Evaluation of alternative disinfectants to formaldehyde for treating broiler eggs in a commercial hatchery

by

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DECLARATION

I, Andrew Jacques van Wijk, hereby declare that this dissertation, which I hereby submit for the MMedVet (Altil) degree to the Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, is my own work and that it has not been previously presented by me for degree purposes at any other tertiary institution.

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A.J. VAN WIJK

DEDICATION

To my wife, Mariëtte, and our daughter, Noelle, for all their support, encouragement and love during my study years. I could not have done it without you.

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SUMMARY

EVALUATION OF ALTERNATIVE DISINFECTANTS TO FORMALDEHYDE FOR TREATING BROILER EGGS IN A COMMERCIAL HATCHERY

by

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Supervisor: Dr. D.B.R. Wandrag

Omphalitis (mushy chick) is a significant cause of early chick mortalities on commercial chicken farms. While there are many factors that affect the incidence of mushy chicks, egg hygiene and handling practices on breeding farms as well as in hatcheries has a significant effect on this incidence. Disinfection of eggs at the breeding farm and/or the hatchery is crucial to decrease the number of bacteria, viruses and fungi on the egg shells which may affect not only the survival of the embryo, but also affects chick quality and performance through chick mortality, leg problems (bacterial femur head necrosis), absorption of the yolk, immune status, growth and feed conversion.

During the 18-week trial period, 17 280 000 broiler eggs were exposed to three different disinfectants during the final three days of incubation in a broiler hatchery. Thirty-seven percent liquid formalin served as the control and were compared to Virocid, a glutaraldehyde and quaternary ammonium compound disinfectant and Imazigard, a disinfectant with polyhexamethylene biguanide and imazilil as the active ingredients. Application time for formalin was continuous through evaporation from days 19 to 21, while Virocid and Imazigard were applied once a day for two minutes as liquids through a cold fogger on days 19 and 20 of incubation.

The trial was performed in 3 hatcher bays in the same hatchery, with each bay receiving a specific chemical treatment for 6 weeks before rotating to another product. Bacterial (Total Viable Counts, *Escherichia coli and Pseudomonas*) as well as fungal (yeasts and

moulds) counts on fluff from hatchers were used as a direct measure of efficacy of disinfection. Seven-day mortality data from broiler chicks were used as an indirect indicator of efficacy of egg disinfection.

Fluff *E. coli* counts from the Virocid group were significantly lower ($p<0.01$) compared to the formalin control group. All other bacteriology and mycology on fluff samples showed no statistically significant differences in the counts between the treatment groups and formalin with p values >0.05.

There was no statistically significant difference in cumulative mortalities up to 7-days between Virocid ($p=0.58$) and Imazigard ($p=0.45$) chicks when compared to chicks emanating from eggs that were treated with formalin.

Comparing the cost of formalin versus the treatment groups was imperative to establish the financial impact of using alternative disinfectants. While the price of a liter of liquid formalin is less than a liter of either Virocid or Imazigard, the price to disinfect an egg during the trial was approximately four times less for each of these disinfectants when compared to formalin. This is because the products are diluted to 2% (Imazigard) and 4% (Virocid) while the formalin is used undiluted.

In conclusion, considering the highly irritant nature of formalin for hatchery personnel as well as newly-hatched chicks, the research has proven that good alternatives exist to effectively and cost effectively disinfect poultry eggs in hatcheries.

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ABBREVIATIONS

ADBAC	Alkyl dimethyl benzyl ammonium chloride
ATSDR	Agency for Toxic Substances and Disease Registry
BAK	Benzalkonium chloride
DDAC	Didecyl dimethyl ammonium chloride
DNA	Deoxyribonucleic acid
DOC	Day old chick
ECHA	European Chemicals Agency
E. coli	*Escherichia coli*
EPA	Environmental Protection Agency (of the United States)
EU	European Union
FAO	Food and Agriculture Organization (of the United Nations)
LPM	Laboratory Procedure Manual
PHMB	Polyhexamethylene biguanide
QAC	Quaternary ammonium compounds
RH	Relative humidity
RNA	Ribonucleic acid
SANAS	South African National Accreditation Scheme
TVC	Total viable count
ULV	Ultra low volume
USA	United States of America

CHAPTER 1: BACKGROUND

1.1 Introduction

It is predicted that the global population will grow from the current 7.9 billion people to 9.7 billion people in 2050. To meet the food demand of this growing global population by producing foods from animal sources in a sustainable way is one of the biggest current and future global challenges (Henchion et al., 2021).

World meat production was forecast to reach 352.7 million tons in 2021, an increase of 4.2 percent from 2020. Of this 352.7 million tons, poultry meat contributes a significant 135 million tons (38.3%). (FAO Meat market review, Dec 2021). The increase in demand for poultry meat relative to other animals is illustrated by the fact that poultry meat consumption accounted for only 12% of all meat consumed in 1962 (Ritchie and Roser, 2019).

To meet this global demand within geographical boundaries, animal production must embrace production efficiencies, genetic gains and technological advances to ensure maximum kilograms of meat are produced per area, without compromising on animal welfare standards.

Broiler meat production across the globe is fully dependent on the constant supply of good quality day-old chicks. Besides the availability of fertile eggs, 2 major parameters impacting day old chick (DOC) availability is fertility and hatchability. Fertility is calculated as the percentage of incubated eggs that are fertile while hatchability is the percentage of fertile eggs that hatch (Macharia King'ori, 2011). Both fertility and hatchability are affected by a multitude of factors, including on-farm factors, nutrition, transport of eggs and incubation parameters.

Hatcheries are a continual potential source of contamination of incubated eggs as well as chicks that have hatched. Contamination of eggs can occur either through the ovary, the oviduct or via the egg shell (Board and Tanter, 1995).

Bacteria can readily penetrate the shells and membranes of intact hatching eggs. This penetration may not only result in infection of the developing embryo, but also of other

eggs incubated in the same incubators. Contamination of the chick with a human enteropathogen like *Salmonella* may also have food safety implications for the consumer. Hatchery practices can't affect infection via the ovary or the oviduct, as these occur on the breeder farms, however, contamination via the egg shell can be influenced by a multitude of factors, from the farm to the hatchery. Immediately after eggs have been laid, they are most vulnerable to infection via the egg shell, as bacteria and fungi can be drawn inside the shell to the membrane during cooling. Therefore, any surface that makes contact with the egg after being laid should be regarded as a potential source of contamination (Berrang et al., 1999).

Processes to disinfect eggs should aim to minimize microbial contamination on the exterior of the eggshell without damaging the cuticle or the developing embryo. A variety of methods are used to sanitize hatching eggs prior to incubation: spraying, dipping, fumigation and radiation. Some of the most commonly used products to disinfect eggs via either of these methods are formaldehyde, peracetic acid, hydrogen peroxide, ozone and ultraviolet light (Cony et al., 2008; Braun et al., 2011; Gottselig et al., 2016; Keïta et al., 2016; Vinayananda et al., 2017).

The conventional method of hatching egg fumigation is by paraformaldehyde fumigation (Kusstatscher et al., 2017). This method very effectively reduces pathogenic microorganisms (Rui et al., 2011) but uses a chemical that is harmful to the embryo and newly hatched chick and is harmful to the health of the hatchery employees (Zeweil et al., 2015, Kusstatscher et al., 2017). Therefore, alternative disinfectants to formaldehyde are needed that can provide satisfactory disinfection without reducing hatchability and not posing a health hazard to the workers applying the products.

1.2 Literature review

Proper biosecurity conditions on poultry breeder farms can reduce microbial contamination of eggs after oviposition. Excessive contamination of these eggs throughout collection, handling and storage on the farms, during transportation to the

hatchery or at the hatchery can lead to a decrease in hatchability as well as a decrease in the quality, growth and performance of the chicks (Scott et al., 1993).

