 1998; Dorman & Deans, 2000; Lambert et al., 2001; Burt, 2004). O mecanismo de ação desses compostos ativos envolve principalmente desestabilizar a membrana celular dos microrganismos e o bloquear suas ações, como produção de energia, transporte ativo e secreção de toxinas (Swamy et al., 2016).

Vários trabalhos publicados indicam diferenças no potencial de ação dos óleos essenciais contra bactérias, com maior potencial de ação contra bactérias gram-positivas e menor potencial de ação contra bactérias gram-negativas, basicamente porque a membrana externa das bactérias gram-negativas possui moléculas de lipopolissacarídeos hidrofílicos em sua composição (Fournomiti et al., 2015). Em contraste, alguns estudos mostraram que alguns óleos essenciais tinham maior potencial contra bactérias gram-negativas do que bactérias gram-positivas (Valeriano et al., 2012; Lopez-Romero et al., 2015; Guimarães et al., 2017) ou o mesmo potencial contra os dois tipos de bactérias (Deans & Ritchie, 1987; Prabuseenivasan et al., 2006; Puškárová et al., 2017). Logo, nenhuma regra geral pode ser estabelecida em relação ao potencial de ação dos óleos essenciais contra bactérias gram-positivas ou gram-negativas. Também foi demonstrado que os óleos essenciais podem agir contra vários fungos (Ooi et al., 2006; Puškárová et al., 2017). Um número significativo de óleos essenciais,

incluindo cravo-da-índia, orégano, canela, gengibre, tomilho e manjericão, são reconhecidos geralmente como seguros (FDA, 2019).

3.5.3. Sanitizantes à base de óleos essenciais para ovos incubáveis

O valor dos óleos essenciais como ingrediente ativo de sanitizantes está principalmente associado à sua eficácia contra um amplo espectro de microrganismos e ao seu reconhecimento como seguro. Por isso, a aplicabilidade desses compostos como alternativas ao formaldeído na sanitização de ovos para incubação tem recebido atenção de pesquisadores (Tabela 1.1) devido às preocupações com a toxicidade de produtos químicos sintéticos (Isman, 2000; Yildirim et al., 2003; Ulucay & Yildirim, 2010; Copur et al., 2010; Oliveira et al., 2020a).

Tabela 1.1. Óleos essenciais e compostos bioativos de óleos essenciais testados em ovos incubáveis.

Óleo essencial e/ou composto bioativo de óleo essencial	Nome científico	Aplicação	Concentração	Ovos incubáveis	País	Referência
Orégano Timol	*Origanum vulgare* *Thymus vulgaris*	Pulverização	0,2 mL/L (v/v)	Codorna	Turquia	Yildirim et al. (2003)
Carvacrol Cinamaldeído	*Origanum vulgare* *Cinnamomum verum*	Imersão	1% (v/v)	Codorna	Turquia	Ulucay & Yildirim (2010)
Orégano	*Origanum onites*	Fumigação	0,55 e 0,75 µl/cm^3; 3 e 6 h exposição	Galinha	Turquia	Copur et al. (2010)
Mirra Gengibre Mirra+Gengibre	*Commiphora molmol* *Zingiber officinale* .	Pulverização	0,2 mL/L (v/v) 0,1+0,1 mL/L (v/v)	Galinha	Egito	Zeweil et al. (2010)
Orégano Gengibre Orégano+Gengibre	*Origanum vulgare* *Zingiber officinale* .	Pulverização	0,2 mL/L (v/v) 0,1+0,1 mL/L (v/v)	Galinha	Egito	Debes & Basyony (2011)
Orégano Cominho Orégano+Cominho	*Origanum vulgare* *Cuminum cyminum* .	Imersão	0,2% e 0,4% (v/v) 0,1+0,1% e 0,2+0,2% (v/v)	Galinha	Egito	Zeweil et al. (2013); Zeweil et al. (2015); Bekhet (2019)
Tomilho	*Thymus vulgaris*	Pulverização	0,5% e 0,7% (v/v)	Galinha	Egito	Shahein & Sedeek (2014)
Trans-cinamaldeído	*Cinnamomum zeylandicum*	Fumigação	1% (v/v)	Galinha	EUA	Upadhyaya et al. (2015)
Eugenol Capim-limão Chá-de-pedestre Capim-limão+Chá-de-pedestre	*Eugenia caryophyllus* *Cymbopogon flexuosus* *Lippia rotundifolia* .	Imersão	1% (v/v)	Galinha	Brasil	Nogueira et al. (2019)
Cravo-da-índia	*Syzygium aromaticum*	Pulverização	0,6 mg/mL (p/v)	Galinha	Brasil	Oliveira et al. (2020a)

A partir dos estudos publicados anteriormente, os óleos essenciais podem diminuir significativamente a microbiota contaminante nas cascas de ovos incubáveis (Tabela 1.2). Essa diminuição pode ser atribuída à capacidade hidrofóbica dos óleos essenciais (Burt, 2004). Além disso, parece que os óleos essenciais possuem um tempo de eficácia antimicrobiana considerável quando aplicados em cascas de ovo, o que pode estar relacionado aos seus constituintes químicos e poder residual. Upadhyaya et al. (2015) sanitizaram ovos incubáveis com trans-cinamaldeído e eugenol a uma concentração de 1% após inocularem as cascas de ovos com *Salmonella enterica* serovar Enteritidis no dia um de incubação. Em cascas de ovo sanitizadas com trans-cinamaldeído, *Salmonella* Enteritidis foi indetectável nos dias um, três, seis e nove de incubação. As contagens de *Salmonella* Enteritidis em ovos sanitizados com eugenol foram reduzidas nos dias um e três e eram indetectáveis nos dias seis e nove. As diferenças entre trans-cinamaldeído, eugenol e os controles de água e álcool foram significativas apenas nos dias um e três, em que trans-cinamaldeído e eugenol foram mais efetivos. No dia 13 de incubação, as cascas dos ovos foram reinoculadas com *Salmonella* Enteritidis e então sanitizadas novamente nas mesmas condições. Trans-cinamaldeído e eugenol reduziram a contagem *Salmonella* Enteritidis na casca do ovo no dia 13. A contagem de *Salmonella* Enteritidis no dia 16 foi igualmente reduzida no tratamento eugenol e indetectável no tratamento trans-cinamaldeído, e no dia 18 não foi mais detectável em ambos os grupos. As diferenças entre trans-cinamaldeído, eugenol e os controles de água e álcool foram significativas nos dias 13, 16 e 18, em que trans-cinamaldeído e eugenol permaneceram mais eficazes.

Tabela 1.2. Efeitos dos óleos essenciais nas contagens de bactérias mesófilas aeróbicas totais e fungos da casca de ovos incubáveis e percentuais de eclodibilidade (continua).

Óleo essencial e/ou composto bioativo de óleo essencial	Efeitos na contagem de bactérias mesófilas aeróbicas totais	Referência
Orégano	A contagem média (10,00 ± 2,50 log_{10} UFC/ovo) foi significativamente menor do que o formaldeído (24,00 ± 2,3 log_{10} UFC/ovo) e ovos não sanitizados (44,70 ± 10,1 log_{10} UFC/ovo).	Yildirim et al. (2003)
Timol Carvacrol Cinamaldeído	As contagens médias (6,73 log_{10} UFC/ovo) foram semelhantes entre si e significativamente menores do que ovos não sanitizados (27,13 log_{10} UFC/ovo).	Ulucay & Yildirim (2010)
Orégano	A contagem média (1,36 log_{10} UFC/ovo) foi significativamente menor do que o formaldeído (1,77 log_{10} UFC/ovo) e ovos não sanitizados (1,83 log_{10} UFC/ovo).	Copur et al. (2010)
Mirra Gengibre Mirra+Gengibre	As contagens médias foram semelhantes entre si e com o formaldeído (variando de 10,6 a 12,4 × 10^3) e significativamente menores do que os ovos não sanitizados (52 × 10^3).	Zeweil et al. (2010)
Tomilho	A contagem média (34,09 ± 1,65 × 10^3 UFC/ovo) foi significativamente maior do que formaldeído (16,62 ± 1,01 × 10^3 UFC/ovo) e significativamente menor do que ovos não sanitizados (45,30 ± 1,81 × 10^3 UFC/ovo).	Shahein & Sedeek (2014)
Orégano Cominho Orégano+Cominho	As contagens médias (1,34 ± 0,33 log_{10} UFC/ovo) foram semelhantes entre si e com o controle de álcool (1,96 ± 0,03 log_{10} UFC/ovo) e significativamente menor do que formaldeído (4,20 ± 0,40 log_{10} UFC/ovo) e controle de água (7,33 ± 0,88 log_{10} UFC/ovo).	Zeweil et al. (2015)

Tabela 1.2. Efeitos dos óleos essenciais nas contagens de bactérias mesófilas aeróbicas totais e fungos da casca de ovos incubáveis e percentuais de eclodibilidade (continuação).

Óleo essencial e/ou composto bioativo de óleo essencial	Efeitos na contagem de bactérias mesófilas aeróbicas totais	Referência
Capim-limão Chá-de-pedestre Capim-limão+Chá-de-pedestre	As contagens médias (3,95 ± 0,27 log_{10} UFC/g) foram semelhantes entre si e significativamente menores do que ovos não sanitizados (5,45 ± 0,7 log_{10} UFC/g).	Nogueira et al. (2019)
	Efeitos na contagem de fungos	**Referência**
Orégano	A contagem média (5,00 ± 1,7 log_{10} UFC/ovo) foi semelhante ao formaldeído (5,50 ± 2,5 log_{10} UFC/ovo) e ovos não sanitizados (11,67 ± 1,7 log_{10} UFC/ovo).	Yildirim et al. (2003)
Timol Carvacrol Cinamaldeído	As contagens médias (4,7 log_{10} UFC/ovo) foram semelhantes entre si e significativamente menores do que ovos não sanitizados (14,02 log_{10} UFC/ovo).	Ulucay & Yildirim (2010)
Orégano	A contagem média (1,13 log_{10} UFC/ovo) foi significativamente menor do que o formaldeído (1,30 log_{10} UFC/ovo) e ovos não sanitizados (1,37 log_{10} UFC/ovo).	Copur et al. (2010)
Mirra Gengibre Mirra+Gengibre	As contagens médias foram semelhantes entre si e com o formaldeído (variando de 4,6 a 5,9 × 10^3) e significativamente menores do que os ovos não sanitizados (14,3 × 10^3).	Zeweil et al. (2010)
Capim-limão Chá-de-pedestre Capim-limão+Chá-de-pedestre	As contagens médias (2,03 ± 0,13 log_{10} UFC/g) foram semelhantes entre si e significativamente menores do que ovos não sanitizados (2,55 ± 0,2 log_{10} UFC/g).	Nogueira et al. (2019)

Tabela 1.2. Efeitos dos óleos essenciais nas contagens de bactérias mesófilas aeróbicas totais e fungos da casca de ovos incubáveis e percentuais de eclodibilidade (conclusão).

Óleo essencial e/ou composto bioativo de óleo essencial	Efeitos na eclodibilidade	Referência
Orégano	O valor médio (73,00 ± 3,7%) foi semelhante aos ovos não sanitizados (65,17 ± 2,50%) e significativamente superior ao formaldeído (50,53 ± 5,7%).	Yildirim et al. (2003)
Orégano	O percentual médio (90,00%) foi semelhante ao formaldeído (89,91%) e ovos não sanitizados (88,02%).	Copur et al. (2010)
Mirra Gengibre Mirra+Gengibre	As médias (90,7%) foram semelhantes entre si e significativamente maiores do que o formaldeído (85%) e ovos não sanitizados (83,5%).	Zeweil et al. (2010)
Orégano Cominho Orégano+Cominho	As médias (96,22 ± 0,80%) foram semelhantes entre si e significativamente maiores do que ovos não sanitizados (87,06 ± 1,54%) e formaldeído (82,05 ± 0,56%).	Zeweil et al. (2013)
Tomilho	O percentual médio (91,45 ± 0,60%) foi significativamente menor do que o formaldeído (95,32 ± 0,54%) e significativamente maior que os ovos não sanitizados (85,62 ± 0,60%).	Shahein & Sedeek (2014)
Cravo	O percentual médio (92,37 ± 3,25%) foi semelhante ao paraformaldeído (94,44 ± 4,54%) e ao álcool de cerais (85,00 ± 2,20%).	Oliveira et al. (2020a)

Altos percentuais de eclodibilidade podem ser alcançados com o uso desses sanitizantes (Tabela 1.2), e se presume que esses resultados estejam diretamente ligados à eficiência dos óleos essenciais em reduzir a carga microbiana da casca do ovo. Em contraste, uma redução no percentual de eclodibilidade de ovos tratados com óleos essenciais foi relatada por Nogueira et al. (2019) e Tebrün et al. (2020). Nogueira et al. (2019) concluíram que a redução da eclodibilidade se deve a uma variedade de fatores não relacionados ao processo de sanitização, com exceção do tempo de imersão dos ovos no sanitizante. Tebrün et al. (2020) especularam que a diminuição da eclodibilidade estava associada à oclusão dos poros da casca do ovo devido à consistência oleosa residual do sanitizante. Portanto, parece que as condições de aplicação dos sanitizantes (preparados com óleo essencial) nos ovos incubáveis, incluindo o tempo de aplicação e a concentração do sanitizante, devem ser consideradas para evitar efeitos negativos na eclodibilidade.

3.5.4. Óleo essencial de cravo-da-índia

O cravo-da-índia é uma árvore de tamanho médio (8-12 m) pertencente à família *Mirtaceae*, nativa da Indonésia, mas que é cultivada em diversos países, incluindo o Brasil (Cortés-Rojas et al., 2014). Os botões florais secos do cravo-da-índia (Figura 1.2a; parte da planta comercializada) pode conter até 18% de óleo essencial (Figura 1.2b), que consistem em aproximadamente 89% de eugenol, de 5% a 15% de acetato de eugenol e β-cariofileno (Jirovetz et al., 2006). O óleo essencial de cravo-da-índia possui propriedades antimicrobianas importantes (Cortés-Rojas et al., 2014), que estão atribuídas comumente a sua principal molécula bioativa, o eugenol, um monoterpeno volátil que varia de incolor a amarelo claro, pouco solúvel em água, altamente solúvel em solventes orgânicos e que apresenta odor forte e sabor ardente (Khalil et al., 2017). Essas características do óleo essencial de cravo-da-índia o tornam um recurso acessível e disponível para sanitização de ovos incubáveis. Ressalta-se que os primeiros resultados obtidos sobre a sua aplicabilidade em ovos incubáveis foram positivos em termos de rendimento de incubação (Oliveira et al., 2020a). No entanto, novas pesquisas foram sugeridas pelos autores com o intuito de confirmar sua eficácia.