Egg hygiene is not only important for the survival of the developing embryo, but it also affects chick quality and performance through chick mortality, leg problems (bacterial femur head necrosis), absorption of yolk, immune status, growth and feed conversion (Meijerhof et al., 2022).

As reported by Mauldin (1999), the total number of bacteria on the surface of the egg at the time of lay may range from 300 to 500. If eggs are dirty, this number can be as high as 80 000.

Typical microbial contaminants are bacteria such as *Escherichia, Salmonella, Pseudomonas, Micrococcus* (North and Bell, 1990) and various types of moulds (Bruce and Johnson, 1978).

It is therefore important to use effective sanitizers on the egg shells to reduce the potential for internal and external microbial contamination. Inadequate application, incorrect sanitizer or dilution of these chemicals may result in microorganisms penetrating the shell pores and infecting the embryo (Araujo and Albino, 2014).

This infection of the embryo may affect incubation efficiency, chick quality and consumer safety (de Faria et al., 2014).

The shell is the outermost covering of the egg and serves as the first line of defense against bacterial contamination. The shell is made up almost entirely of calcium carbonate and has approximately 17 000 pores that are between 5 and 10 µm in size. It is important to note that most bacteria are between 0.5 and 2 µm and can thus pass through the pores. However, through the cuticle the egg has a thin outermost protective coating which blocks the pores and prevents bacterial contamination (Biology of eggs, USDA, 2016).

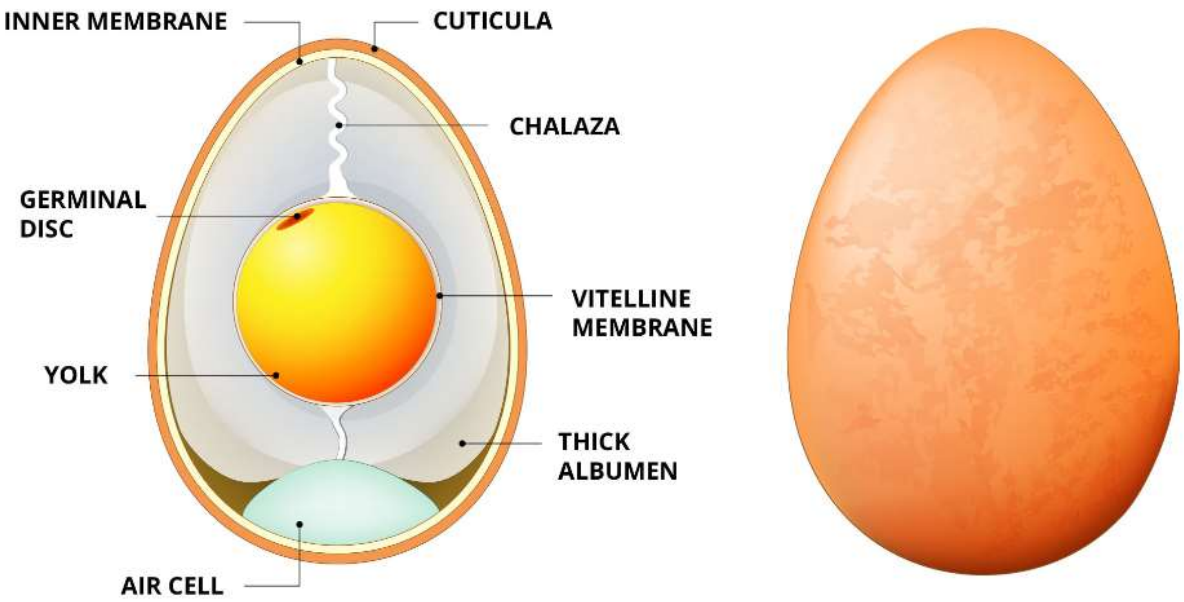

Figure 1: Anatomy of an egg (The onsen egg temperature curve, 2022)

After 18 days of incubation, eggs are transferred from setter incubators (eggs stored in trays) to a different type of incubator called a hatcher (eggs stored in baskets) for the final stage of incubation. Hatchers and hatcher baskets are a potential source of infection for newly hatched chicks, even if they originate form clean eggs (Furuta and Muruyama, 1982).

Fumigation of eggs with a disinfectant after transfer to the hatchers can decrease microbial levels in these incubators and increase the chance of healthy chicks (Cadirci, 2009).

Under commercial conditions it is essential that the shells of hatching eggs are disinfected at some point between the farm and the hatchery. Not only is this a good practice, but often also a legal requirement (Aviagen- Hatchery tips 2017).

Formaldehyde is available commercially as a solid polymer, paraformaldehyde $OH(CH_2O)_nH(n=8\text{-}100)$, and as formalin, which is a 37% aqueous solution. The most common route of occupational exposure to formalin or formaldehyde is via inhalation, with other routes being contact via the skin and eyes and possibly ingestion (Wartew, 1983).

Formaldehyde is not an easy disinfectant to replace. It has a wide microbiological spectrum, it forms a dry gas so does not dampen the eggshell and it is cost-effective (Aviagen- Hatchery tips 2017).

Williams (1970) had shown the efficacy of formalin in killing bacteria on the egg shells when doing early on-farm preincubation fumigation of hatching eggs. It confirmed the efficacy of early preincubation fumigation of eggs on the farm.

Other research on egg fumigation at the hatchery such as done by Keïta et al. (2016) predominantly focused on egg fumigation prior to incubation, which is done at the farm after egg collection and/or at the hatchery prior to incubating the eggs. The authors' research specifically addresses the use of alternatives to formaldehyde during the final stage of incubation.

The biocidal effect of formaldehyde is due to its ability to act on the proteins and nucleic acid bases of microorganisms (Russel, 1976).

It has the ability to form stable methylene bridges and inter-molecular cross linkages. It also alkylates the nitrogen atoms of purine and pyrimidine bases in DNA and RNA (Habeeb and Hiramoto, 1968).

The potential health hazard for lab workers exposed to formaldehyde through inhalation was emphasized as long ago as 1928 by Sabrazeo et al. (Cadirci, 2009).

Airborne formaldehyde is a potent eye and respiratory tract irritant (Andersen et al., 2019). Even though it has been demonstrated almost 40 years ago that formaldehyde increased the incidence of squamous cell carcinoma in the nasal tissue of rats (Kerns et al., 1983), the ability of formaldehyde to cause human nasopharyngeal and lymphohematopoietic cancers and the required exposure dose and duration for this has not been conclusively proven despite many toxicological and epidemiological cohort studies (Andersen et al., 2019).

The risk estimates as well as the hazard classification for formaldehyde varies widely between regulatory authorities across the globe (Andersen et al., 2019).

European Union (EU) authorities have regulated formaldehyde use because of its carcinogenic potential. It has also been shown that using formaldehyde pre-incubation and during incubation has a negative effect by both the shortening as well as the loss of tracheal cilia in both 18-day embryos as well as in day-old chicks (Hayretdag and Kolankaya, 2008).

However, according to Cadirci (1997) it is yet to be determined what the toxic level of formaldehyde is for the developing chicken embryo.

The EU has banned the use of formaldehyde as a biocide (including embalming) under the Biocidal Products Directive (98/8/EC) due to its carcinogenic properties. There are currently no such restrictions in South Africa (August 2022) and it is still commonly used for egg disinfection on poultry breeder farms as well as in hatcheries.

Glutaraldehyde is a colourless, oily liquid with a sharp, pungent odour. Besides its use in fogging and cleaning of poultry houses, it has many other industrial, laboratory, agricultural, medical, and some household purposes, most commonly for disinfecting and sterilization of various surfaces and equipment (National Library of Medicine, 2020).

Quaternary ammonium compounds (QACs) are among the most commonly used disinfectants in the food and agricultural industries, and it is available as numerous commercial products and formulations. They are cationic surfactants that impact cell walls and membranes of bacteria. QACs are positively charged and this makes them readily bind to the negatively charged surfaces of most microorganisms (Chauret, 2014).