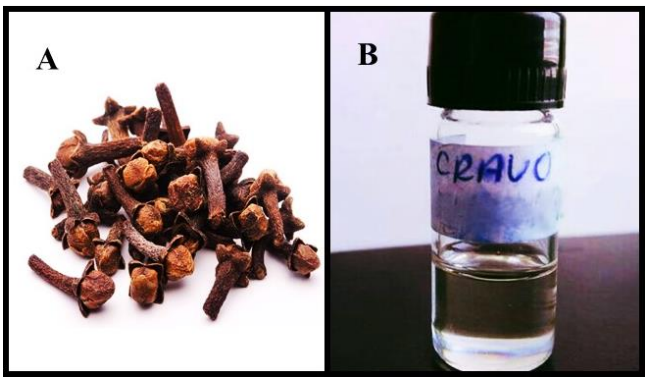

Figura 1.2. (A) Botões florais secos do cravo-da-índia; (B) Óleo essencial de cravo-da-índia. **Fonte**: Arquivo pessoal (2019).

3.6. Parâmetros físicos da incubação artificial de ovos incubáveis

Durante a produção avícola, além de ser importante considerar as etapas de pré-incubação, como a sanitização de ovos incubáveis, acima mencionada, é fundamental entendermos quais os fatores físicos estão envolvidos e afetam a etapa de incubação artificial de ovos.

A incubação artificial de ovos é um processo pelo qual são fornecidas condições controladas de temperatura, umidade, viragem e ventilação para o adequado desenvolvimento do embrião, nos quais, em aproximadamente 21 dias, estará pronto para eclodir e iniciar sua vida produtiva (Visschedijk, 1991). Por isso, vale destacar que alterações de um desses parâmetros físicos pode inviabilizar a embriogênese, resultando em perdas produtivas e econômicas (Visschedijk, 1991; Ramli et al., 2015).

O monitoramento constante da temperatura no interior da incubadora é crucial para fornecer o calor necessário para o desenvolvimento embrionário e para a manutenção das duas funções metabólicas normais (Noiva et al., 2014). A temperatura ideal dentro da incubadora deve variar entre 37,5 e 37,8 °C (Tullett, 1990), pois variações fora desse intervalo podem provocar um impacto significativo na temperatura do embrião (French, 1997) e, consequentemente, no seu crescimento ao longo da incubação. De acordo com a literatura, conclui-se que alterações da temperatura durante a incubação podem reduzir a qualidade e afetar os órgãos internos de pintos neonatos (Leksrisompong et al., 2007; Molenaar et al., 2011).

À medida que o embrião se desenvolve e os nutrientes são absorvidos e metabolizados, há perda de água para o ambiente por evaporação como resultado do processo metabólico (Noiva et al., 2014). A perda de água do embrião para o ambiente externo permite aumento no volume da câmara de ar, para que no momento da bicagem da membrana interna o embrião faça a transição com sucesso da respiração corioalantóica para a pulmonar (Ar & Rahn, 1980). Portanto, essa perda tem que ser adequada durante a incubação, com valores variando entre 11 e 14% (Rosa & Avila, 2000), pois se for muito baixa poderá provocar a super-hidratação embrionária e alterações no tamanho da câmara de ar

ou se for muito alta ocasionará a desidratação embrionária, o que em ambos os casos resultará em mortalidade. Sabe-se que a umidade relativa influencia na perda de água do ovo e, por esta razão, esse parâmetro precisa ser controlado na incubadora (entre 56 e 60%) para garantir o correto desenvolvimento embrionário (Tullett, 1990; Barbosa et al., 2013).

A viragem de ovos desempenha um papel fundamental no crescimento embrionário (Yoshizaki & Saito, 2002), pois facilita a absorção e metabolização dos nutrientes do albúmen e da gema pelo embrião, impede a aderência do embrião à membrana interna da casca e faz com que o embrião se mantenha na posição correta para eclodir no final da incubação (Eycleshymer, 1906; Wilson, 1991). É recomendado que as incubadoras funcionem com uma frequência de viragem de 24 vezes ao dia até o 18º dia de incubação em um ângulo de 45º (Funk & Forward, 1960; Oliveira et al., 2020b), visto que virar os ovos mais vezes aumenta os custos de produção e na prática a melhora nas taxas de eclodibilidade são mínimas; e ao virar menos vezes, essas taxas são reduzidas (Freeman & Vince, 1974; Oliveira et al., 2020b). Quanto ao ângulo, foi relatado que ovos submetidos a ângulos maiores ou menores que 45º durante o período de incubação apresentaram redução das taxas de eclodibilidade (Funk & Forward, 1960).

A ventilação dentro das incubadoras é fundamental para garantir o fornecimento de oxigênio e consequente remoção de dióxido de carbono, durante o processo de crescimento do embrião (Tullett & Burton, 1982). Além disso, a ventilação mantém a circulação adequada de ar na incubadora, previne a inadequada concentração de gases tóxicos, controla a proliferação de microrganismos e promove a troca constante de ar para a extração do excesso de calor e para ajudar a manter a correta umidade relativa (Tullett & Burton, 1982; Taylor, 1997).

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CAPÍTULO 2 – CLOVE ESSENTIAL OIL IN THE SANITATION OF FERTILE EGGS[1]

[1]Paper published in ***Poultry Science***, v. 99, n. 11, p. 5509-5516, 2020.

ABSTRACT

The aim of this study was to evaluate the efficacy of sanitizing fertile eggs with clove essential oil as an alternative to paraformaldehyde; effects on the reduction in eggshell microbial count, incubation yield, and neonatal chick quality were measured. A total of 1,460 brown fertile eggs with a mean weight of 58.64 ± 0.49 g (from 37-wk-old CPK [Pesadão Vermelho] breeder hens) were collected under aseptic conditions and randomly distributed into 4 treatments (nonsanitized and sanitized with grain alcohol, clove essential oil, and paraformaldehyde) before incubation. The count of total aerobic mesophilic bacteria was significantly lower after spraying with clove essential oil (2.30 ± 0.24 $\log_{10}$ CFU/mL) than on nonsanitized eggs (3.49 ± 0.34 $\log_{10}$ CFU/mL) or on eggs sprayed with grain alcohol (3.09 ± 0.14 $\log_{10}$ CFU/mL) but did not differ significantly from the count in the paraformaldehyde group (2.23 ± 0.29 $\log_{10}$ CFU/mL). The mean value hatchability of fertile eggs for the eggs treated with clove essential oil (84.69 ± 1.65%) was statistically similar to paraformaldehyde (81.87 ± 3.92%), but significantly higher than the negative control (74.03 ± 3.58%) and grain alcohol (73.59 ± 2.87%). In the Pasgar© score assessment, it was determined that the clove essential oil (9.21 ± 0.89) had a superior effect on the physical quality of the chicks compared with the effects of the other treatments. Clove essential oil is effective and safe for eggs intended for incubation. Its use as an alternative to paraformaldehyde in the sanitation of fertile eggs is recommended.

Key words: Bacterial enumeration, clove essential oil, fertile eggs, hatching results, sanitizers.

RESUMO

O objetivo deste estudo foi avaliar o efeito da sanitização de ovos férteis com óleo essencial de cravo-da-índia como alternativa ao paraformaldeído sobre a redução na contagem microbiana da casca do ovo, rendimento de incubação e qualidade dos pintos neonatos. Um total de 1.460 ovos férteis marrons com peso médio de 58,64 ± 0,49 g (de matrizes da linhagem CPK [Pesadão Vermelho] de 37 semanas) foram coletados sob condições assépticas e distribuídos aleatoriamente em 4 tratamentos (não sanitizados e sanitizados com álcool de cereais, óleo essencial de cravo-da-índia e paraformaldeído) antes da incubação. A contagem de bactérias mesófilas aeróbias totais da casca foi significativamente menor após a pulverização com óleo essencial de cravo-da-índia (2,30 ± 0,24 $\log_{10}$ UFC/mL) do que em ovos não sanitizados (3,49 ± 0,34 $\log_{10}$ UFC/mL) ou em ovos pulverizados com álcool de cereais (3,09 ± 0,14 $\log_{10}$ UFC/mL), mas não diferiu significativamente da contagem no grupo paraformaldeído (2,23 ± 0,29 $\log_{10}$ UFC/mL). O valor médio de eclodibilidade para os ovos tratados com óleo essencial de cravo-da-índia (84,69 ± 1,65%) foi estatisticamente semelhante ao paraformaldeído (81,87 ± 3,92%), mas significativamente superior ao controle negativo (74,03 ± 3,58%) e álcool de cereais (73,59 ± 2,87%). Na avaliação do escore Pasgar © foi determinado que o óleo essencial de cravo-da-índia (9,21 ± 0,89) teve um efeito superior na qualidade física dos pintos em comparação com os efeitos dos outros tratamentos. O óleo essencial de cravo-da-índia é eficaz e seguro para ovos destinados à incubação. Seu uso como alternativa ao paraformaldeído na sanitização de ovos férteis é recomendado.

Palavras-chave: Contagem bacteriana, óleo essencial de cravo-da-índia, ovos férteis, resultados de incubação, sanitizantes.

1. INTRODUCTION

There is a constant challenge to improve the productivity of the poultry production chain, whether in the prehatch, hatch, or posthatch stage. In this sense, maximizing the efficiency of incubation processes and maximizing the quality of day-old chicks are among the main objectives of poultry farming. For these goals to be achieved, we must identify the critical steps that may result in production losses.

One of the main strategic points at which the poultry industry can optimize the efficiency of production is the sanitation of fertile eggs. Reducing the microbial load of eggshells can minimize the occurrence and prevalence of pathogenic microorganisms, which are severely harmful to embryonic development, and maximize hatchability and chick quality (Shahein & Sedeek, 2014). The sanitizing compound commonly used in farms and hatcheries is paraformaldehyde, which is effective for maintaining low contamination levels of eggshells (Williams, 1970; Whistler & Sheldon, 1989). However, paraformaldehyde is highly toxic to the health of the professionals who handle it and to chick embryos and harmful to the environment (Casteel et al., 1987; Roca et al., 2008; Cadirci, 2009; Unsaldi & Ciftci, 2010; Zeweil et al., 2015; Rhomberg, 2015).

Clove essential oil (*Syzygium aromaticum*) may be an alternative for sanitizing fertile eggs (Oliveira & Santos, 2018; Oliveira et al., 2020). Usually, odoriferous and liquid essential oils are mixtures of lipophilic substances derived from secondary metabolites of plants. Chemically, most essential oils are composed of terpenoids, phenylpropanoids or linear alkanes and alkenes (Dhifi et al., 2016). Clove essential oil consists of a mixture of aliphatic and cyclic volatile terpenes and phenylpropanoids (Oliveira et al., 2016), with eugenol being the major component, and has high antimicrobial activity (Dhara & Tripathi, 2013).

Further studies demonstrating the effectiveness of clove essential oil in the sanitation of fertile eggs are necessary, considering its chemical composition and antimicrobial activity, as well as the first promising results regarding the artificial incubation process of eggs sanitized with this oil (Oliveira et al., 2020). In addition, the development of new products to replace those considered harmful is funda-

mental for the advancement of the poultry sector. This study aimed to evaluate the efficacy of sanitizing fertile eggs with clove essential oil as an alternative to paraformaldehyde, measuring the reduction in eggshell microbial count, incubation yield, and neonatal chick quality.

2. MATERIALS AND METHODS

2.1. Ethics approval

The present study was approved by the Ethics Committee on Animal Use of the University of Brasília under opinion No. 33/2019.

2.2. Experimental procedure

A total of 1,460 brown fertile eggs with a mean weight of 58.64 ± 0.49 g (from 37-week-old CPK [Pesadão Vermelho] breeder hens) were collected under aseptic conditions and randomly distributed into four treatments before incubation, as described in Table 2.1.

Table 2.1. Description of treatments, chemical concentrations, and methods of application to eggs.

Treatment	Concentration	Application	Number of eggs
Nonsanitized*	-	-	365
Grain alcohol	93.5%	Spraying	365
Clove essential oil	0.39%	Spraying	365
Paraformaldehyde	6 g/m³	Fumigation	365

* Negative control.

Internal egg quality was measured using the Haugh unit (HU) and yolk index (YI) of 100 eggs (25 per treatment). There was no significant difference among treatments, preventing egg quality from interfering with the incubation results. The mean HU of the eggs was 85.65 ± 7.31 (P = 0.2830; coefficient of variation (CV) = 8.30%), and they were classified as "AA", i.e., of excellent quality (USDA, 2000). The calculated YI was 0.39 ± 0.03 (P = 0.6647; CV = 7.33%). These analyses were also essential because the internal structure of the egg has the potential to meet the nutrient and energy demands of embryos until hatching.

2.3. Acquisition and preparation of clove essential oil-based sanitizing agent

Dried clove flower buds were obtained from a commercial market in Brasília, Federal District, Brazil. The essential oil was extracted in a laboratory of natural product chemistry (Federal Institute of Brasília, Gama, Federal District, Brazil) by a method adapted from Ascenção & Filho (2013) involving hydrodistillation with the Clevenger extraction system (Vidrolabor, Poá, São Paulo, Brazil). The chemical analysis of the clove essential oil by means of gas chromatography coupled to mass spectrometry (GC-MS) allowed the identification of three components, with eugenol (89.97%) being the main component (Table 2.2). The structures were defined on the basis of retention times, calculation of the Kovats index, the Wiley7, FFNSCl.3 and NIST08 databases and comparison with data from Adams (2017).

Table 2.2. Chemical compounds identified in the clove essential oil.

Peak	CRT (min)	Area (%)	CKI	TKI*	Compound
1	22.730	89.97	1,363	1,359	Eugenol
2	25.248	2.22	1,422	1,419	β-Caryophyllene
3	29.602	7.81	1,530	1,522	Acetyleugenol

Abbreviations: CTR, compound retention time; CKI, calculated Kovats index; TKI, tabulated Kovats index, *Adams (2017).

To prepare the sanitizer, the clove essential oil was diluted in grain alcohol (93.5%; Cromoline Química Fina, Diadema, São Paulo, Brazil) to a concentration of 0.39%. This concentration was chosen because it was the lowest concentration of the oil tested in vitro by the disc diffusion method adapted from Bauer et al. (1966) that showed an inhibitory effect against standard strains of *Escherichia coli* (ATCC 25,922), *Pseudomonas aeruginosa* (ATCC 27,853), and *Staphylococcus aureus* (ATCC 25,923) (Figure 2.1).