Both the Environmental Protection Agency (EPA) of the United States as well as the European Chemicals Agency (ECHA) have concluded that QACs are not carcinogenic. The two-main antimicrobial sub-classes of QACs are alkyldimethylbenzylammonium chloride (ADBAC) also known as benzalkonium chloride (BAK) and didecyldimethylammonium chloride (DDAC). The EPA's Cancer Assessment Review Committee classified the ADBAC as Group D "not likely to be carcinogenic to humans" and DDAC as Group E "evidence of non-carcinogenicity for humans" (Osimitz, 2021).

Rodgers et al. (2001) has shown the anti-microbial efficacy of QAC based disinfectants against *Staphylococcus* when applied in the hatchers during egg incubation. It was compared against non-formaldehyde based disinfectants.

Is glutaraldehyde carcinogenic? The Environmental Protection Agency's (EPA) cancer assessment review committee classified glutaraldehyde as "not likely to be carcinogenic to humans." This classification is based on the evidence that it does not cause cancer in animals. The National Toxicology Program has established that there was "no evidence of carcinogenic activity" of glutaraldehyde in rodents exposed to glutaraldehyde for 24 months (ATSDR, 1999). However, glutaraldehyde does have several toxic properties for humans when exposed through skin contact or inhalation and therefore the necessary preventative measures need to be taken when handling products containing glutaraldehyde. Exposure may cause the following symptoms: throat and lung irritation, asthma and difficulty breathing, dermatitis, nasal irritation, sneezing, wheezing, burning eyes, and conjunctivitis (U.S. Department of Health and Human Services, 2017).

Even though glutaraldehyde readily biodegrades in both freshwater and marine environments, it is acutely toxic to aquatic organisms (Leung, 2001).

Polyhexamethylene biguanide (PHMB), also known as polyhexanide is a disinfectant with antiviral and antibacterial properties. It is commonly used in cosmetics and personal care products, wound care dressings, contact lens cleaning solutions, perioperative cleansing products, and swimming pool cleaners (Schnuch et al., 2007).

According to the Australian Government's Human Health Risk Assessment of Polyhexanide that was published in 2018, there were some evidence of a tumorigenic response in rats but polyhexanide does not pose a carcinogenic risk to humans.

Therefore, QAC and/or glutaraldehyde based disinfectants do not pose the same health risks to people as formaldehyde and from this aspect it would be beneficial to use as an alternative to formaldehyde for disinfection of eggs.

CHAPTER 2: HYPOTHESIS

2.1 Problem

Omphalitis (yolk sac infection, mushy chick) remains one of the most common causes of chick mortalities in the first seven days of a chicken's life. In an intensive breeder chicken farming operation, hatching eggs are removed from the breeder houses, either manually by hand or automated through conveyer belts transporting eggs from the next boxes. These eggs are temporarily stored in the farm's egg room before being transported to the hatchery by truck where it is incubated. Infection of eggs on the breeder farm after being laid in the nest boxes, during handling, transportation or incubation in the hatchery by bacteria, viruses or fungi increase the chance of embryonic infection during incubation or of the chick after it has hatched. An infected egg may explode ("banger") during incubation and in the process, infect many other eggs in close proximity.

Chicken eggs have a total incubation time of 21 days. After 18 days the eggs are moved from trays in setter incubators to baskets in hatcher incubators as the eggs need to hatch in the baskets for the chicks to move freely.

It is common practice in South African poultry hatcheries to use formaldehyde to disinfect the hatcher environment before transfer of eggs from the setter incubators to the hatcher incubators after 18 days of incubation as well as for the first 24 to 48 hours after egg transfer, during which time the chicks start to hatch. Formalin (liquid) is typically applied through a trickle fumigation process, whereby formalin liquid is converted to formaldehyde (a colourless, pungent gas) at incubation temperatures of 37°C. Formaldehyde is a very effective disinfectant, it is readily available and cost effective, but it is potentially carcinogenic for people exposed to the fumes over time.

2.2 Hypothesis

Formalin is a superior disinfectant to other, non-formalin based disinfectants, Virocid (Cidlines) and Imazigard (Techniblend) for disinfection of eggs during incubation in a commercial broiler hatchery.

2.3 Objectives

The objectives of this research were to prove that:

2.3.1 There is a difference in total viable bacterial (TVC), *E. coli* and *Pseudomonas* counts on fluff between formalin, Virocid and Imazigard.

2.3.2 There is a difference in yeast and mould counts on fluff between formalin, Virocid and Imazigard.

2.3.3 Cumulative 7-day mortalities of chicks that are treated with formalin are lower than those from Virocid and Imazigard.

2.3.4 Formalin is more cost effective than Virocid and Imazigard.

CHAPTER 3: EXPERIMENTAL DESIGN

3.1 Materials

3.1.1 Rainbow Chickens' Worcester Hatchery

The trial hatchery was the Rainbow Chickens' Worcester Broiler Hatchery in De Wet, Worcester, South Africa. The hatchery has 20 multi-stage setter machines and 30 hatcher machines. The Worcester hatchery hatches 5 days per week and sets 192 000 eggs per day for each of these 5 days, producing approximately 800 000 broiler chicks per week, depending on the hatchability. It is one of three hatcheries supplying chicks to Rainbow Chickens' broiler farms in the Western Cape.

3.1.2 Hatchers

Three hatcher bays (passages), each with 10 incubation machines (hatchers). Each hatcher has dimensions of 3.2m x 2.2m x 2.1m (volume of 14.8m^3) and a set capacity of 19 200 eggs, therefore one hatcher bay with 10 hatchers has a total capacity to set 192 000 eggs per hatch. Temperature and relative humidity (RH) were controlled in the hatchers through Chick Master hatcher controls. The temperature in the hatchers was maintained at 36.6°C and the RH was 50%. The total incubation time was 506 hours.

Cooling of air in the hatchers were achieved through cooling coils with cold water and electrical elements were used for heating the hatchers.

3.1.3 Hatching eggs

Hatching eggs were supplied from various breeder flocks to the trial hatchery. Most of the eggs were supplied from six breeder farms in Worcester that are owned by Rainbow Chickens, while eggs were also transferred internally from Rainbow Chickens in Kwa-Zulu Natal and bought in from external suppliers. The total number of eggs used per week were therefore 960 000 and total eggs used during the 18-week trial period were 17 280 000. Eggs were predominantly from hens of the Cobb breed, but also from Ross and Arbor Acre breeds.

3.1.4 Ultra Low Volume (ULV) fogger

The ULV or cold fogger was used to apply the two treatment chemicals while the formalin (control) disinfects through evaporation to a formaldehyde gas. The fogger has a tank size of 7 liters and droplet size of 5-20 micron.

3.1.5 Stainless-steel pans

The stainless-steel pans have dimensions of 50cm long x 16cm wide x 8cm high and were filled with formalin liquid as described under methods. Each hatcher was supplied with one pan.

3.1.6 Disinfectants

Table 1: A comparison of the 3 disinfectants used during the trial

Product	Physical state	pH	Microbial spectrum	Active ingredients	Concentration
Virocid	Liquid	4	Viruses Bacteria Fungi Fungal spores	Didecyldimethylammoniumchloride Alkyldimethylbenzylammoniumchloride Glutaral	7.8% 17.1% 10.7%
Imazigard	Liquid	6-8	Viruses Bacteria Fungi Fungal spores	Polyhexamethylene biguanide (Vantocil) Imazalil sulphate	Undisclosed
Formalin	Liquid	3-4	Viruses Bacteria Fungi	Formalin	37%

3.2 Methods

3.2.1 Introduction

A total of 17 280 000 eggs were incubated during the 18-week trial period. The trial hatchery hatched 5 days per week, from Thursdays to Mondays with no hatches on Tuesdays and Wednesdays. Eggs were transferred by vacuum suction cups from 20 multi-stage setter machines to the 3 hatcher bays, 5 days a week. Eggs were transferred to only one of the three hatcher bays on any given day. The hatcher bays were run as

all-in-all-out units, with each bay receiving 192 000 eggs on one specific day and all 10 hatchers in that bay being cleared 72 hours later. Hatcher bays received eggs sequentially, therefore a hatcher bay received eggs for 2 days per week for 2 weeks, and then only once during week 3. Therefore, each hatcher received eggs five times during a 3-week period.

3.2.2 Assignment of disinfectants to different hatcher bays

The trial duration was determined to be 18 weeks to gather enough data that would be of statistical significance. Treatments and controls were alternated between the 3 bays every 6 weeks, as per Table 2. This rotation of treatment and control chemicals between the 3 bays was done to compensate for any possible inherent difference between the 3 bays which may affect the outcome if one bay had received the same treatment over the 18-week trial period.

Table 2: Assignment of disinfectants to different hatcher bays

	Monday	Tuesday	Wednesda	Thursday	Friday	Saturday	Sunday		
Week 1	Bay 1			Bay 2	Bay 3	Bay 1	Bay 2		Formaldehyde
Week 2	Bay 3			Bay 1	Bay 2	Bay 3	Bay 1		Virocid
Week 3	Bay 2			Bay 3	Bay 1	Bay 2	Bay 3		Imazigard
Week 4	Bay 1			Bay 2	Bay 3	Bay 1	Bay 2		
Week 5	Bay 3			Bay 1	Bay 2	Bay 3	Bay 1		
Week 6	Bay 2			Bay 3	Bay 1	Bay 2	Bay 3		
Week 7	Bay 1			Bay 2	Bay 3	Bay 1	Bay 2		
Week 8	Bay 3			Bay 1	Bay 2	Bay 3	Bay 1		
Week 9	Bay 2			Bay 3	Bay 1	Bay 2	Bay 3		
Week 10	Bay 1			Bay 2	Bay 3	Bay 1	Bay 2		
Week 11	Bay 3			Bay 1	Bay 2	Bay 3	Bay 1		
Week 12	Bay 2			Bay 3	Bay 1	Bay 2	Bay 3		
Week 13	Bay 1			Bay 2	Bay 3	Bay 1	Bay 2		
Week 14	Bay 3			Bay 1	Bay 2	Bay 3	Bay 1		
Week 15	Bay 2			Bay 3	Bay 1	Bay 2	Bay 3		
Week 16	Bay 1			Bay 2	Bay 3	Bay 1	Bay 2		
Week 17	Bay 3			Bay 1	Bay 2	Bay 3	Bay 1		
Week 18	Bay 2			Bay 3	Bay 1	Bay 2	Bay 3		

3.2.3 Application of disinfectants

3.2.3.1 Formalin

Before eggs were transferred to the hatchers, one stainless steel pan of 50 x 16 x 8cm was placed on the floor of each hatcher. The pans were filled with 1 000ml of 37% liquid formalin, which evaporated spontaneously at the hatcher temperatures of 36.6°C. Twenty-four hours after eggs were transferred, the pans were each topped up with 500ml of 37% liquid formalin and again with another 500ml of the same formalin solution after another 24-hours. Formalin was used undiluted.

3.2.3.2 Virocid

Virocid was diluted to 4% (40ml per 1 000ml water) by mixing 280ml Virocid with 7 liters of tap water. The hatcher doors were opened, and it was applied as a cold mist spray with a mist fogger (ULV fogger) at 10-micron droplet size (setting 3) between the trolleys and in the spaces above the trolleys. It was applied continuously for 2 minutes per hatcher. This equated to 700ml diluted product used per hatcher. The hatcher ventilation was not turned off during the fogging. After fogging, the hatcher doors were closed again. The Virocid was applied directly after transfer and repeated 24-hours later.

3.2.3.3 Imazigard

Imazigard was diluted to 2% (20ml per 1 000ml water) by mixing 140ml Imazigard with 7 liters of tap water. The hatcher doors were opened, and it was applied as a cold mist spray with a mist fogger (ULV fogger) at 10-micron droplet size (setting 3) between the trolleys and in the spaces above the trolleys. It was applied continuously for 2 minutes per hatcher. This equated to 700ml diluted product used per hatcher. The hatcher ventilation was not turned off during the fogging. After fogging, the hatcher doors were closed again. The Imazigard was applied directly after transfer and repeated 24-hours later.

3.2.4 Metrices

3.2.4.1 Bacteriology and mycology on fluff samples

Fluff samples were collected on the day of hatch before take-off of chicks. This was done by using a sterile forceps and sterile 20ml specimen containers. Each container holds approximately one gram of fluff. Fluff was sampled from the floor of each hatcher, placed in a sterile specimen container and sent to the Rainbow Chickens Worcester Laboratory on the same day for testing. This laboratory is accredited by the South African National Accreditation Scheme (SANAS) under accreditation number V0006 (Addendum B). The following tests were performed on the fluff as per Laboratory Procedure Manual (LPM) on 0.1g of fluff:

Total Viable Count (TVC) (BAC 20.1)

Escherichia coli (BAC 20.2)

Pseudomonas (BAC 20.9)

Yeasts and moulds (BAC 20.6)

For each hatcher, there was one fluff sample and four microbiological results: three bacterial and one fungal count. Fungal or bacterial plate counts were done per 0.1g of fluff, and the results were converted to colonies per gram of fluff.

3.2.4.2 Seven-day chick mortalities

After chicks in baskets were removed from the hatcher machines, the chicks were graded, and first grade chicks were sent to broiler farms in chick baskets. The hatchability percentage per flock was then calculated as percentage of first grade chicks that hatched from the number of eggs that were set.

Chicks were placed in broiler houses where the capacity varied from 30 000 to 40 000 chicks per house. If the 192 000 eggs that were transferred to a specific hatcher bay had a hatchability percentage of 80%, that bay would produce 153 600 chicks. These chicks would be placed in 4-5 houses. Houses were heated to 33°C air temperature for 48 hours before chicks were placed. Chicks had access to crumble feed ad lib as well as water through nipple drinker systems. The lighting program for the 1st week consisted out of 24

hours of light for the first day, followed by 23 hours light and 1 hour of darkness for the following 6 days.

Cumulative losses up to 7 days were calculated and expressed as a percentage of chicks placed in a specific house. Losses include chicks that died naturally as well as birds that were humanely culled. The Rainbow Chickens' Worcester Hatchery together with two other hatcheries hatches chicks for Rainbow Chickens Western Cape. In some instances, more than one hatchery supplied chicks into a specific broiler house. For statistical analyses of 7-day mortalities, only broiler houses that received chicks exclusively from the Worcester hatchery were used and houses that received a split placement were discarded.

The four microbiological parameters were expressed as a number of bacterial or fungal colonies per gram of fluff. One fluff sample was collected from each hatcher with 19 200 eggs. This equates to 300 fluff samples per group (control or treatment) during the 18-week trial period. The number of TVC, *E. coli and Pseudomonas* bacteria as well as yeasts and moulds were determined on every fluff sample.

Seven-day mortality data was calculated as described under 3.2.4.2. The 7-day mortality percentages were then compared between the formalin group as well as the Virocid and Imazigard groups and interpreted together with the microbiological fluff data as an indirect parameter of fumigation efficacy in the hatcher incubators.

Table 3: Treatment groups and sampling

	Hatcher disinfectant		
	Formalin	**Virocid**	**Imazigard**
Number of bays treated over 18-weeks	30	30	30
Number of hatchers treated over 18-weeks	300	300	300
Number of eggs treated	5 760 000	5 760 000	5 760 000
Fluff samples tested for TVC	270	246	262
Fluff samples tested for *E. coli*	270	256	262
Fluff samples tested for *Pseudomonas*	270	256	262
Fluff samples tested for yeasts and moulds	270	255	262

Counts of <10, <100 and <1000 were recorded as 0 for the analysis. Medians, 25th and 75th percentiles of each outcome (TVC, *E. coli, Pseudomonas* and yeasts and moulds) were tabulated by treatment group and depicted using box plots.