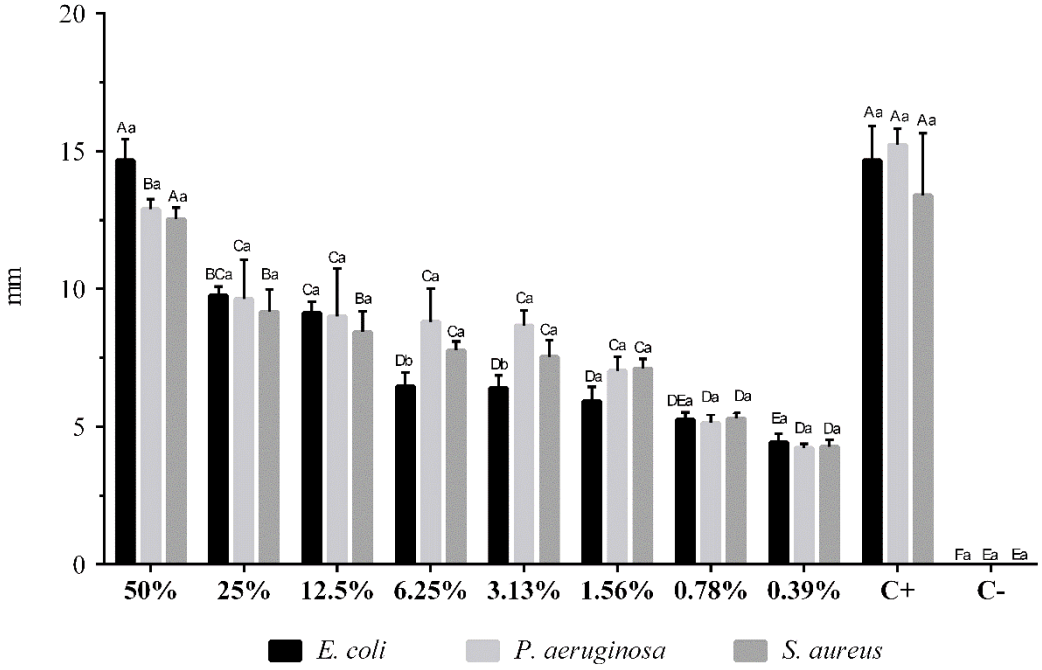

Figure 2.1. Determination of the antimicrobial activity of clove essential oil against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* by the disk diffusion method.[1] [A-F]; [a,b] Means with different uppercase (bacteria) or lowercase (oil concentration) letters differ significantly ($P < 0.05$). **Abbreviations**: C+, positive control (30 µg of chloramphenicol); C−, negative control (5% DMSO); DMSO, dimethyl sulfoxide. [1]Results are expressed as the mean diameter of the inhibition halos in millimeters (mm) for each concentration of clove oil (%) tested.

For this test, bacterial strains (ATCC, Manassas, Virginia, USA) were activated in brain heart infusion broth (Neogen, Lansing, Michigan, USA) and incubated for approximately 24 h at 36 °C. Subsequently, they were standardized in sterile saline (NaCl 0.85%) until a turbidity compatible with grade 0.5 of the McFarland scale (1.5 x 10^8 CFU/mL) was obtained. The oil was weighed to determine the volume that comprised 100 mg. This amount corresponded to the full-strength (100%) concentration and was then serially diluted in dimethyl sulfoxide (DMSO 5%; Sigma-Aldrich, San Luis, Missouri, USA). The sterile filter paper discs (4 mm) were impregnated with 10 µL of clove essential oil in concentrations ranging from 50 to 0.39% (w/v) and then deposited with sterile forceps on the surface of the Petri dishes containing the culture medium (Mueller-Hinton agar, Himedia, Mumbai, Maharashtra, India), which was previously inoculated with 100 µL of bacteria. DMSO was used as a negative control. Chloramphenicol (30 µg/disc; Sigma-Aldrich, San Luis, Missouri, USA) was used as a positive control. Plates were then inverted and incubated for approximately 24 h at 36 °C, and the diameter of the inhibition zones was measured in millimeters using a digital caliper with 0.001-mm precision (Mitutoyo, Suzano, São Paulo, Brazil). Each test was performed with three replicates.

The grain alcohol used in this study served as the carrier vehicle of the clove essential oil. Therefore, its isolated effect on the sanitation of fertile eggs was also tested.

2.4. Egg sanitation

Sanitation procedures were performed in a room at a commercial hatchery (Planaltina, Federal District, Brazil) 20 minutes after egg collection. The eggs from negative control treatment were kept in the same room as the other treatments and did not receive any sanitation procedure. In the grain alcohol and clove essential oil treatments, eggs were homogeneously sprayed over their entire surface with manual sprayers. After spraying, the eggs were placed in sterile trays for drying at room temperature for 30 to 50 minutes. At the same time, the eggs from paraformaldehyde treatment were sent for fumigation. In this process, a concentration of 6 g/m³ paraformaldehyde was used. Burning of the product, fumigation, and gas exhaustion proceeded for 20 minutes in a completely closed chamber, according to the guidelines of the commercial hatchery. The relative humidity and temperature in the chamber were 70% and 25 °C, respectively.

2.5. Eggshell microbial count

A method adapted from Fasenko et al. (2009) was used to count microbes on the eggshell. Twenty eggs from each treatment, one hour after sanitation, were placed into sterile plastic bags (pooled sample of 4 eggs per bag) labeled according to treatment and transported under cooling to the laboratory of microbiology (Federal Institute of Brasília, Planaltina), where the analyses of eggshell microbial count were performed. In the laboratory, 220 mL of 0.1% peptone saline solution (Kasvi, São José dos Pinhais, Paraná, Brazil) were added to each bag. The whole-egg washing technique was used to remove the eggshell microbial load. Then, a 1.0-mL aliquot was removed from each bag, and serial decimal dilutions in 0.1% peptone saline solution were performed for each sample. A 1.0-mL aliquot of each dilution was plated on standard plate count agar (PCA) (Neogen, Lansing, Michigan, USA), violet red bile glucose agar (Kasvi, São José dos Pinhais, Paraná, Brazil), and potato dextrose agar (PDA) (Himedia, Mumbai, Maharashtra, India) to count aerobic mesophilic microorganisms, Enterobacteriaceae, and molds and yeasts, respectively. The plates containing PCA and violet red bile glucose agar were incubated at 37 °C for 48 hours, and the plates with PDA were incubated at 29 °C for 6 days. After the incubation period, the colonies formed were counted, and the results are expressed in $\log_{10}$ CFU/mL of pooled sample of four eggs.

2.6. Incubation and hatching

After the sanitation process, the eggs were transported to a laboratory of poultry science (Federal Institute of Brasília, Planaltina). The eggs were stored for 3 days at a temperature of 16°C to 18°C and a 55 to 60% relative humidity. The eggs were then separated by treatment into incubation trays with a capacity of 80 eggs each. For each treatment, 4 incubation trays were used, totaling 320 eggs. The incubation trays containing the eggs were individually weighed and randomly distributed into 4 single-stage setters (Luna 480, Chocmaster, Curitiba, Paraná, Brazil), as shown in Figure 2.2. The setters were in an air-conditioned room at 22°C to 24°C and 50 to 55% humidity. These meteorological variables were monitored by 2 thermohygrometers (Testo 608-H1, Campinas, São Paulo, Brazil) to ensure the proper functioning of the setters.

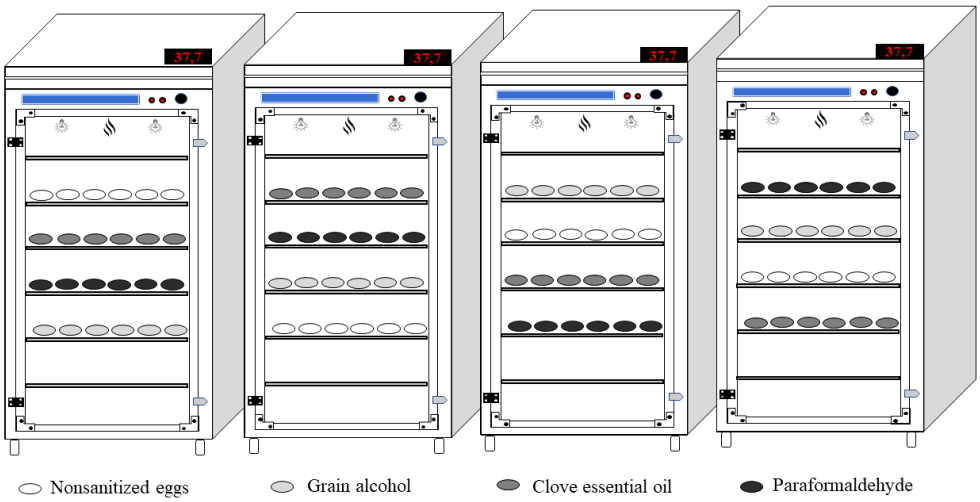

Figure 2.2. Distribution of treatments in setters.

The setters were operated at a mean temperature of 37.7°C (99.86°F), a mean relative humidity of 60%, and with automatic turning every hour at a 45° angle for the first 18 days of incubation. On the eighth day (192 h of incubation), all eggs were candled to remove infertile eggs and eggs with early embryonic mortality. Starting on day 19 (456 h of incubation), the incubation trays were weighed again, and the setters were operated at a mean temperature of 36.6°C (97.88°F) and a 65% relative humidity. After 21 days (504 h of incubation), the unhatched eggs were counted, opened, and evaluated to determine the number of infertile eggs, the period of embryonic mortality (early [0–7 days], mid [8–18 days], and late [19–21 days plus pipped]), and the number of contaminated eggs.

After the end of the incubation process, the percentages of egg weight loss (%), fertility (%), hatchability of set eggs (%), hatchability of fertile eggs (%), early dead (%), mid dead (%), late dead (%), contaminated eggs (%), and chick yield (%) were calculated per Aviagen (2011) and Baylan et al. (2018) using equations 1 to 9, respectively.

1) Egg weight loss (%) = [(initial egg weight − egg weight measured on the transfer day)/initial egg weight] × 100.
2) Fertility (%) = (number of fertilized eggs/number of eggs set) × 100.
3) Hatchability of set eggs (%) = (number of hatched chicks/total number of set eggs) × 100.
4) Hatchability of fertile eggs (%) = (number of hatched chicks/number of fertile eggs) × 100.
5) Early dead (%) = (number of dead embryos on days 0–7 of incubation/number of fertile eggs) × 100.
6) Mid dead (%) = (number of dead embryos on days 8–18 of incubation/number of fertile eggs) × 100.
7) Late dead (%) = (number of dead embryos on days 19–21 of incubation/number of fertile eggs) × 100.
8) Contaminated eggs (%) = (number of contaminated eggs/number of fertile eggs) × 100.
9) Chick yield (%) = (chick weight on the day of hatch/initial egg weight) × 100.

2.7. Evaluation of eggshell thickness

A method adapted from Barbosa et al. (2012) was used to evaluate eggshell thickness. After the chicks hatched, 50 eggshells from each treatment were separated and dried at room temperature for 3 days. Then, the thickness of each eggshell was measured without removing its internal membranes, and means were obtained from three different points at the equatorial plane of the eggshell using a digital caliper with 0.001-mm precision (Mitutoyo, Suzano, São Paulo, Brazil).

2.8. Evaluation of chick quality

After removal of the chicks from the incubators, their quality was visually assessed using the Pasgar© score method adapted from Boerjan (2006). Typically, each bird started the evaluation with 10 points and lost one point for each trait (Table 2.3) considered to be poor by the examiner. This subjective assessment was performed by a single person to avoid interexaminer variation.

Table 2.3. Assessment of chick quality according to the Pasgar© score.

Observed parameter	Assessment
Navel area	Healing level
Legs	Presence of injury
Eyes	Brightness and wideness of the gape of the eyelid
Beak	Presence of injury
Abdomen	Degree of absorption of the yolk sac
Reflex	Ability to react to stimuli

Source: Adapted from Boerjan (2006).

2.9. Experimental design and statistical analysis

The experiment followed a randomized block design with four treatments. The analysis of incubation yield was based on four replicates per treatment, in which each tray of 80 eggs constituted a replicate. To analyze eggshell thickness and chick yield and quality, each egg and chick were considered a replicate. A completely randomized experimental design was used for eggshell microbial count, with five replicates per treatment, in which each pooled sample of four eggs was considered a replicate. Data were subjected to analysis of variance and the means were compared by the Tukey's test when the assumptions of normality and homoscedasticity were met. The Kruskal-Wallis test was used when the test for normality distribution or homogeneity of variances failed. Statistical significance was considered at $P < 0.05$ for all tests. All data were analyzed using the SAS Studio® University Edition software (SAS Inst. Inc., Cary, NC).

3. RESULTS AND DISCUSSION

3.1. Eggshell microbial count

The count of total aerobic mesophilic bacteria was significantly lower ($P < 0.0001$; CV = 9.54%; Figure 2.3) after spraying with clove essential oil (2.30 ± 0.24 $\log_{10}$ CFU/mL) than on nonsanitized eggs (3.49 ± 0.34 $\log_{10}$ CFU/mL) or on eggs sprayed with grain alcohol (3.09 ± 0.14 $\log_{10}$ CFU/mL) but did not differ significantly from the count in the paraformaldehyde group (2.23 ± 0.29 $\log_{10}$ CFU/mL).

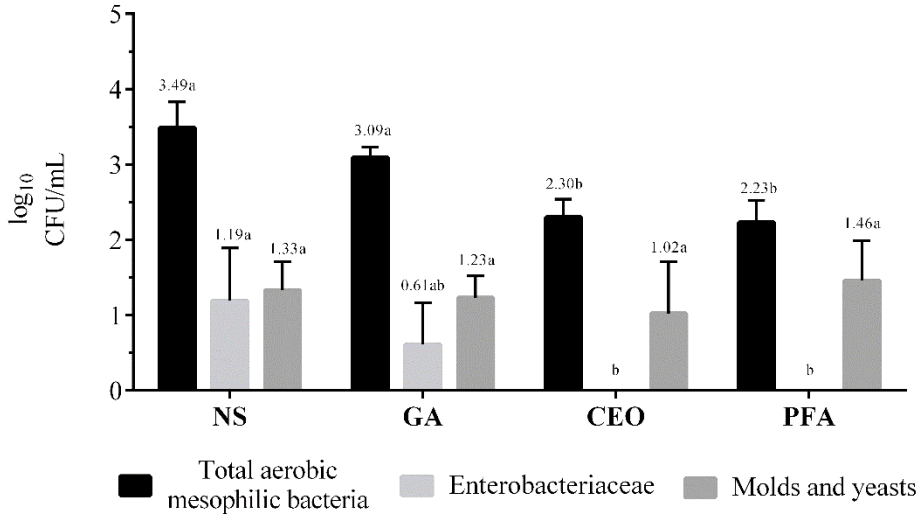

Figure 2.3. Counts of total aerobic mesophilic bacteria ($P < 0.0001$; CV = 9.54%), Enterobacteriaceae ($P = 0.0066$; CV = 98.24%), and molds and yeasts ($P = 0.6544$; CV = 37.59%) on eggshell surfaces according to different treatments.[1] [a,b]Means with different letters differ significantly ($P < 0.05$). **Abbreviations**: CEO, clove essential oil; CV, coefficient of variation; GA, grain alcohol; NS, nonsanitized; PFA, paraformaldehyde. [1]Results are expressed in $\log_{10}$ CFU/mL pooled over 4 eggs.

Essential oils are effective in reducing the microbial contamination of eggshells intended for incubation (Copur et al., 2010; Ulucay & Yildirim, 2010), which is reinforced by our results. In this

study, the potent antimicrobial activity of clove essential oil was mainly because of its high phenolic compound content (Chaieb et al., 2007). Phenolic compounds react with the phospholipids of the bacterial cell membrane, making them more permeable. This change in membrane permeability leads to the loss of ions, a reduction in membrane potential, depletion of the function of proton pumps, and a reduction in adenosine triphosphate, causing cell death (Burt, 2004).

The count of Enterobacteriaceae was significantly affected (P = 0.0066; CV = 98.24%; Figure 2.3) by the treatments and ranged from 0.00 in the clove essential oil and paraformaldehyde groups to 1.19 ± 0.70 $\log_{10}$ CFU/mL for the negative control. These results show that the presence of *Salmonella enterica* serovar Enteritidis and pathogenic *Escherichia coli* was not observed in the eggshells after sanitation with clove essential oil or paraformaldehyde. Prabuseenivasan et al. (2006) reported that clove essential oil exhibited bioactivity mainly against gram-negative bacteria, which was confirmed by the results of this study. In general, the low count of Enterobacteriaceae indicated good hygienic conditions of the farm that provided the eggs (Musgrove et al., 2014).

The total count of molds and yeasts ranged from 1.02 ± 0.69 (clove essential oil) to 1.46 ± 0.53 $\log_{10}$ CFU/mL (paraformaldehyde). No significant differences were observed (P = 0.6544; CV = 37.59%; Figure 2.3) between any of the treatments. The low fungal load on the surface of the eggshells may be explained by the fact that the eggs were not exposed to a very humid environment on the farm (Board & Tranter, 1995).

3.2. Hatching results

The percentage of egg weight loss during incubation did not differ between treatments in this study (P = 0.1495; CV = 3.00%, Table 2.4). This parameter is more strongly influenced by physical factors essential for incubation, such as temperature and relative humidity (Barott, 1937; Tullet & Burton, 1982; Meijerhof & van Beek, 1993). In this experiment, as the eggs were subjected to the same incubation conditions, no difference between the treatments was expected. In addition, evaluating egg weight loss during incubation allowed us to indirectly estimate the level of sanitizers damage to the cuticle, and, consequently, embryonic development (Brake & Sheldon, 1990; Peebles et al.,1998). Our findings suggest that the cuticle was not damaged by any of the sanitation processes.

Table 2.4. Egg weight before setting and during transfer and the percentage of egg weight loss in eggs treated with different treatments.[1]

Treatment	Egg weight before setting (g)	Egg weight during transfer (g)	Egg weight loss (%)
Nonsanitized	58.55 ± 0.86	51.55 ± 0.90	11.95 ± 0.36
Grain alcohol	58.82 ± 0.30	52.13 ± 0.49	11.38 ± 0.45
Clove essential oil	58.66 ± 0.36	51.78 ± 0.32	11.72 ± 0.46
Paraformaldehyde	58.53 ± 0.45	52.09 ± 0.35	11.01 ± 0.29
P value	0.7310	0.2920	0.1495
Coefficient of variation (%)	0.85	0.99	3.00

No significant differences existed between means ($P > 0.05$).
[1]Results are expressed as the means ± Standard deviation (SD).

There was no significant difference ($P = 0.0546$, CV = 1.76%) in the percentage of fertility (Table 2.5). The mean fertility was 82.62 ± 2.02%. This result was observed because the eggs were obtained from breeder hens of the same age that received the same management at the poultry house.

Table 2.5. Fertility, hatchability of set eggs, hatchability of fertile eggs, early, mid and late embryonic mortality, and contaminated eggs according to different treatments.[1]

Treatment	Fert (%)	Hatch (%)	Hatch fert (%)	Early dead (%)	Mid dead (%)	Late dead (%)	Cont. (%)
Nonsanitized	83.27 ± 2.31	61.63 ± 3.32^{b}	74.03 ± 3.58^{b}	7.09 ± 1.54	2.35 ± 1.99	13.41 ± 2.24^{a}	3.13 ± 1.25^{a}
Grain alcohol	81.96 ± 2.02	60.32 ± 2.89^{b}	73.59 ± 2.87^{b}	7.60 ± 1.46	2.40 ± 0.93	13.60 ± 1.00^{a}	2.81 ± 0.84^{ab}
Clove essential oil	83.60 ± 1.77	70.81 ± 2.40^{a}	84.69 ± 1.65^{a}	4.32 ± 2.68	1.56 ± 1.24	8.67 ± 3.86^{b}	0.38 ± 0.76^{c}
Paraformaldehyde	81.63 ± 1.96	66.87 ± 4.48^{ab}	81.87 ± 3.92^{a}	6.46 ± 1.46	1.48 ± 1.38	9.63 ± 2.92^{ab}	0.81 ± 0.94^{bc}
P value	0.0546	0.0027	0.0043	0.0915	0.5443	0.0197	0.0330
Coefficient of variation (%)	1.76	4.07	3.86	25.68	69.77	18.69	57.54

[a,b,c] Means in the same column with different superscript letters differ significantly ($P < 0.05$).
Abbreviations: Fert, fertility; Hatch, hatchability of set eggs; Hatch fert, hatchability of fertile eggs; Cont., contaminated**.**
[1]Results are expressed as the means ± SD.

A significant difference was observed for the hatchability of set eggs (P = 0.0027, CV = 4.07%; Table 2.5). The eggs treated with clove essential oil hatched at a mean rate of 70.81 ± 2.40%, which was similar to the mean of 66.87 ± 4.48% observed in the group fumigated with paraformaldehyde and significantly higher than that in the negative control (61.63 ± 3.32%) and grain alcohol (60.32 ± 2.89%) groups. This result can be attributed to the effects of the treatments, as there was no significant difference in the fertility rate.

The hatchability of fertile eggs (P = 0.0043; CV = 3.86%) differed significantly between the studied treatments (Table 2.5). The mean values for the eggs treated with clove essential oil (84.69 ± 1.65%) and paraformaldehyde (81.87 ± 3.92%) were statistically similar but were higher than those for the negative control (74.03 ± 3.58%) and the eggs treated with grain alcohol (73.59 ± 2.87%). Oliveira et al. (2020) compared the hatchability of fertile eggs between clove essential oil treatment at a concentration of 0.6 mg/mL (92.37 ± 3.25%) and paraformaldehyde treatment (94.44 ± 4.54%) and did not observe significant differences. Copur et al. (2010) evaluated the effect of oregano essential oil at two concentrations (0.55 and 0.75 mL/cm^3) and two exposure times (3 and 6 hours) on the sanitation of eggs intended for incubation and observed that the mean hatchability of these eggs (90.00%) did not differ significantly from the hatchability of formaldehyde-treated eggs (89.91%). In this study, sanitation with clove essential oil increased hatchability compared to that of nonsanitized eggs, possibly due to control of the bacterial load on the eggshell surface.

No significant differences in mortality were found between the treatments during the early (P = 0.0915; CV = 25.68%; Table 2.5) or middle (P = 0.5443; CV = 69.77%) incubation stage. However, there was a significant reduction (P = 0.0197; CV = 18.69%) in embryonic mortality during the late incubation stage in eggs sprayed with clove essential oil (8.67 ± 3.86%) compared to eggs sprayed with grain alcohol (13.60 ± 1.00%) and eggs in the negative control group (13.41 ± 2.24%). In this context, Copur et al. (2011) and Baylan et al. (2018) reported that a decrease in early and late mortality may be the result of reduced eggshell contamination. Therefore, the lower mortality percentage observed in the late incubation stage of eggs treated with clove essential oil may be associated with decreased microbial populations on the eggshell due to the action of the chemical constituents present in the oil.

The rate of egg contamination during incubation was affected by the treatments (P = 0.0330; CV = 57.54%; Table 5). The largest percentage of contaminated eggs was observed in the negative control group (3.13 ± 1.25%), followed by the grain alcohol (2.81 ± 0.84%), paraformaldehyde (0.81 ± 0.94%), and clove essential oil (0.38 ± 0.76%) groups. These results reinforce that the clove essential oil-based sanitizing agent presents a broad spectrum of antimicrobial activity and may have been able to maintain low levels of microorganisms on the eggs during incubation, since according to Magwood (1964), there is a significant increase in the bacterial count on the eggshell during this period.

Chick initial weight (P = 0.0723; CV = 0.91%) and yield (P = 0.2122; CV = 1.05%) did not differ between treatments (Table 2.6). However, there was a significant difference (P < 0.0001; CV = 2.48%) in the physical quality score of the chicks. Chick weight is strongly associated with the weight of the egg from which the chick hatches (Morris et al., 1968). As no differences were observed in initial egg weight or weight loss, differences in the initial weight of the chicks were not expected.

Tabela 2.6. Chick weight, percentage of chick yield, and chick physical quality score assessment according to different treatments.[1]

Treatment	Chick weight (g)	Chick yield (%)	Pasgar© Score
Nonsanitized	40.00 ± 0.68	68.34 ± 0.32	9.09 ± 0.81[bc]
Grain alcohol	40.51 ± 0.20	68.87 ± 0.54	9.06 ± 0.96[c]
Clove essential oil	39.93 ± 0.40	68.08 ± 1.08	9.21 ± 0.89[a]
Paraformaldehyde	40.32 ± 0.48	68.89 ± 1.08	9.13 ± 0.90[b]
P value	0.0723	0.2122	<0.0001
Coefficient of variation (%)	0.91	1.05	2.48

[a,b,c]Means in the same column with different superscript letters differ significantly (P < 0.05).
[1]Results are expressed as the means ± SD.

The ideal chick yield ranges from 67 to 68% (Aviagen, 2011). In the present experiment, all treatments presented yields classified as "slightly high" (68.55 ± 0.76%) but close to acceptable. The data observed in this study differ from those reported by Oliveira et al. (2020), who observed that eggs sanitized with grain alcohol (67.50 ± 1.92%), clove essential oil (67.90 ± 1.87%), or paraformaldehyde (67.80 ± 1.85%) showed chick yields classified as ideal. However, because in both experiments the eggs were subjected to ideal temperature and humidity conditions, a possible explanation for these contradictory results may be the egg weight loss observed in the present study, which, despite being within the ideal range reported in the literature (Molenaar et al., 2010), was lower and resulted in heavy chicks.

In the Pasgar© score assessment, it was determined that the clove essential oil (9.21 ± 0.89) had a superior effect on the physical quality of the chicks compared with that of the other treatments. It is known that if eggs intended for incubation are not sanitized with effective products before being placed in the incubators, chick quality may decrease because of the high bacterial contamination that may occur, which may cause infection of the yolk sac (Harry, 1957; Cortés et al., 2004). Therefore, more viable chicks were obtained in the clove essential oil treatment possibly because this sanitizer does not adversely affect embryos or chicks. In addition, we hypothesized that chicks from eggs treated with clove essential oil had fewer microorganisms compared with chicks from nonsanitized eggs, which may also have contributed to obtaining more viable chicks. In this study, the sanitation of eggs

with paraformaldehyde in the concentration and microclimate conditions used did not affect (negatively or positively) the quality of birds.

Eggshell thickness is one of the factors that affects gas exchange and moisture loss during embryonic development (Ar et al., 1974; Veldsman et al., 2020). Therefore, any undesirable changes in this parameter may be harmful to embryos. In the present study, the similarity (P = 0.8502; CV = 7.79%; Table 2.7) between the means of eggshell thickness (0.365 ± 0.026 mm) showed that the tested treatments did not negatively affect this variable, even though the application of sanitizing agents to eggs can affect eggshell structure (Kim & Slavik, 1996). Oliveira et al. (2020) also did not observe significant differences in the thickness of eggshells treated with grain alcohol, clove essential oil, ethanolic extract of propolis, or paraformaldehyde (mean of 0.370 ± 0.029 mm).

Table 2.7. Eggshell thickness according to different treatments.[1]

Treatment	**Eggshell thickness (mm)**
Nonsanitized	0.364 ± 0.024
Grain alcohol	0.365 ± 0.019
Clove essential oil	0.363 ± 0.031
Paraformaldehyde	0.368 ± 0.028
P value	0.8502
Coefficient of variation (%)	7.79

No significant differences existed between means ($P > 0.05$).
[1]Results are expressed as the means ± SD.

4. CONCLUSIONS

Clove essential oil is effective and safe for eggs intended for incubation. Its use as an alternative to paraformaldehyde in the sanitation of fertile eggs is recommended because it reduces the eggshell microbial load, resulting in good incubation parameters and better neonatal chick quality. Furthermore, our data indirectly suggest that the application of clove essential oil does not negatively affect the structural integrity of the cuticle on the eggshell surface or the development of the embryo.

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CAPÍTULO 3 – SPRAYING HATCHING EGGS WITH CLOVE ESSENTIAL OIL DOES NOT COMPROMISE THE QUALITY OF EMBRYOS AND ONE-DAY-OLD CHICKS OR BROILER PERFORMANCE[1]

[1]Paper accepted for publication in ***Animals***, 2020.

ABSTRACT

The objective of this study was to evaluate whether sanitizing hatching eggs with clove essential oil in the preincubation phase affects broiler performance and influences the hatch window and quality of embryos and one-day-old chicks. Hatching eggs (n = 1280; mean weight = 58.64 ± 0.49 g) from a batch of 37-week-old broiler breeder hens of the CPK (Pesadão Vermelho) lineage were randomly distributed into four treatments in the preincubation phase. The treatments consisted of three different sanitization procedures (spraying with grain alcohol, spraying with clove essential oil, and fumigation with paraformaldehyde) and a control treatment (nonsanitized). The lengths of the embryos and one-day-old chicks (one of the parameters used to assess bird quality) were not significantly different among the treatments, with means of 15.30 ± 1.41 and 18.37 ± 0.76 mm, respectively. Body weight, body weight gain, feed consumption, and feed conversion rate in different rearing periods did not differ significantly among the treatments. However, there was a significant difference in the percentage of survivability during the initial period (1 to 28 days) among the treatments. In conclusion, clove essential oil treatment did not negatively affect the quality of embryos and one-day-old chicks or the performance of broilers.

Key words: Broilers; chick quality; clove essential oil; embryo development; hatching eggs.

RESUMO

O objetivo deste estudo foi avaliar se a sanitização de ovos para incubação com óleo essencial de cravo-da-índia na fase de pré-incubação afeta o desempenho de frangos de corte e influencia a janela de nascimento e a qualidade de embriões e pintos de um dia de idade. Ovos para incubação (n = 1280; peso médio = 58,64 ± 0,49 g) de um lote de matrizes de corte de 37 semanas da linhagem CPK (Pesadão Vermelho) foram distribuídos aleatoriamente em quatro tratamentos na fase de pré-incubação. Os tratamentos consistiram em três diferentes procedimentos de sanitização (pulverização com álcool de grãos, pulverização com óleo essencial de cravo-da-índia e fumigação com paraformaldeído) e um tratamento controle (não sanitizado). Os comprimentos dos embriões e pintos de um dia (um dos parâmetros usados para avaliar a qualidade das aves) não diferiram significativamente entre os tratamentos, com médias de 15,30 ± 1,41 e 18,37 ± 0,76 mm, respectivamente. O peso corporal, ganho de peso corporal, consumo de ração e a conversão alimentar nos diferentes períodos de criação não diferiram significativamente entre os tratamentos. No entanto, houve diferença significativa no percentual de sobrevivência durante o período inicial (1 a 28 dias) entre os tratamentos. Em conclusão, o tratamento com óleo essencial de cravo-da-índia não afetou negativamente a qualidade dos embriões e pintos de um dia de idade ou o desempenho dos frangos de corte.