The outcomes were assessed for normality within treatment groups using the Shapiro-Wilk test and were then log-transformed to achieve normality. To avoid the problem of log-transforming zero values, 1 was added to each count before transforming. Each outcome was then compared between treatment groups, using the formalin-treated group as the reference group, using linear mixed models, with month of hatching as a covariate. For bacterial and fungal count outcomes, the experimental unit was the hatcher, and hatcher nested within bay was included as a random effect. For mortality percentage, the experimental unit was the bay, which was included as a random effect. Statistical analysis was done using Stata 17 (StataCorp, College Station, Texas, U.S.A.). Statistical significance was assessed at $p<0.05$.

CHAPTER 4: RESULTS

4.1 Bacterial Total Viable Counts (TVC) per gram of fluff

Table 4: Means, standard deviations and percentiles of TVC per gram of fluff

Group	Observations	Mean	Std. deviation	25th percentile	50th percentile	75th percentile
Virocid	246	16818.9	29797.2	1000	3900	19200
Imazigard	262	26930.5	53901.5	1100	5700	24000
Formalin	270	21569.8	32920.2	900	5000	31200

Table 5: Statistical analysis of TVC on fluff across the 3 groups of disinfectants

Group	Coefficient	Std. error	z	P>z	95% confidence interval	
Virocid	-0.104	0.79	-1.31	0.19	-.259	.051
Imazigard	0.009	0.079	0.11	0.911	-0.146	0.163
Formalin	0 (base)					

The results of TVC bacterial counts on fluff are shown in Tables 4 and 5 as well as in Figure 2. As can be seen in Figure 2, there was not a statistically significant difference in log TVC counts when comparing the two treatment groups to formalin as p-values were 0.19 (Virocid) and 0.911 (Imazigard).

The correlation coefficients of -0.104 and 0.009 point towards very weak correlations between the outcome of either chemical when compared to the base (formalin).

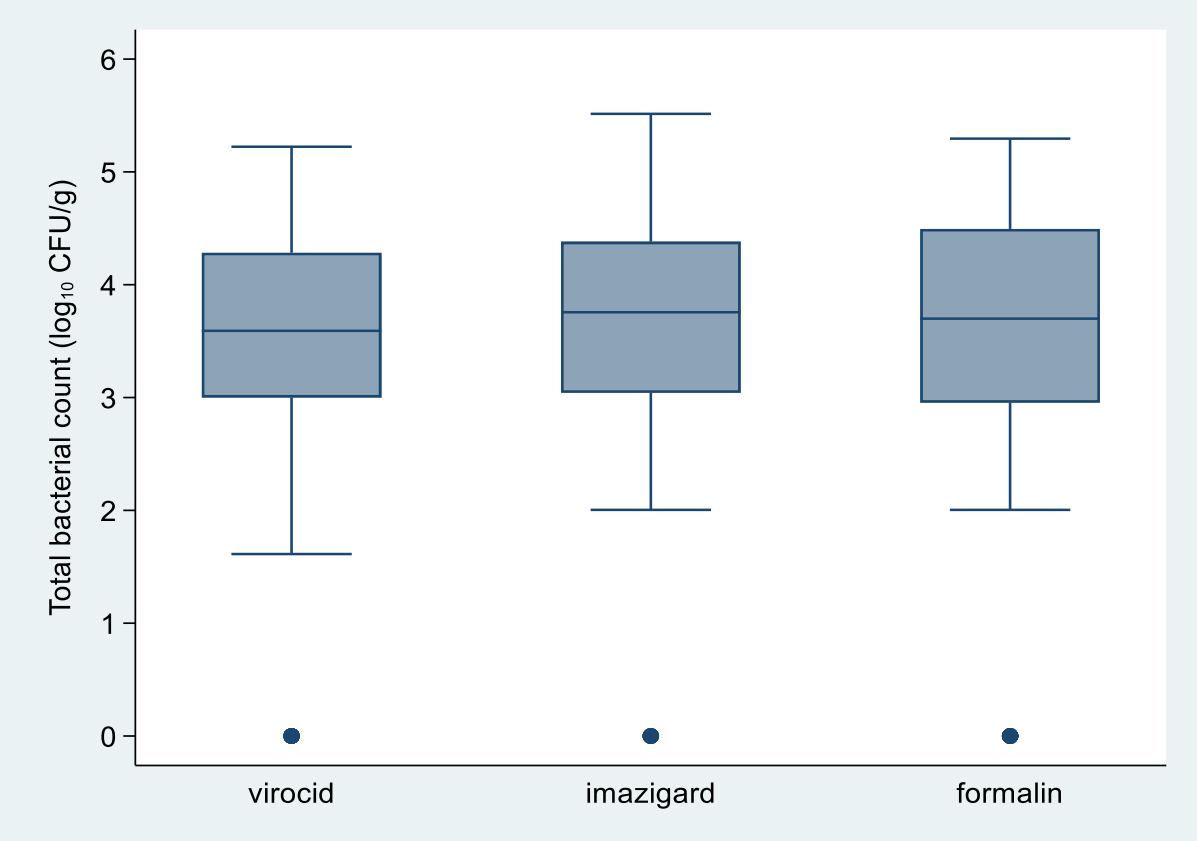

Figure 2: Log Total Viable Counts (TVC) counts per gram of fluff

The 3 vertical lines in the box section of the graph when read from bottom to top represents the 25th percentile, median (or 50th percentile) and the 75th percentile. The minimum (bottom) and maximum (top) values are represented by the vertical bars at the ends of the whiskers, while outliers are depicted as dots.

4.2 *E. coli* counts per gram of fluff

Table 6: Means, standard deviations and percentiles of *E. coli* per gram of fluff

Group	Observations	Mean	Std. deviation	25th percentile	50th percentile	75th percentile
Virocid	256	501.6	2844.4	0	0	0
Imazigard	262	2273.3	13798.3	0	0	100
Formalin	270	668.7	3508.2	0	0	100

Table 7: Statistical analysis of *E. coli* on fluff across the 3 groups of disinfectants

Group	Coefficient	Std. error	z	P>z	95% confidence interval	
Virocid	-0.281	0.104	-2.7	0.007	-0.485	-0.077
Imazigard	-0.002	0.105	-0.02	0.986	-0.207	0.203
Formalin	0 (base)					

E. coli counts on fluff collected from hatchers treated with Virocid were significantly lower ($p<0.01$) than *E. coli* counts on fluff collected from the formalin (control) hatchers.

E. coli counts on fluff collected from hatchers treated with Imazigard did not differ significantly ($p=0.986$) from the formalin group.

The negative correlation coefficients between Virocid (-0.28) and formalin and between Imazigard (-0.002) and formalin are negligible.

The results are depicted in Tables 6 and 7 and Figure 3.