Palavras-chave: Desenvolvimento embrionário, frangos de corte, óleo essencial de cravo-da-índia, ovos para incubação, qualidade do pinto.

1. INTRODUCTION

The surface of the shells of newly laid eggs can be colonized by distinct microorganisms, which can negatively affect the productive and economic capacity of poultry farming by increasing embryo mortality rates and reducing the quality of one-day-old chicks (Reid et al., 1961; Arhienbuwa et al., 1980; Board & Tranter, 1995; Oliveira et al., 2020b). Therefore, to mitigate mortality and reduce the eggshell microbiota that are potentially pathogenic to embryos, the poultry industry adopts a variety of strategies, including the implementation of biosafety practices.

The sanitization of hatching eggs is a common biosafety practice performed in farms and hatcheries and mainly involves the use of paraformaldehyde (Furuta & Sato, 1977; Samberg & Meroz, 1995; Jabbar et al., 2019), a product that, although effective against microorganisms, is carcinogenic and teratogenic (Nielsen & Wolkoff, 2010; Duong et al., 2011; Zeweil et al., 2015), presenting a risk to the health of chicken embryos and chicken egg handlers (Casteel et al., 1987; Nielsen & Wolkoff, 2010; Zeweil et al., 2015). Therefore, researchers have sought to develop and evaluate potentially safe products that reduce the pathogenic microbial load of hatching eggshells, with the goal of minimizing impacts on human and animal health.

The application of clove essential oil in sanitizing formulations used on hatching eggs is safe and recommended (Oliveira et al., 2020b). Studies have shown that clove essential oil is effective in reducing the microbial load of hatching eggshells, provides good results in terms of incubation performance, and improves the quality of neonate chicks (Oliveira et al., 2020a; Oliveira et al., 2020b). This oil is an aromatic hydrophobic extract of *Syzygium aromaticum* (family: Myrtaceae) and is mainly composed of phenylpropanoids, such as eugenol ($C_{10}H_{12}O_2$), which constitutes 90% of the oil and is the main compound responsible for its antimicrobial activity (Craveiro & Queiroz, 1993; Atanasova-Pancevska et al., 2017).

Considering that clove essential oil is a promising sanitizing compound for hatching eggs and that no studies have investigated the performance of broilers hatched from eggs treated with this oil, the objective of this study was to evaluate whether the sanitization of hatching eggs with clove essential

oil in the preincubation phase affects broiler performances. Furthermore, this study investigated whether this oil affects the hatch window and the quality of embryos and one-day-old chicks.

2. MATERIALS AND METHODS

2.1. Experimental procedure

Hatching eggs (n = 1280; mean weight = 58.64 ± 0.49 g) from 37-week-old broiler breeder hens of the CPK line (known as Pesadão Vermelho) were randomly distributed into four treatments in the preincubation phase. CPK is a slow-growing broiler breeder line with red plumage that is active, resistant, and adapted to free-range systems. The treatments consisted of three different sanitization procedures (spraying with grain alcohol, spraying with clove essential oil, and fumigation with paraformaldehyde) and a control treatment (nonsanitized). The experimental protocol was approved by the Animal Use Ethics Committee of the University of Brasília (Document number 33/2019).

2.2. Sanitizers and sanitization methods

2.2.1. Nonsanitized

The eggs used for this treatment were not subjected to any sanitization process.

2.2.2. Grain alcohol

Grain alcohol (93.5%) (Cromoline Química Fina, Diadema, São Paulo, Brazil) served as the carrier vehicle for clove essential oil; therefore, it was tested to ensure that there was no synergism. Eggs were sprayed individually with grain alcohol using a manual sprayer. After spraying, the eggs were placed in sterile trays (30 to 50 min) to dry at room temperature. The trays were sterilized by ultraviolet light at 254 nm for 15 min in a laminar flow cabinet (OptiMair, ESCO, Horsham, PA, USA) at a microbiology laboratory (Federal Institute of Brasília, Planaltina, Federal District, Brazil).

2.2.3. Clove essential oil

The essential oil was extracted from dried clove flower buds by hydrodistillation according to a method adapted from Ascenção & Filho (2013) using the Clevenger extractor system (Vidrolabor,

Poá, São Paulo, Brazil). Subsequently, the oil was diluted in 93.5% grain alcohol at a concentration of 0.39% (w/v) (Oliveira et al., 2020b). The spraying and drying procedures used were similar to those performed on eggs treated with grain alcohol.

2.2.4. Paraformaldehyde

In this treatment, eggs were sanitized by fumigation for 20 min with 6 g/m^3 paraformaldehyde volatilized on a metal plate in a sanitization chamber (temperature: 25 °C; relative humidity: 70%), according to the guidelines of the commercial hatchery.

All sanitization processes were conducted in a commercial hatchery (Planaltina, Federal District, Brazil) 20 min after egg collection.

2.3. Storage and incubation

The eggs were stored for a period of three days in a poultry science laboratory (Federal Institute of Brasília, Planaltina, Federal District, Brazil). During storage, the temperature was maintained between 16 and 18 °C, and the relative air humidity was maintained between 55 and 60%. After storage, the egg trays were weighed and distributed in different trays in four single-stage setters (Luna 480, Chocmaster, Curitiba, Paraná, Brazil). For each treatment, four incubation trays were used, with one tray in each setter (tray position, and therefore, the treatment, was distributed randomly) for a total of 320 eggs for each treatment. The setters were sanitized with a lysoform-based (SC Johnson, Racine, Wisconsin, USA) liquid sanitizer before incubation according to the manufacturer's guidelines. The temperature of the incubation room was maintained between 22 and 24 °C, and the relative humidity was maintained between 50 and 55%. All microclimatic variables were monitored by thermohygrometers (608-H1, Testo, Campinas, São Paulo, Brazil).

From the beginning of incubation until the 18th day, the mean temperature and relative humidity of the setters were 37.7 °C and 60%, respectively. During this same period, the eggs were turned by a 45° angle every hour. On the eighth day, all eggs were subjected to candling and the infertile eggs were removed and opened to confirm infertility according to Aviagen (2009). No egg was replaced. On the 19th day, the incubation trays were weighed again, and the setters were operated at a mean temperature and relative humidity of 36.6 °C and 65%, respectively. The incubation process was terminated on the 21st day.

2.4. Hatch window

The egg hatching time was defined by video recordings using four infrared bullet cameras (VHD 1010B G4, Intelbras, Campinas, São Paulo, Brazil). After 462 h of incubation, the number of

chicks that hatched was recorded every 6 h. The chicks were counted, weighed, and removed from the setters so that the cameras maintained good visibility. The hatch window comprised the period between the first and last hatched chick in each tray.

2.5. Length and weight of embryos and one-day-old chicks and weight of residual yolk, and relative organ weight

On the 18th day of incubation, 15 eggs from each treatment were randomly selected and removed from the setters. The eggs were opened, embryo length was recorded and, then, the embryos were euthanized by cervical dislocation. The length (mm) of the embryo, wing, beak, and leg were measured with a 0.01-mm precision digital calliper (Mitutoyo, Suzano, São Paulo, Brazil). The embryo, residual yolk, heart, liver and gallbladder, proventriculus and gizzard, breast, and intestine were weighed (g) using a 0.0001-g precision analytical scale (Gehaka, São Paulo, São Paulo, Brazil). Relative organ weights were calculated as a percentage of the weight of the embryo without residual yolk. On the first day post-hatching, 15 one-day-old chicks from each treatment were randomly selected and the same procedures used for embryo measurements were conducted. However, the relative organ weights were calculated as a percentage of the chick weight. These analyses were used as parameters to assess the quality of birds.

2.6. Broiler house and management

After 21 days of incubation, 90 healthy chicks (45 females/45 males; sexed according to the wing feather characteristics) from each treatment with similar weights (mean = 40.23 ± 0.72 g) were subdivided into five replicates. Each replicate group (18 birds = 9 females/9 males) was randomly housed in a 2.40 m² pen (experimental unit) equipped with feeders and drinkers suitable for each growout period, in addition to poultry bedding made of rice straw. The birds had ad libitum access to feed and water and received the same diet, which was formulated as follows according to Prado (2019) for each rearing period: initial, corn 63%, soybean meal 33%, and minerals and vitamins 4%; growth, corn 68%, soybean meal 28%, and minerals and vitamins 4%; final, corn 82%, soybean meal 14%, and minerals and vitamins 4%. The feed was supplied twice a day. The vaccination schedule followed the guidelines in the free-range chicken farming manual (Santos et al., 2009).

During the first 14 days, infrared lamps provided heat to the birds, which were exposed to 24 h of light. From the 15th day, a lighting schedule of 16 h of light:8 h of dark was initiated. The birds were subjected to similar temperature and humidity conditions throughout the study. The broiler house was equipped with a ventilation system with two manually operated fans, nebulization, and side curtains.

The birds (pen weight) and wasted feed from each experimental unit were weighed weekly on a precision scale (Triunfo, São Paulo, São Paulo, Brazil) until the 70th day; however, only the weights of days 28, 56, and 70 were used to evaluate performance (body weight, weight gain, feed consumption, and feed conversion), representing the three rearing periods (initial (day 1 to day 28), growth (day 29 to day 56), and final (day 57 to day 70)]. The scale was observed by 15 s approximately. During this period, the highest and lowest values were recorded and then the mean between these values was used as weight of birds. Mortality was recorded throughout the experimental period to calculate survivability. Birds were not replaced in the house; moreover, all dead birds were weighed so that it was possible to adjust the feed consumption and feed conversion during the period.

2.7. Experimental design and statistical analysis

The experiment followed a completely randomized design with four treatments (nonsanitized, grain alcohol, clove essential oil, and paraformaldehyde). In the analysis of embryo and chick quality, each embryo and chick was considered a replicate. The analysis of posthatching performance was based on five replicates per treatment, in which each pen of 18 birds constituted a replicate. All analyses were performed with SAS Studio University Edition (Inst. Inc., Cary, NC, USA). The data were analyzed by analysis of variance (PROC GLM), and means were compared using Tukey's test. The hatching curves were subjected to survival analysis using the PROC LIFETEST command and the Kaplan–Meier method combined with log-rank analysis with subsequent comparison by Tukey's test. The hatch window (period between the first and last hatching) was compared by the Kruskal–Wallis test using the PROC NPAR1WAY procedure. Statistical significance was considered at $P < 0.05$ for all tests.

3. RESULTS

3.1. Hatch window

Chick hatching began between hours 462 and 468 and ended between hours 492 and 498 for all treatments (Figure 3.1). There were no significant differences in the hatch window among the treatments (P = 0.8348). However, the hatching curves of the grain alcohol, clove essential oil, and paraformaldehyde treatments differed (P = 0.006) from those of the nonsanitized treatment (Figure 1).

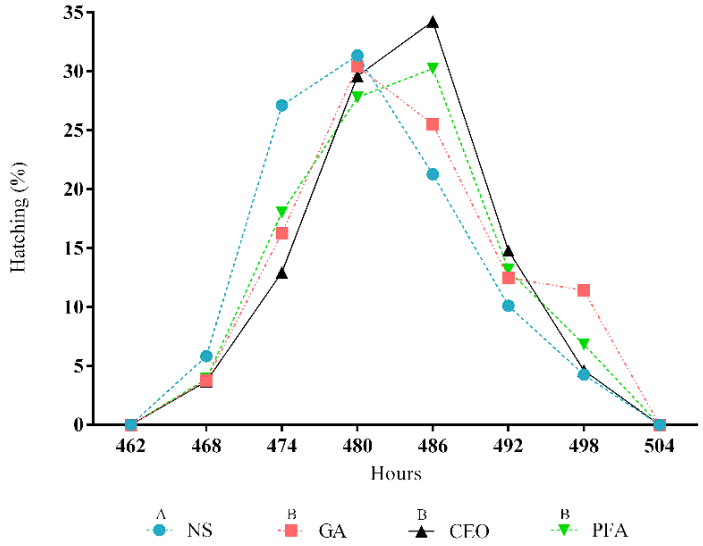

Figure 3.1. Hatching curves for different sanitization treatments. NS, nonsanitized; GA, grain alcohol; CEO, clove essential oil; PFA, paraformaldehyde. [A,B] Curves with different letters differ significantly (P < 0.05).

3.2. Quality of embryos and one-day-old chicks

The lengths of embryos (15.30 ± 1.41 mm), wings, beaks, and leg at 18 days of incubation as well as the lengths of chicks (18.37 ± 0.76 mm), wings, beaks, and legs at 1 day of age were not significantly different (P > 0.05) among the treatments (Tables 3.1 and 3.2). The absolute weights of

the embryo (mean = 29.75 ± 3.02 g) and residual yolk at 18 days of incubation (11.79 ± 2.58 g) as well as the weight of chicks (40.44 ± 4.20 g) and residual yolk at 1 day of age (3.44 ± 1.05 g) were not affected ($P > 0.05$) by the sanitization treatments (Tables 3.1 and 3.2).

The relative organ weights of the embryos at 18 days of development and those of one-day-old chicks were similar ($P > 0.05$) among the treatments, except for the relative weights of the liver and gallbladder, breast, and intestine of the embryos at 18 days of age ($P < 0.05$) (Tables 3.1 and 3.2). The weights of the liver and gallbladder, breast, and intestine of the embryos subjected to the paraformaldehyde treatment were significantly higher than those of the embryos subjected to the grain alcohol treatment; however, the values for the three treatments did not differ from those for the nonsanitized treatment.

Table 3.1. Mean values for lengths of the embryo, wings, beaks, and legs, weight of the embryo without residual yolk and of the residual yolk, and the relative organ weight in the different sanitization treatments.

Items	**Treatments**					
	Nonsanitized	**Grain Alcohol**	**Clove Essential Oil**	**Paraformaldehyde**		
	Length (cm)				*P*	CV (%)
Embryo	15.44 ± 1.78	14.87 ± 1.20	15.75 ± 1.07	15.14 ± 1.59	*	9.21
Wing	2.65 ± 0.37	2.73 ± 0.35	2.84 ± 0.28	2.62 ± 0.33	*	12.38
Beak	1.11 ± 0.18	1.15 ± 0.15	1.18 ± 0.24	1.10 ± 0.14	*	14.91
Leg	3.96 ± 0.39	3.80 ± 0.46	4.14 ± 0.36	3.75 ± 0.70	*	13.10
	Weight (g)					
Embryo without residual yolk	29.85 ± 4.05	29.05 ± 1.94	29.57 ± 2.80	30.51 ± 3.27	*	10.20
Residual yolk	11.87 ± 2.23	11.48 ± 2.74	11.97 ± 1.82	11.82 ± 3.51	*	22.72
	Relative organ weight (%)					
Heart	0.67 ± 0.05	0.69 ± 0.08	0.74 ± 0.16	0.85 ± 0.14	*	55.11
Liver and gallbladder	1.84 ± 0.17^{ab}	1.65 ± 0.14^{b}	1.99 ± 0.12^{ab}	2.16 ± 0.18 ^{a}	0.0021	24.85
Proventriculus and gizzard	5.80 ± 0.43	5.05 ± 0.38	5.55 ± 0.41	5.64 ± 0.37	*	24.33
Breast	4.25 ± 0.22^{ab}	4.06 ± 0.22^{b}	4.33 ± 0.18^{ab}	4.82 ± 0.21 ^{a}	0.0039	15.42
Intestine	2.71 ± 0.28^{ab}	2.44 ± 0.30^{b}	2.80 ± 0.28^{ab}	3.05 ± 0.62 ^{a}	0.0134	44.41

^{a,b} Means in the same row with different superscript letters differ significantly ($P < 0.05$). * nonsignificant; CV, coefficient of variation.

Table 3.2. Mean values for lengths of the chick, wings, beaks, and legs, weight of the chick and residual yolk, and relative organ weight in the different sanitization treatments.

Items	**Treatments**					
	Nonsanitized	**Grain Alcohol**	**Clove Essential Oil**	**Paraformaldehyde**		
	Length (cm)				*P*	CV (%)
Chick	18.53 ± 0.59	18.13 ± 0.96	18.63 ± 0.72	18.17 ± 0.75	*	4.40
Wing	3.37 ± 0.19	3.29 ± 0.37	3.55 ± 0.52	3.34 ± 0.31	*	10.72
Beak	1.03 ± 0.08	1.07 ± 0.09	1.09 ± 0.09	1.06 ± 0.12	*	9.10
Leg	4.18 ± 0.13	4.13 ± 0.26	4.28 ± 0.20	4.12 ± 0.25	*	5.17
	Weight (g)					
Chick	40.61 ± 3.23	40.04 ± 5.28	40.98 ± 4.83	40.14 ± 3.45	*	10.58
Residual yolk	3.38 ± 0.78	3.46 ± 1.18	3.43 ± 1.20	3.47 ± 1.02	*	30.77
	Relative organ weight (%)					
Heart	0.81 ± 0.09	0.80 ± 0.09	0.88 ± 0.11	0.90 ± 0.10	*	28.51
Liver and gallbladder	3.42 ± 0.28	3.25 ± 0.32	3.32 ± 0.37	3.29 ± 0.31	*	23.91
Proventriculus and gizzard	6.57 ± 0.52	6.39 ± 0.44	6.98 ± 0.61	6.72 ± 0.56	*	19.95
Breast	2.19 ± 0.27	2.17 ± 0.25	2.27 ± 0.25	2.19 ± 0.26	*	29.76
Intestine	7.36 ± 0.81	7.04 ± 0.58	7.76 ± 1.10	7.20 ± 0.82	*	28.37

No significant differences existed between means ($P > 0.05$). * nonsignificant; CV, coefficient of variation.

3.3. Broiler performance

Body weight, body weight gain, feed consumption, and feed conversion ratio in the different rearing periods did not differ significantly ($P > 0.05$; Table 3.3) among the treatments; however, there was a significant difference ($P = 0.0397$) in the percentage of survivability during the initial period (1 to 28 days). The survivability of birds hatched from eggs treated with clove essential oil (97.96%) did not differ significantly from that of birds hatched from eggs treated with paraformaldehyde (96.94%), but it was significantly higher than that of birds in the grain alcohol (92.84%), and nonsanitized (92.48%) treatments.

Table 3.3. Mean values for the body weight, body weight gain, feed consumption, feed conversion ratio, and survivability of broilers from eggs sanitized with different sanitizers.

Parameters	Treatments					
	Nonsanitized	**Grain Alcohol**	**Clove Essential Oil**	**Paraformaldehyde**	***P***	**CV (%)**
1 to 28 days (initial period)						
Body weight (g)	541.68	544.45	550.51	553.31	*	3.71
Body weight gain (g)	501.07	513.50	510.06	514.44	*	4.27
Feed consumption (g)	861.72	875.59	861.34	871.58	*	4.86
Feed conversion ratio	1.720	1.704	1.689	1.689	*	3.21
Survivability (%)	92.48[b]	92.84[b]	97.96[a]	96.94[ab]	0.0397	4.01
29 to 56 days (growth period)						
Body weight (g)	1975.88	2015.84	2002.92	2010.38	*	2.11
Body weight gain (g)	1434.20	1461.38	1452.41	1457.07	*	2.18
Feed consumption (g)	3821.57	3907.88	3896.01	3795.28	*	6.67
Feed conversion ratio	2.664	2.674	2.683	2.603	*	6.36
Survivability (%)	100.00	100.00	100.00	100.00	*	0.00
57 to 70 days (final period)						
Body weight (g)	2667.92	2742.08	2731.84	2728.97	*	2.36
Body weight gain (g)	692.04	726.26	728.75	718.59	*	5.38
Feed consumption (g)	2643.98	2842.03	2741.80	2745.06	*	6.49
Feed conversion ratio	3.830	3.915	3.765	3.819	*	5.71
Survivability (%)	98.82	98.82	98.82	100.00	*	1.82
P	<0.0001	<0.0001	<0.0001	<0.0001		
1 to 70 days (overall period)						
Total survivability (%)	97.10	97.22	98.93	98.98	*	3.33

[a,b] Means in the same row with different lowercase letters differ significantly ($P < 0.05$). * nonsignificant; CV, coefficient of variation

4. DISCUSSION

Essential oils have been used as effective sanitizers for hatching eggs (Ulucay & Yildirim, 2010; Copur et al., 2010; Oliveira et al., 2020b). Positive effects on the sanitization of hatching eggs with essential oils, such as clove essential oil, have been described in terms of both antimicrobial efficiency and incubation performance and in terms of bird quality (Oliveira et al., 2020a; Oliveira et al., 2020b). In this sense, to contribute to the results already described in the literature associated with the applicability of this oil for hatching eggs, the present study investigated the effects of clove essential oil on post-hatch performance, also considering the effect on the hatch window and bird quality.

4.1. Hatch window

The mean hatch window was 27.75 h, corroborating the results described by Decuypere et al. (2001), who reported a hatch window range of 24 to 48 h in commercial hatcheries. However, the mean hatching time of the nonsanitized eggs was 2.5 h shorter than the mean hatching time of eggs treated with grain alcohol, clove essential oil, or paraformaldehyde. In addition, Figure 3.1 shows that a high number of eggs in this treatment hatched earlier, before the middle of the hatch window period, which is in fact a disadvantage because it increases the duration that chicks remain in the hatcher, which consequently increases the likelihood of animal dehydration (Hodgetts, 2006). On the other hand, more eggs sanitized with clove essential oil and paraformaldehyde hatched in the middle of the hatch window period, indicating the importance of good sanitization of hatching eggs to maintain adequate embryonic development.

4.2. Quality of embryos and one-day-old chicks

The quality of embryos and one-day-old chicks is of great importance for poultry production. The lengths of embryos and one-day-old chicks were measured to evaluate the effects of the treatments on bird quality. Length measurements (Tables 3.1 and 3.2) showed that the quality of the embryos and one-day-old chicks was not affected, confirming that the tested sanitizers did not negatively impact

bird development, possibly because they did not alter the properties of the cuticle, since the application of sanitizers in the eggshell can affect the cuticle and alter the permeability of the eggshell and embryonic development (Brake & Sheldon, 1990).

The weights of the embryos and one-day-old chicks were also used to assess bird quality. The different sanitization treatments did not significantly alter the weights of the embryos and one-day-old chicks or the weights of the respective residual yolks (Tables 3.1 and 3.2). The yolk is the main source of energy for the growth and maintenance of the body during embryonic development (Noble & Cocchi, 1990; Speake et al., 1998). Therefore, the results suggest that the amount of nutrients absorbed from the yolk and converted into body tissue by the birds during development was proportional among the treatments, which may have contributed to the similar weights of these birds. In addition, the amount of residual yolk and the weight of embryos and one-day-old chicks are mainly affected by the age and lineage of the broiler breeder hens and the duration and conditions of storage and incubation (Alsobayel et al., 2013; Zuidhof et al., 2014; Van der Wagt et al., 2020). This finding may explain the similarity among treatments for these variables in this experiment because all eggs were from broiler breeders of the same age and lineage, subjected to the same storage conditions and time, and exposed to the same incubation conditions.

The relative internal organ weights (Tables 3.1 and 3.2) served as an indicator of the responses of the embryos and one-day-old chicks to the possible toxicity of the sanitizers. In this experiment, no macroscopic alterations, such as atrophy or hypertrophy, were observed in the internal organs. Therefore, it can be stated that the tested sanitizers did not negatively affect the development of organs during embryogenesis and the post-hatching period. The results suggest that clove essential oil at 0.39% is safe for embryos and does not negatively affect their survival, growth, or health. In addition, the results suggest that adequate sanitization of eggs with paraformaldehyde can avoid the adverse effects of this compound on the development of birds. The adverse effects of paraformaldehyde in chick embryos described in the literature, include malformations, low weight, and underdevelopment (Zeweil et al., 2015).

4.3. Broiler performance

Fasenko et al. (2009) did not observe significant differences in body weight or feed conversion between broilers from eggs sanitized with electrolyzed oxidizing water and those not sanitized during 39 days of growth, which is in accordance with our results. In this study, the percentage of survivability for the first 28 days of broiler rearing was higher in the clove essential oil treatment group than in the nonsanitized treatment group (Table 3.3). Although chick quality did not differ among the treatments in terms of weight and length, we can hypothesize that chicks from eggs sanitized with clove essential

oil had a lower microbial load than chicks from eggs that were not sanitized, which may have resulted in greater survivability in the initial period in the clove oil treatment group. Therefore, studies that measure the internal microbial load of chicks from eggs sanitized with clove essential oil need to be carried out to support this hypothesis.

5. CONCLUSIONS

Clove essential oil treatment did not impair the hatch window, quality, or development of embryos and one-day-old chicks, or the performance parameters evaluated (body weight, body weight gain, feed consumption, feed conversion ratio, and survivability). Therefore, considering the factors evaluated here, this oil can be used as a sanitizer for incubating eggs safely for the birds.

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CAPITULO 4 – CONSIDERAÇÕES FINAIS

Para garantir que o desafio microbiano do ambiente de incubação seja mínimo e, assim, não afete o rendimento de incubação, deve ser adotada a prática de sanitização de ovos na fase de pré-incubação. Há um movimento crescente para sanitizar ovos incubáveis com produtos que sejam menos ofensivos do que à fumigação química, convencional e tóxica com o formaldeído. Para este propósito, o óleo essencial de cravo-da-índia é um sanitizante alternativo potencial e recomendado, pois é seguro, reduz a carga microbiana da casca, não afeta a qualidade da casca em termos de espessura, proporciona alta taxa de eclodibilidade, não afeta negativamente a qualidade de embriões e pintos e nem o desempenho de frangos de corte. Estudos adicionais que visam à avaliação de óleos essenciais na sanitização de ovos incubáveis são necessários, uma vez que reduzir a dependência e o uso de produtos químicos sintéticos e seus efeitos tóxicos é primordial. Além disso, é necessário que esses estudos avaliem e confirmem se a estrutura da casca (cutícula) de ovos incubáveis é danificada ou não pela pulverização de óleos essenciais.

Os ovos utilizados neste estudo foram provenientes de ninhos e são considerados limpos. Portanto, observou-se que ovos limpos possuem carga microbiana capaz de reduzir a eclodibilidade, o que reforça a necessidade do uso de sanitizantes nesses ovos. Considerando que o óleo essencial de cravo-da-índia foi eficiente para ovos limpos, é fundamental a avaliação desse composto em ovos sujos, devido à contaminação da casca ser consideravelmente maior. Por fim, é importante enfatizar que o óleo essencial de cravo-da-índia é uma opção viável para sanitizar ovos incubáveis.

ANEXO A

Clove essential oil in the sanitation of fertile eggs

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ABSTRACT The aim of this study was to evaluate the efficacy of sanitizing fertile eggs with clove essential oil as an alternative to paraformaldehyde; effects on the reduction in eggshell microbial count, incubation yield, and neonatal chick quality were measured. A total of 1,460 brown fertile eggs with a mean weight of 58.64 ± 0.49 g (from 37-wk-old CPK [Pesadão Vermelho] breeder hens) were collected under aseptic conditions and randomly distributed into 4 treatments (nonsanitized and sanitized with grain alcohol, clove essential oil, and paraformaldehyde) before incubation. The count of total aerobic mesophilic bacteria was significantly lower after spraying with clove essential oil (2.30 ± 0.24 $\log_{10}$ CFU/mL) than on nonsanitized eggs (3.49 ± 0.34 $\log_{10}$ CFU/mL) or on eggs sprayed with grain alcohol (3.09 ± 0.14 $\log_{10}$ CFU/mL) but did not differ significantly from the count in the paraformaldehyde group (2.23 ± 0.29 $\log_{10}$ CFU/mL). The hatchability of fertile eggs differed significantly between the studied treatments. The mean values for the eggs treated with clove essential oil (84.69 ± 1.65%) and paraformaldehyde (81.87 ± 3.92%) were statistically similar but were higher than the negative control (74.03 ± 3.58%) and grain alcohol (73.59 ± 2.87%) values. In the Pasgar© score assessment, it was determined that the clove essential oil (9.21 ± 0.89%) had a superior effect on the physical quality of the chicks compared with the effects of the other treatments. Clove essential oil is effective and safe for eggs intended for incubation. Its use as an alternative to paraformaldehyde in the sanitation of fertile eggs is strongly recommended.

Key words: bacterial enumeration, clove essential oil, fertile eggs, hatching results, sanitizers

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INTRODUCTION

There is a constant challenge to improve the productivity of the poultry production chain, whether in the prehatch, hatch, or posthatch stage. In this sense, maximizing the efficiency of incubation processes and maximizing the quality of day-old chicks are among the main objectives of broiler farming. For these goals to be achieved, we must identify the critical steps that may result in production losses.

One of the main strategic points at which the poultry industry can optimize the efficiency of production is the sanitation of fertile eggs. Reducing the microbial load of eggshells can minimize the occurrence and prevalence of pathogenic microorganisms, which are severely harmful to embryonic development and maximize hatchability and chick quality (Shahein and Sedeek, 2014). The sanitizing compound commonly used in farms and hatcheries is paraformaldehyde, which is effective for maintaining low contamination levels of eggshells (Williams, 1970; Whistler and Sheldon, 1989). However, paraformaldehyde is highly toxic to the health of the professionals who handle it and to chick embryos and harmful to the environment (Casteel et al., 1987; Roca et al., 2008; Cadirci, 2009; Unsaldi and Ciftci, 2010; Rhomberg, 2015). Zeweil et al. (2015), for example, observed that paraformaldehyde can cause malformations in developing chick embryos.