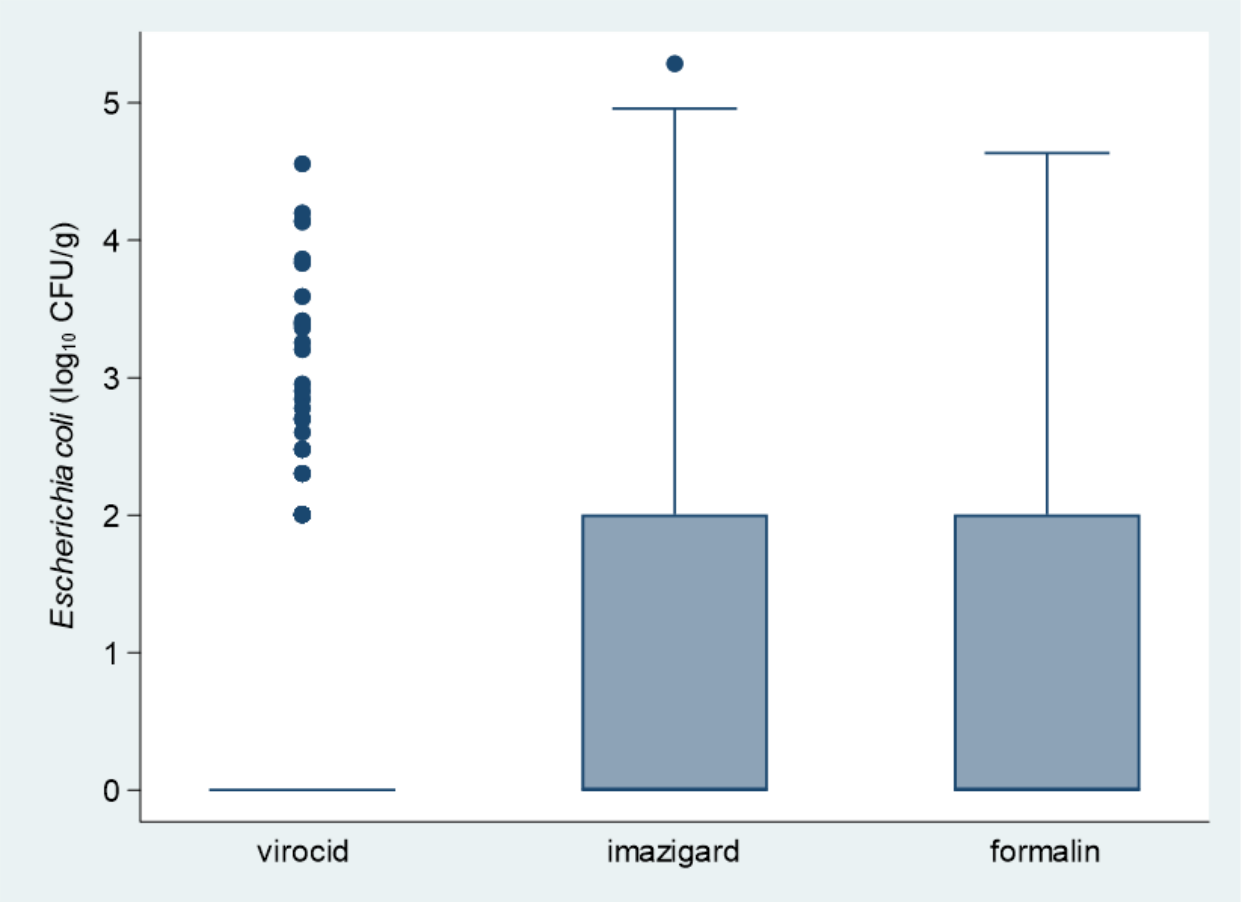

Figure 3: Log *E. coli* counts per gram of fluff

4.3 *Pseudomonas* counts per gram of fluff

Table 8: Means, standard deviations and percentiles of *Pseudomonas* per gram of fluff

Group	Observations	Mean	Std. deviation	25th percentile	50th percentile	75th percentile
Virocid	256	10915.4	30049.4	100	1050	9000
Imazigard	262	18001.5	53690.3	300	1850	10600
Formalin	270	21936.7	48963.4	100	1450	18200

Table 9: Statistical analysis of *Pseudomonas* on fluff across the 3 groups of disinfectants

Group	Coefficient	Std. error	z	P>z	95% confidence interval	
Virocid	-0.26	0.136	-1.92	0.055	-0.526	0.006
Imazigard	0.11	0.14	0.84	0.4	-0.153	0.381
Formalin	0 (base)					

There was no statistically significant difference in *Pseudomonas* counts on fluff when comparing either the Virocid group (p=0.055) or the Imazigard group (p=0.4) to the control group (formalin).

The correlation coefficients of -0.26 (Virocid) and 0.11 (Imazigard) are too small to be significant.

These findings are depicted in Tables 8 and 9 and Figure 4.

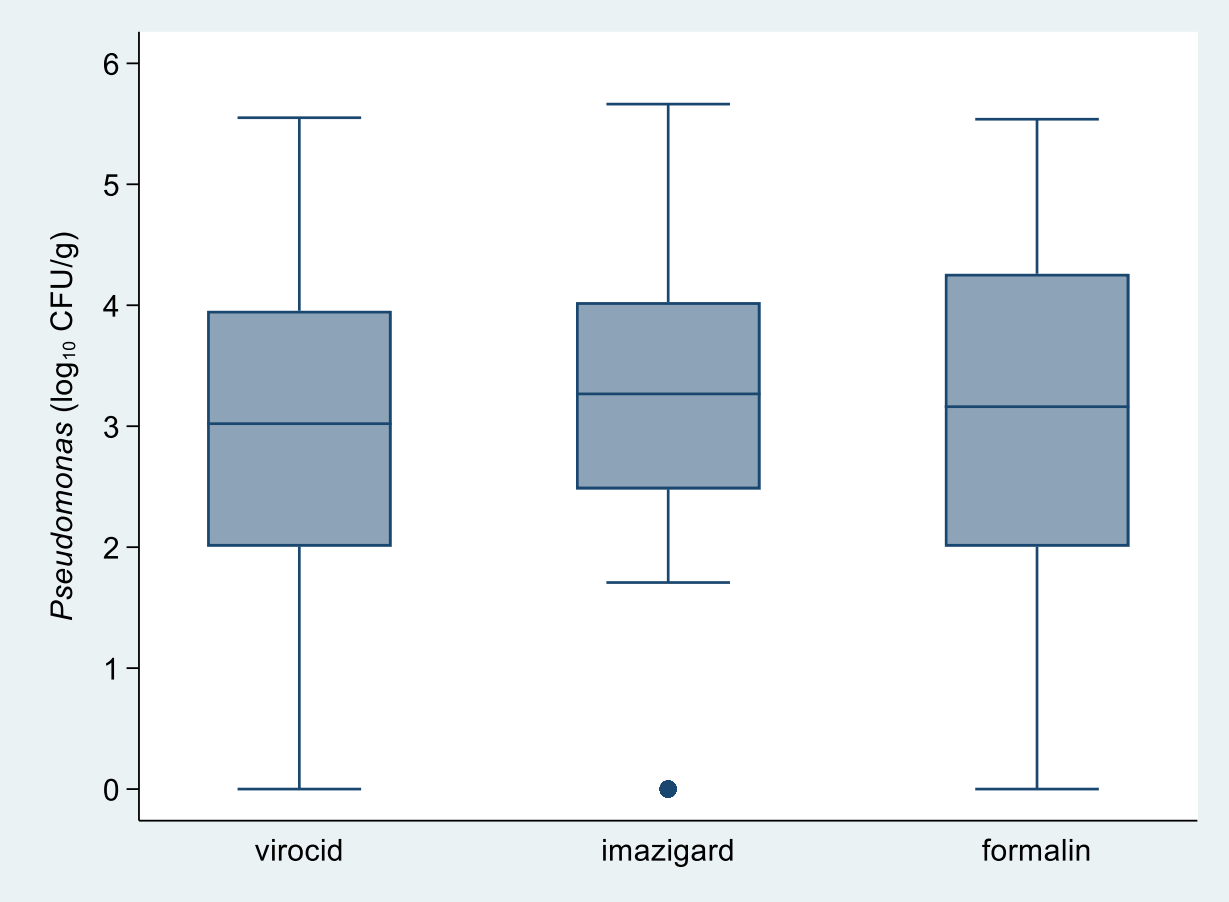

Figure 4: Log *Pseudomonas* counts per gram of fluff

4.4 Yeasts and moulds counts per gram of fluff

Table 10: Means, standard deviations and percentiles of yeasts and moulds per gram of fluff

Group	Observations	Mean	Std. deviation	25th percentile	50th percentile	75th percentile
Virocid	255	342	1696.2	0	0	100
Imazigard	262	313	1269.6	0	0	100
Formalin	270	356.3	2130	0	0	100

Table 11: Statistical analysis of yeasts and moulds on fluff across the 3 groups of disinfectants

Group	Coefficient	Std. error	z	P>z	95% confidence interval	
Virocid	-0.025	0.098	-0.25	0.8	-0.216	0.167
Imazigard	.063	0.1	0.65	0.515	-0.129	0.256
Formalin	0 (base)					

The log yeasts and moulds counts as depicted in Tables 10 and 11 and Figure 5 shows that there is not a statistically significant difference in yeasts and moulds counts on fluff between either of the two treatment groups and formalin as indicated by very high p-values of 0.8 (Virocid) and 0.515 (Imazigard).