Clove essential oil (*Syzygium aromaticum*) may be an alternative for sanitizing fertile eggs (Oliveira and Santos, 2018; Oliveira et al., 2020). Usually, odoriferous and liquid essential oils are mixtures of lipophilic substances derived from secondary metabolites of plants. Chemically, most essential oils are composed of terpenoids, phenylpropanoids, or linear alkanes and alkenes (Dhifi et al., 2016). Clove essential oil consists of a mixture of aliphatic and cyclic volatile terpenes and

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Table 1. Description of treatments, chemical concentrations, and methods of application to eggs.

Treatment		Concentration	Application	Number of eggs
T1	Nonsanitized[1]	-	-	365
T2	Grain alcohol	93.5%	Spraying	365
T3	Clove essential oil	0.39%	Spraying	365
T4	Paraformaldehyde	6 g/m^3	Fumigation	365

[1]Negative control.

phenylpropanoids (Oliveira et al., 2016), with eugenol being the major component, and has high antimicrobial activity (Dhara and Tripathi, 2013).

Further studies demonstrating the effectiveness of clove essential oil in the sanitation of fertile eggs are necessary, considering its chemical composition and antimicrobial activity, as well as the first promising results regarding the artificial incubation process of eggs sanitized with this oil (Oliveira et al., 2020). In addition, the development of new products to replace those considered harmful is fundamental for the advancement of the poultry sector. This study aimed to evaluate the efficacy of sanitizing fertile eggs with clove essential oil as an alternative to paraformaldehyde, measuring the reduction in eggshell microbial count, incubation yield, and neonatal chick quality.

MATERIALS AND METHODS

Ethics Approval

The present study was approved by the Ethics Committee on Animal Use of the University of Brasília under opinion No. 33/2019.

Experimental Procedure

A total of 1,460 brown fertile eggs with a mean weight of 58.64 ± 0.49 g (from 37-wk-old CPK [Pesadão Vermelho] breeder hens) were collected under aseptic conditions and randomly distributed into 4 treatments before incubation, as described in Table 1.

Internal egg quality was measured using the Haugh unit and yolk index of 80 eggs (20 per treatment). There was no significant difference among treatments, preventing egg quality from interfering with the incubation results. The mean Haugh unit of the eggs was 85.65 ± 7.31 (P = 0.2830; coefficient of variation [**CV**] = 8.30%), and they were classified as "AA", that is, of excellent quality (USDA, 2000). The calculated yolk index was 0.39 ± 0.03 (P = 0.6647; CV = 7.33%).

Table 2. Chemical compounds identified in the clove essential oil.

Peak	CRT (min)	Area (%)	CKI	TKI[1]	Compound
1	22.730	89.97	1,363	1,359	Eugenol
2	25.248	2.22	1,422	1,419	β-Caryophyllene
3	29.602	7.81	1,530	1,522	Acetyleugenol

Abbreviations: CTR, compound retention time; CKI, calculated Kovats index; TKI, tabulated Kovats index.
[1]Adams (2017).

These analyses were also essential because the internal structure of the egg has the potential to meet the nutrient and energy demands of embryos until hatching.

Acquisition and Preparation of Clove Essential Oil-Based Sanitizing Agent

Dried clove flower buds were obtained from a commercial market in Brasília, Federal District, Brazil. The essential oil was extracted in a laboratory of natural product chemistry (Federal Institute of Brasília, Gama, Federal District, Brazil) by a method adapted from Ascenção and Filho (2013) involving hydrodistillation with the Clevenger extraction system (Vidrolabo, Poá, São Paulo, Brazil). The chemical analysis of the clove essential oil by means of gas chromatography coupled to mass spectrometry allowed the identification of 3 components, with eugenol (89.97%) being the main component (Table 2). The structures were defined on the basis of retention times, calculation of the Kovats index, the Wiley7, FFNSCl.3, and NIST08 databases and comparison with data from Adams (2017).

To prepare the sanitizer, the clove essential oil was diluted in 93.5% grain alcohol (Cromoline Química Fina, Diadema, São Paulo, Brazil) to a concentration of 0.39%. This concentration was chosen because it was the lowest concentration of the oil tested in vitro by the disc diffusion method recommended by Bauer et al. (1966) that showed an inhibitory effect against standard strains of *Escherichia coli* (ATCC 25,922), *Pseudomonas aeruginosa* (ATCC 27,853), and *Staphylococcus aureus* (ATCC 25,923) (Figure 1).

For this test, bacterial strains (ATCC, Manassas, VA) were activated in brain heart infusion broth (Neogen, Lansing, MI) and incubated for approximately 24 h at 36°C. Subsequently, they were standardized in sterile saline (NaCl 0.85%) until a turbidity compatible with grade 0.5 of the McFarland scale (1.5 × 10^8 CFU/mL) was obtained. The oil was weighed to determine the volume that comprised 100 mg. This amount was used in testing as the full-strength (100%) concentration and was then serially diluted in dimethyl sulfoxide (**DMSO** 5%; Sigma-Aldrich, Saint Louis, MO). The sterile filter paper discs (4 mm) were impregnated with 10 µL of clove essential oil in concentrations ranging from 50 to 0.39% (p/v) and then deposited with sterile forceps on the surface of the Petri dishes containing the culture medium (Mueller-Hinton agar, HiMedia, Mumbai, Maharashtra, India), which was previously inoculated with 100 µL of bacteria. A pure DMSO control was included with each test to ensure

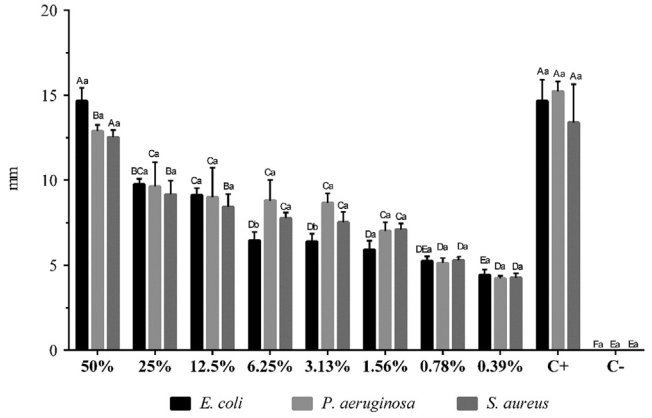

Figure 1. Determination of the antimicrobial activity of clove essential oil against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* by the disk diffusion method.[1 A-F; a,b] Means with different uppercase (bacteria) or lowercase (oil concentration) letters differ significantly ($P < 0.05$). Abbreviations: C+, positive control (30 µg of chloramphenicol); C−, negative control (5% DMSO); DMSO, dimethyl sulfoxide. [1]Results are expressed as the mean diameter of the inhibition halos in millimeters (mm) for each concentration of clove oil (%) tested.

that microbial growth was not inhibited by DMSO itself. Chloramphenicol (30 µg/disc; Sigma-Aldrich) was used as a positive control. Plates were then inverted and incubated for approximately 24 h at 36°C, and the diameter of the inhibition zones was measured in millimeters using a digital caliper with 0.001-mm precision (Mitutoyo, Suzano, São Paulo, Brazil). Each test was performed with 3 replicates.

The grain alcohol used in this study served as the carrier vehicle of the clove essential oil. Therefore, its isolated effect on the sanitation of fertile eggs was also tested.

Egg Sanitation

Sanitation procedures were performed in a room at a commercial hatchery (Planaltina, Federal District, Brazil) 20 min after egg collection. The eggs from treatment T1 (negative control) were kept in the same room as the other treatments and did not receive any sanitation procedure. In the T2 (grain alcohol) and T3 (clove essential oil) treatments, eggs were homogeneously sprayed over their entire surface with manual sprayers. After spraying, the eggs were placed in sterile trays for drying at room temperature for 30 to 50 min. At the same time, the eggs from treatment T4 (paraformaldehyde) were sent for fumigation. In this process, a concentration of 6 g/m^3 paraformaldehyde was used. Burning of the product, fumigation, and gas exhaustion proceeded for 20 min in a completely closed chamber, according to the guidelines of the commercial hatchery. The relative humidity and temperature in the chamber were 70% and 25°C, respectively.

Eggshell Microbial Count

A method adapted from Fasenko et al. (2009) was used to count microbes on the eggshell. One hour after sanitation, 20 eggs from each treatment were placed into sterile plastic bags (pooled sample of 4 eggs per bag) labeled according to treatment and transported under cooling to the laboratory of microbiology (Federal Institute of Brasília, Planaltina), where the analyses of eggshell microbial count were performed. Each bag containing a pooled sample of 4 eggs was reopened, and 220 mL of 0.1% peptone saline solution (Kasvi, São José dos Pinhais, Paraná, Brazil) was added. The eggs were manually massaged for 5 min to extract the microbial load. Then, a 1.0-mL aliquot was removed from each bag, and serial decimal dilutions in 0.1% peptone saline solution were performed for each sample. A 1.0-mL aliquot of each dilution was plated on standard plate count agar (Neogen), violet red bile glucose agar (Kasvi), and potato dextrose agar (HiMedia) to count of total aerobic mesophilic bacteria, Enterobacteriaceae, and molds and yeasts, respectively. The plates containing plate count agar and violet red bile glucose agar were incubated at 37°C for 48 h, and the plates with potato dextrose agar were incubated at 29°C for 6 D. After the incubation period, the colonies formed were counted, and the results are expressed in $\log_{10}$ CFU/mL of pooled sample of 4 eggs.

Incubation and Hatching

After the sanitation process, the eggs were transported to a laboratory of poultry science (Federal Institute of Brasília, Planaltina). The eggs were stored for 3 D at a temperature of 16°C to 18°C and a 55 to 60% relative humidity. The eggs were then separated by treatment into incubation trays with a capacity of 80 eggs each. For each treatment, 4 incubation trays were used, totaling 320 eggs. The incubation trays containing the eggs were individually weighed and randomly distributed into 4 single-stage setters (Luna 480, Chocmaster, Curitiba, Paraná, Brazil), as shown in Figure 2. The setters were in an air-conditioned room at 22°C to 24°C and 50 to 55% humidity. These meteorological variables were monitored by 2 thermohygrometers (Testo 608-H1, Campinas, São Paulo, Brazil) to ensure the proper functioning of the setters.

The setters were operated at a mean temperature of 37.7°C (99.86°F), a mean relative humidity of 60%, and with automatic turning every hour at a 45° angle for the first 18 D of incubation. On the eighth day (192 h of incubation), all eggs were candled to remove infertile eggs and eggs with early embryonic mortality. Starting on day 19 (456 h of incubation), the incubation trays were weighed again, and the incubators were operated at a mean temperature of 36.6°C (97.88°F) and a 65% relative humidity. After 21 D (504 h of incubation), the unhatched eggs were counted, opened, and evaluated to determine the number of infertile eggs, the period of embryonic mortality (early [0–7 D], mid [8–18 D], and late [19–21 D plus pipped]), and the number of contaminated eggs.

After the end of the incubation process, the percentages of egg weight loss (%), fertility (%), hatchability of set eggs (%), hatchability of fertile eggs (%), early dead (%), mid dead (%), late dead (%), contaminated eggs (%), and chick yield (%) were calculated per

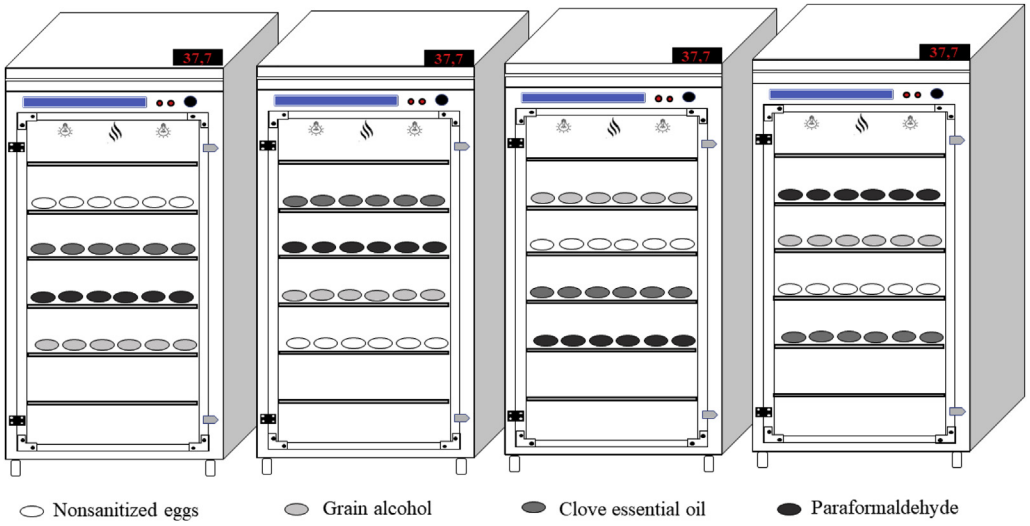

Figure 2. Distribution of treatments in setters.

Aviagen (2011) and Baylan et al. (2018) using equations 1 to 9, respectively.

1) Egg weight loss (%) = [(initial egg weight − egg weight measured on the transfer day)/initial egg weight] × 100.
2) Fertility (%) = (number of fertilized eggs/number of eggs set) × 100.
3) Hatchability of set eggs (%) = (number of hatched chicks/total number of set eggs) × 100.
4) Hatchability of fertile eggs (%) = (number of hatched chicks/number of fertile eggs) × 100.
5) Early dead (%) = (number of dead embryos on days 0–7 of incubation/number of fertile eggs) × 100.
6) Mid dead (%) = (number of dead embryos on days 8–18 of incubation/number of fertile eggs) × 100.
7) Late dead (%) = (number of dead embryos on days 19–21 of incubation/number of fertile eggs) × 100.
8) Contaminated eggs (%) = (number of contaminated eggs/number of fertile eggs) × 100.
9) Chick yield (%) = (chick weight on the day of hatch/initial egg weight) × 100.

Evaluation of Eggshell Thickness A method adapted from Barbosa et al. (2012) was used to evaluate eggshell thickness. After the chicks hatched, 50 eggshells from each treatment were separated and dried at room temperature for 3 D. Then, the thickness of each eggshell was measured without removing its internal membranes, and means were obtained from 3 different points at the equatorial plane of the eggshell using a digital caliper with 0.001-mm precision (Mitutoyo).

Table 3. Assessment of chick quality according to the Pasgar© score.

Observed parameter	Assessment
Navel area	Healing state
Legs	Presence of injury
Eyes	Brightness and wideness of the gape of the eyelid
Beak	Presence of injury
Venter	Degree of absorption of the yolk sac
Reflex	Ability to react to stimuli

Source: Adapted from Boerjan (2006).

Evaluation of Chick Quality After removal of the chicks from the incubators, their quality was visually assessed using the Pasgar© score method adapted from Boerjan (2006). Typically, each bird started the evaluation with 10 points and lost 1 point for each trait (Table 3) considered to be poor by the examiner. This subjective assessment was performed by a single person to avoid interexaminer variation.

Experimental Design and Statistical Analysis The experiment followed a randomized block design with 4 treatments. The analysis of incubation yield was based on 4 replicates per treatment, in which each tray of 80 eggs constituted a replicate. To analyze eggshell thickness and chick yield and quality, each egg and chick were considered a replicate. A completely randomized experimental design was used for eggshell bacterial count, with 5 replicates each, in which each pooled sample of 4 eggs was considered a replicate. Data were subjected to analysis of variance in SAS Studio University Edition software (SAS Inst. Inc., Cary, NC). Means were tested for significant differences by Tukey's test when the assumptions of normality and homoscedasticity were met. When the test for normality distribution or homogeneity of variances failed, the Kruskal-Wallis test was used. Statistical significance for all tests was considered at $P < 0.05$.

RESULTS AND DISCUSSION

Eggshell Microbial Count

The count of total aerobic mesophilic bacteria was significantly lower ($P < 0.0001$; CV = 9.54%; Figure 3) after spraying with clove essential oil (2.30 ± 0.24 $\log_{10}$ CFU/mL) than on nonsanitized eggs (3.49 ± 0.34 $\log_{10}$ CFU/mL) or on eggs sprayed with grain alcohol

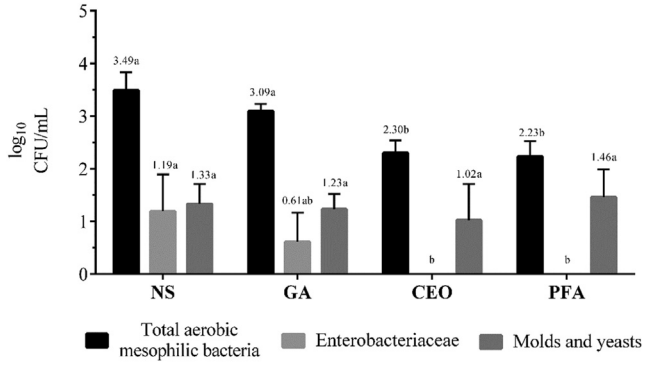

Figure 3. Counts of total aerobic mesophilic bacteria ($P < 0.0001$; CV = 9.54%), Enterobacteriaceae ($P = 0.0066$; CV = 98.24%), and molds and yeasts ($P = 0.6544$; CV = 37.59%) on eggshell surfaces according to different treatments.[1] [a,b]Means with different letters differ significantly ($P < 0.05$). Abbreviations: CEO, clove essential oil; CV, coefficient of variation; GA, grain alcohol; NS, nonsanitized; PFA, paraformaldehyde. [1]Results are expressed in $\log_{10}$ CFU/mL pooled over 4 eggs.

(3.09 ± 0.14 $\log_{10}$ CFU/mL) but did not differ significantly from the count in the paraformaldehyde group (2.23 ± 0.29 $\log_{10}$ CFU/mL).

Essential oils are effective in reducing the microbial contamination of eggshells intended for incubation (Copur et al., 2010; Ulucay and Yildirim, 2010), which is reinforced by the present results. In this study, the potent antimicrobial activity of clove essential oil was mainly because of its high phenolic compound content (Chaieb et al., 2007). Phenolic compounds react with the phospholipids of the bacterial cell membrane, making them more permeable. This change in membrane permeability leads to the loss of ions, a reduction in membrane potential, depletion of the function of proton pumps, and a reduction in adenosine triphosphate, causing cell death (Burt, 2004).

The count of Enterobacteriaceae was significantly affected ($P = 0.0066$; CV = 98.24%; Figure 3) by the treatments and ranged from 0.00 in the clove essential oil and paraformaldehyde groups to 1.19 ± 0.70 $\log_{10}$ CFU/mL for the negative control. These results show that the presence of *Salmonella enterica* serovar Enteritidis, and pathogenic *E. coli* was not observed in the eggshells after sanitation with clove essential oil or paraformaldehyde. Prabuseenivasan et al. (2006) reported that clove essential oil exhibited bioactivity mainly against gram-negative bacteria, which was confirmed by the results of this study. In general, the low count of Enterobacteriaceae indicated good hygienic conditions of the farm that provided the eggs (Musgrove et al., 2014).

The total count of molds and yeasts ranged from 1.02 ± 0.69 (clove essential oil) to 1.46 ± 0.53 $\log_{10}$ CFU/mL (paraformaldehyde). No significant differences were observed ($P = 0.6544$; CV = 37.59%; Figure 3) between any of the treatments. The low fungal load on the surface of the eggshells may be explained by the fact that the eggs were not exposed to a very humid environment on the farm (Board and Tranter, 1995).

Hatching Results

The percentage of egg weight loss during incubation did not differ between treatments in this study ($P = 0.1495$; CV = 3.00%, Table 4). This parameter is more strongly influenced by physical factors essential for incubation, such as temperature and relative humidity (Barott, 1937; Tullet and Burton, 1982; Meijerhof and van Beek, 1993). In this experiment, as the eggs were subjected to the same incubation conditions, no difference between the treatments was expected. In addition, evaluating egg weight loss during incubation allowed us to indirectly estimate the level of sanitizers damage to the cuticle, and, consequently, embryonic development (Brake and Sheldon, 1990; Peebles et al., 1998). Our findings suggest that the cuticle was not damaged by any of the sanitation processes.

There was no significant difference ($P = 0.0546$, CV = 1.76%) in the percentage of fertility (Table 5). The mean fertility was 82.62 ± 2.02%. This result was observed because the eggs were obtained from breeder hens of the same age that received the same management at the poultry house.

A significant difference was observed for the hatchability of set eggs ($P = 0.0027$, CV = 4.07%; Table 5). The eggs treated with clove essential oil hatched at a mean rate of 70.81 ± 2.40%, which was similar to the mean of 66.87 ± 4.48% observed in the group fumigated with paraformaldehyde and significantly higher than that in the negative control (61.63 ± 3.32%) and grain alcohol (60.32 ± 2.89%) groups. This result can be attributed to the effects of the treatments, as there was no significant difference in the fertility rate.

Table 4. Egg weight before setting and during transfer and the percentage of egg weight loss in eggs treated with different treatments.[1]

Treatment	Egg weight before setting (g)	Egg weight during transfer (g)	Egg weight loss (%)
Nonsanitized	58.55 ± 0.86	51.55 ± 0.90	11.95 ± 0.36
Grain alcohol	58.82 ± 0.30	52.13 ± 0.49	11.38 ± 0.45
Clove essential oil	58.66 ± 0.36	51.78 ± 0.32	11.72 ± 0.46
Paraformaldehyde	58.53 ± 0.45	52.09 ± 0.35	11.01 ± 0.29
P-value	0.7310	0.2920	0.1495
Coefficient of variation (%)	0.85	0.99	3.00

No significant differences existed between means ($P > 0.05$).
[1]Results are expressed as the means ± SD.

Table 5. Fertility, hatchability of set eggs, hatchability of fertile eggs, early, mid, and late embryonic mortality, and contaminated eggs according to different treatments.[1]

Treatment	Fert (%)	Hatch (%)	Hatch fert (%)	Early dead (%)	Mid dead (%)	Late dead (%)	Cont. (%)
Nonsanitized	83.27 ± 2.31	61.63 ± 3.32^{b}	74.03 ± 3.58^{b}	7.09 ± 1.54	2.35 ± 1.99	13.41 ± 2.24^{a}	3.13 ± 1.25^{a}
Grain alcohol	81.96 ± 2.02	60.32 ± 2.89^{b}	73.59 ± 2.87^{b}	7.60 ± 1.46	2.40 ± 0.93	13.60 ± 1.00^{a}	2.81 ± 0.84^{a,b}
Clove essential oil	83.60 ± 1.77	70.81 ± 2.40^{a}	84.69 ± 1.65^{a}	4.32 ± 2.68	1.56 ± 1.24	8.67 ± 3.86^{b}	0.38 ± 0.76^{c}
Paraformaldehyde	81.63 ± 1.96	66.87 ± 4.48^{a,b}	81.87 ± 3.92^{a}	6.46 ± 1.46	1.48 ± 1.38	9.63 ± 2.92^{a,b}	0.81 ± 0.94^{b,c}
P-value	0.0546	0.0027	0.0043	0.0915	0.5443	0.0197	0.0330
Coefficient of variation (%)	1.76	4.07	3.86	25.68	69.77	18.69	57.54

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

The hatchability of fertile eggs ($P = 0.0043$; CV = 3.86%) differed significantly between the studied treatments (Table 5). The mean values for the eggs treated with clove essential oil (84.69 ± 1.65%) and paraformaldehyde (81.87 ± 3.92%) were statistically similar but were higher than those for the negative control (74.03 ± 3.58%) and the eggs treated with grain alcohol (73.59 ± 2.87%).

Oliveira et al. (2020) compared the hatchability of fertile eggs between clove essential oil treatment at a concentration of 0.6 mg/mL (92.37 ± 3.25%) and paraformaldehyde treatment (94.44 ± 4.54%) and did not observe significant differences. Copur et al. (2010) evaluated the effect of oregano essential oil at 2 concentrations (0.55 and 0.75 mL/cm^3) and 2 exposure times (3 and 6 h) on the sanitation of eggs intended for incubation and observed that the hatchability of these eggs (90.00%) did not differ significantly from the hatchability of formaldehyde-treated eggs (89.91%). Thus, sanitation with clove essential oil increased hatchability compared with that of nonsanitized eggs, possibly because of control of the bacterial load on the eggshell surface.

No significant differences in mortality were found between the treatments during the early ($P = 0.0915$; CV = 25.68%; Table 5) or middle ($P = 0.5443$; CV = 69.77%) incubation stage. However, there was a significant reduction ($P = 0.0197$; CV = 18.69%) in embryonic mortality during the late incubation stage in eggs sprayed with clove essential oil (8.67 ± 3.86%) compared with eggs sprayed with grain alcohol (13.60 ± 1.00) and eggs in the negative control group (13.41 ± 2.24%). In this context, Copur et al. (2011) and Baylan et al. (2018) reported that a decrease in early and late mortality may be the result of reduced eggshell contamination. Therefore, the lower mortality percentage observed in the late incubation stage of eggs treated with clove essential oil may be associated with decreased microbial populations on the eggshell because of the action of the chemical constituents present in the oil.

The rate of egg contamination during incubation was affected by the treatments ($P = 0.0330$; CV = 57.54%; Table 5). The largest percentage of contaminated eggs was observed in the negative control group (3.13 ± 1.25%), followed by the grain alcohol (2.81 ± 0.84%), paraformaldehyde (0.81 ± 0.94%), and clove essential oil (0.38 ± 0.76%) groups. These results reinforce that the clove essential oil-based sanitizing agent presents a broad spectrum of antimicrobial activity and may have been able to maintain low levels of microorganisms on the eggs during incubation, because according to Magwood (1964), there is a significant increase in the bacterial count on the eggshell during this period.

Chick initial weight ($P = 0.0723$; CV = 0.91%) and yield ($P = 0.2122$; CV = 1.05%) did not differ between treatments (Table 6). However, there was a significant difference ($P < 0.0001$; CV = 2.48%) in the physical quality score of the chicks. Chick weight is strongly associated with the weight of the egg from which the chick hatches (Morris et al., 1968). As no differences were observed in initial egg weight or weight loss, differences in the initial weight of the chicks were not expected.

Chick yield allows us to infer whether the incubation time and parameters were correct, with ideal chick yield values ranging between 67 and 68% (Aviagen, 2011). In the present experiment, all treatments presented yields classified as "slightly high" (68.55 ± 0.76%) but close to acceptable. The data observed in this study differ from those reported by Oliveira et al. (2020), who

Table 6. Chick weight, percentage of chick yield, and chick physical quality score assessment according to different treatments.[1]

Treatment	Chick weight (g)	Chick yield (%)	Pasgar Score©
Nonsanitized	40.00 ± 0.68	68.34 ± 0.32	9.09 ± 0.81^{b,c}
Grain alcohol	40.51 ± 0.20	68.87 ± 0.54	9.06 ± 0.96^{c}
Clove essential oil	39.93 ± 0.40	68.08 ± 1.08	9.21 ± 0.89^{a}
Paraformaldehyde	40.32 ± 0.48	68.89 ± 1.08	9.13 ± 0.90^{b}
P-value	0.0723	0.2122	<0.0001
Coefficient of variation (%)	0.91	1.05	2.48

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

Table 7. Eggshell thickness according to different treatments.[1]

Treatment	Eggshell thickness (mm)
Nonsanitized	0.364 ± 0.024
Grain alcohol	0.365 ± 0.019
Clove essential oil	0.363 ± 0.031
Paraformaldehyde	0.368 ± 0.028
P-value	0.8502
Coefficient of variation (%)	7.79

No significant differences existed between means ($P > 0.05$).
[1]Results are expressed as the means ± SD.

observed that eggs sanitized with grain alcohol (67.50 ± 1.92%), clove essential oil (67.90 ± 1.87%), or paraformaldehyde (67.80 ± 1.85%) showed chick yields classified as ideal. However, because in both experiments the eggs were subjected to ideal temperature and humidity conditions, a possible explanation for these contradictory results may be the egg weight loss observed in the present study, which, despite being within the ideal range reported in the literature (Molenaar et al., 2010), resulted in heavy chicks.

In the Pasgar© score assessment, it was determined that the clove essential oil (9.21 ± 0.89%) had a superior effect on the physical quality of the chicks compared with that of the other treatments. It is known that if eggs intended for incubation are not sanitized with effective products before being placed in the incubators, chick quality may decrease because of the high bacterial contamination that may occur, which may cause infection of the yolk sac (Harry, 1957; Cortés et al., 2004). Therefore, the microbial contamination level may be an indicator of chick quality. Thus, more viable chicks were obtained in the clove essential oil treatment because this sanitizer reduces microbial populations and does not adversely affect embryos or chicks.

Eggshell thickness is one of the factors that affects gas exchange and moisture loss during embryonic development (Ar et al., 1974; Veldsman et al., 2020). Therefore, any undesirable changes in this parameter may be harmful to embryos. In the present study, the similarity ($P = 0.8502$; CV = 7.79%; Table 7) between the means of eggshell thickness (0.365 ± 0.026 mm) showed that the tested treatments did not negatively affect this variable, even though the application of sanitizing agents to eggs can affect eggshell structure (Kim and Slavik, 1996). Oliveira et al. (2020) also did not observe significant differences in the thickness of eggshells treated with grain alcohol, clove essential oil, ethanolic extract of propolis, or paraformaldehyde (mean of 0.37 ± 0.029 mm).

CONCLUSIONS

Clove essential oil is effective and safe for eggs intended for incubation. Its use as an alternative to paraformaldehyde in the sanitation of fertile eggs is strongly recommended because it reduces the eggshell microbial load, resulting in good incubation parameters and better neonatal chick quality. Furthermore, our data indirectly suggest that the application of clove essential oil does not negatively affect the structural integrity of the cuticle on the eggshell surface or the development of the embryo.

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CERTIFICATE OF ACCEPTANCE

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