The correlation coefficients of -0.025 (Virocid) and 0.063 (Imazigard) are negligible and do not point to a meaningful positive or negative correlation between either of the treatment groups and formalin.

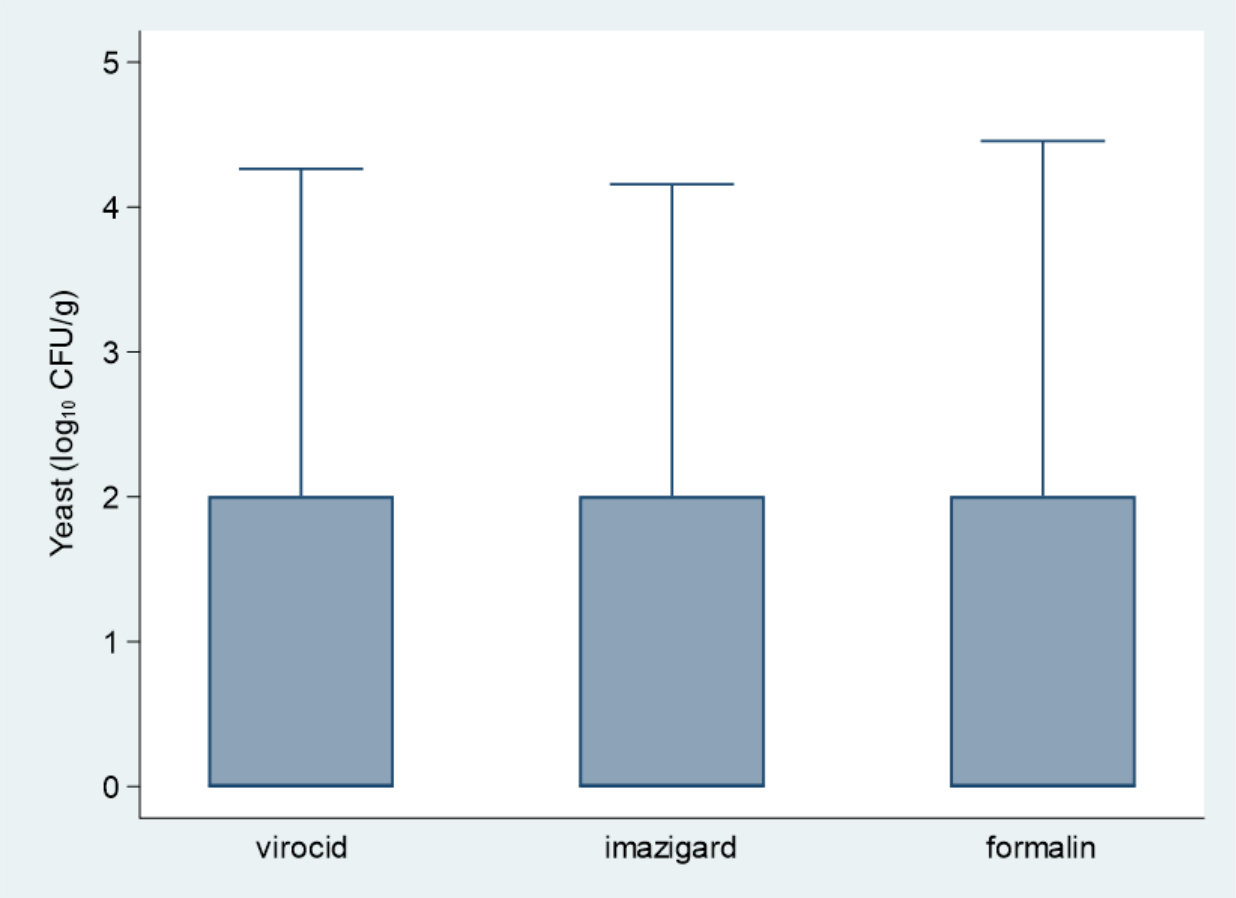

Figure 5: Log yeast and mould counts per gram of fluff

4.5 Seven-day chick mortalities

Table 12: Means, standard deviations and percentiles of cumulative 7-day chick mortalities

Group	Observations	Mean	Std. deviation	25th percentile	50th percentile	75th percentile
Virocid	30	1.06	0.38	0.84	0.915	1.34
Imazigard	30	1.03	0.35	0.85	0.91	1.2
Formalin	31	1.16	0.67	0.81	0.91	1.29

Table 13: Statistical analysis of 7-day chick mortalities across the 3 groups of disinfectants

Group	Coefficient	Std. error	z	P>z	95% confidence interval	
Virocid	-0.02	0.037	-0.55	0.583	-0.094	0.053
Imazigard	-0.03	0.038	-0.76	0.448	-0.102	0.045
Formalin	0 (base)					

The 7-day mortality % did not differ significantly between either the Virocid group (p=0.583) and the Imazigard group (p=0.448).

The negative correlation coefficients of -0.002 and -0.003 for Virocid and Imazigard respectively points to negligible correlations between either of the two treatment groups and formalin. Generally, correlations <-0.8 or >0.8 are considered strong correlations.

These results are depicted in Tables 12 and 13 and in Figure 6.

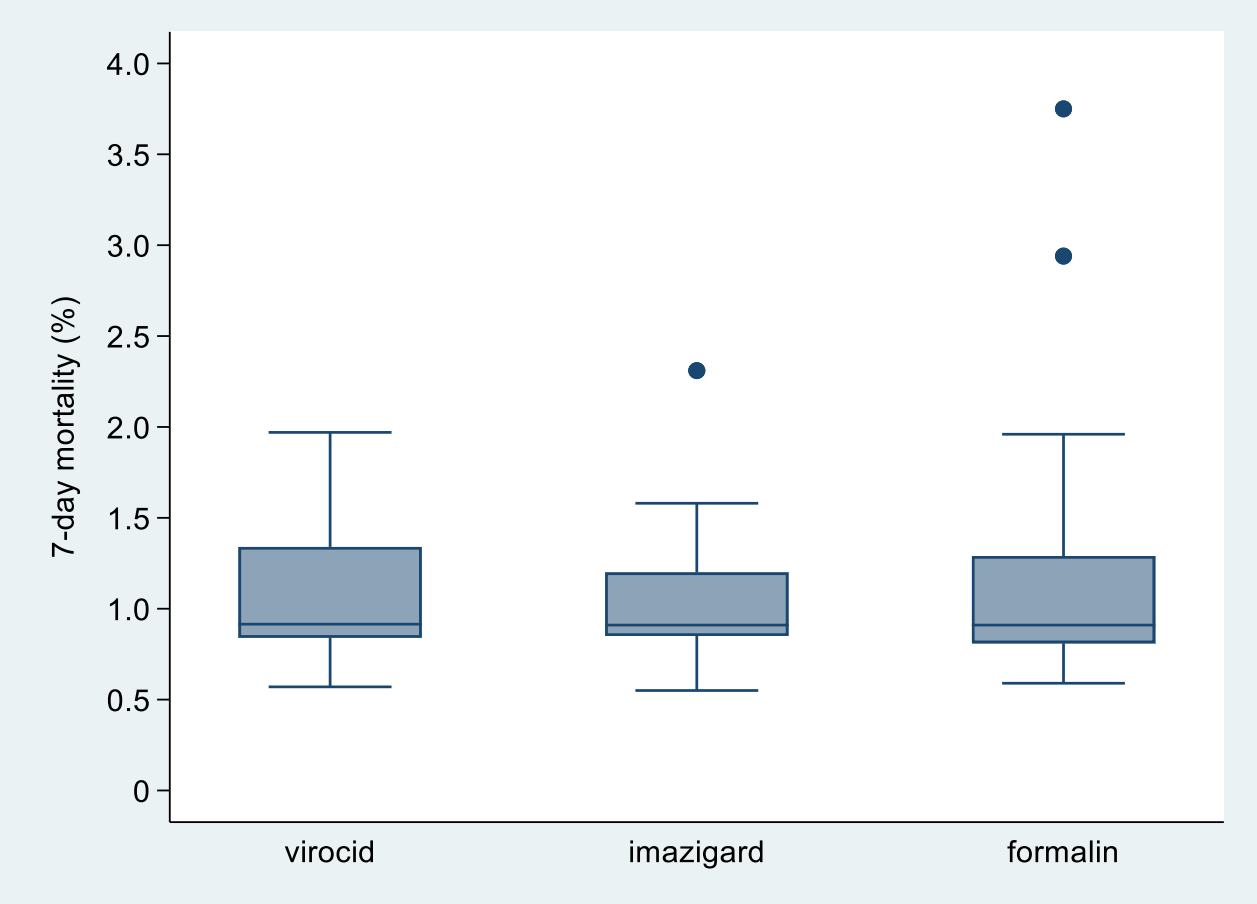

Figure 6: Seven-day chick mortality percentages from the 3 groups of disinfectants

4.6 Cost analysis

Considering the large size of commercial broiler hatcheries and the significant expenditure on hygiene programs, it is imperative to consider cost as a factor when comparing disinfectants. The cost per egg is calculated by taking the total amount of product used per hatcher between transfer of eggs and take-off of chicks, divided by the number of eggs per hatcher.

Table 14: A comparison of the cost between the 3 disinfectants

Product	Cost per liter	Dilution rate	Cost per liter diluted	Application rate per hatcher (diluted)	Cost per hatcher (19 200 eggs)	Cost per egg
Formalin (37%)	R15.98	Undiluted	R15.98	2 000ml	R31.96	0.17c
Virocid	R159.70	4%	R6.38	1 400ml	R8.93	0.046c
Imazigard	R332.00	2%	R6.64	1 400ml	R9.30	0.048c

As per Table 14, the cost per liter of undiluted formalin is significantly lower than either of the 2 trial products. However, as the trial products are diluted at 2-4% and the formalin is used neat, the cost after dilution is lower than formalin and the total cost per egg is almost four times less than formalin. It thus showed that Virocid and Imazigard, if used at the dilution and application rate as per this trial, is more cost-effective than formalin for disinfecting eggs during the final stages of incubation in a hatchery.

CHAPTER 5: DISCUSSION AND CONCLUSION

Many other studies have investigated alternatives to formaldehyde for disinfection of hatching eggs, which has long been used as an effective disinfectant in hatcheries and is still commonly used in South African hatcheries. Most of these studies have focused on disinfecting eggs prior to incubation, by using various products and through various routes of application (Keïta et al., 2016).

On the contrary, the intention of this research was to specifically evaluate alternatives to formalin during the final stage of incubation, when eggs hatch and a large number of bacteria and fungi are released in the incubators with the potential to infect other eggs and chicks.

The negative effect of formaldehyde gas on the tracheal epithelial cells of chicks during incubation has already been demonstrated (Hayretdag and Kolankaya, 2008). Based on the chemical and physical characteristics as well as the safety profile of both Virocid and Imazigard and its user-friendliness compared to formaldehyde, it can be assumed that these products will not cause the same tracheal epithelial damage to chicks as formaldehyde.

It would have been ideal to also incorporate a negative control in the trial, where a fourth group of eggs were exposed to water only at the same volume and route of application as the two treatments. However, the associated risk of not disinfecting eggs during incubation is too large for a commercial broiler hatchery of this size as it may jeopardize chick quality, but in a smaller scale trial it would have been possible.

It must also be stated that the route of application of the chemicals during the trial, with formalin being poured in pans and left to evaporate and the 2 trial products being fogged via a ULV fogger, does make applying the formalin much easier and less labour intensive than using a ULV fogger. Opening the doors of the hatcher machines to apply the chemical with a ULV fogger also may affect hatcher temperature and RH, although during the trial this was only done for 2 minutes at a time which is too short to negatively impact hatcher parameters. If alternative disinfectants are to replace formalin for disinfection of

eggs in a hatchery, it would be preferred to install automatic mist foggers in the hatchers to eliminate the need to apply the products manually.

For all microbiological parameters assessed, it was shown that there were not statistically significant differences between bacterial and yeasts and moulds counts between the formalin control group as well as the 2 treatment groups. The only exception was suppression of *E. coli* by Virocid, which had highly significant reduction compared to formalin as indicated by $p<0.01$. Based on this it can be stated that the trial had established that the 2 disinfectants evaluated both managed to achieve similar or better reduction of bacteria and yeasts and moulds when compared to formalin.

Broiler chick performance as measured by 7-day cumulative mortality was also not impacted using the 2 alternative disinfectants compared to the positive control. It can therefore also be concluded that this metrics was not affected by using alternative glutaraldehyde/QAC based disinfectants.

The aim of the study was to establish if alternative disinfectants can be used to replace formalin for disinfecting eggs during the final stage of incubation. The results for all metrices showed that the Virocid and Imazigard treatments performed either similar to or better than formalin. It is therefore concluded that both Virocid and Imazigard can be used safely and cost effectively as alternatives to formaldehyde for disinfecting eggs during the final stages of incubation in a commercial hatchery.

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ADDENDUM A: Animal ethics committee approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

12 July 2021

Approval Certificate
New Application

AEC Reference No.:	REC137-20
Title:	Evaluation of alternative disinfectants to formaldehyde for disinfecting broiler eggs during incubation in a commercial hatchery
Researcher:	Dr AJ van Wijk
Student's Supervisor:	Dr DBR Wandrag

Dear Dr AJ van Wijk,

The **New Application** as supported by documents received between 2021-02-24 and 2021-07-02 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2021-07-02.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
Eggs to chicks (Cobb and Ross breeds)	17280000
Samples Fluff, collected from floor (Broilers)	900

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2022-07-12.
3. Please remember to use your protocol number (REC137-20) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

ADDEDNUM B: SANAS accreditation certificate for Rainbow Chickens' Worcester Laboratory

CERTIFICATE OF ACCREDITATION

In terms of section 22(2)(b) of the Accreditation for Conformity Assessment, Calibration and Good Laboratory Practice Act, 2006 (Act 19 of 2006), read with sections 23(1), (2) and (3) of the said Act, I hereby certify that:-

RCL FOODS CONSUMER (PTY) LTD

Co. Reg. No.: 1960/002377/07

WESTERN CAPE

Facility Accreditation Number: **V0006**

is a South African National Accreditation System accredited facility
provided that all conditions and requirements are complied with

This certificate is valid as per the scope as stated in the accompanying scope of accreditation, Annexure "A", bearing the above accreditation number for

MICROBIOLOGY

The facility is accredited in accordance with the recognised International Standard

ISO/IEC 17025:2017

The accreditation demonstrates technical competency for a defined scope and the operation of a quality management system

While this certificate remains valid, the Accredited Facility named above is authorised to use the relevant accreditation symbol to issue facility reports and/or certificates

Mr M Phaloane
Acting Chief Executive Officer

Effective Date: 19 August 2020
Certificate Expires: 07 June 2025

We wish you the best with your research.
Yours sincerely

H. Lutermann

Dr Heike Lutermann
DEPUTY CHAIRMAN: UP-Animal Ethics Committee