The evaluation of a continual disinfection program on a commercial broiler chicken farm

By

Deon Beauzec

Submitted in accordance with the requirements for a Master of Science degree in Microbiology

In the

Faculty of Natural and Agricultural Sciences

Department of Microbiology and Biochemistry

University of the Free State

Bloemfontein 9300

South Africa

December 2022

Supervisor: Prof R. Bragg

Co-supervisor: Ms S Mc Carlie

Declaration

I, Deon Beauzec, declare that the research dissertation that I herewith submit for the master's degree qualification in Microbiology at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

Table of Contents

Abstract

The rise of antibiotic resistance as a global health threat has been linked to overuse of antibiotics, in particular, in the agricultural sector. A post-antibiotic world is fast approaching where antibiotics may be completely ineffective. To delay and prepare for this reality, alternative solutions to the use of antibiotics in animal production are required. Biosecurity describes some of the oldest techniques related to animal health, including infectious agent destruction and exclusion. Novel solutions to biosecurity must be investigated, however the possible loss of production related to halting antibiotic use is a major risk to food security. This study focused on assessing a continuous disinfection program using Virukill®, a modified- didecyldimethylammonium chloride-based disinfectant, for post-placement cleaning and disinfection, direct spray, and administration in water supply, at a commercial scale. This work provides a proof of concept for industrial application of a continuous disinfection program as a biosecurity measure in poultry production. Through bacterial and viral evaluation methods it was established that the continuous disinfection program was equal or better than standard aldehyde disinfectants in reducing viral loads. Organisms that survived the cleaning and disinfection process were also isolated and identified with molecular methods. The possibility of disinfectant resistance was also investigated but no evidence of resistance was observed. It was established that Virukill® was more effective than DDAC and BC in inhibiting the growth of organism isolated. Through growth performance studies this work establishes for the first time that the Virukill® continuous disinfection program improves performance of broilers, and a correlation was established between cleaning efficacy and performance. This work provides a viable alternative biosecurity-based alternative to the use of antibiotics in broiler production.

List of Abbreviations

AI – Avian Influenza

APEC – Avian Pathogenic *Escherichia coli*

BC – Benzalkonium Chloride

CIA – Critically Important Antibiotic

DAFF – Department of Agriculture, Forestry and Fisheries

DDAC – didecyldimethylammonium chloride

FCR – Feed Conversion Ratio

IB – Infectious Bronchitis

IBD – Infectious Bursal Disease

MDR – Multidrug Resistance

MIC – Minimum Inhibitory Concentration

ND – Newcastle Disease

PEF – Production Efficiency Factor

QAC – Quaternary ammonium compound

SAPA – South African Poultry Association

WHO – World Health Organization

List of Figures

List of Tables

Acknowledgements

To my supervisor Prof Bragg, thank you for being brave and willing enough to answer a stray request to do a Masters in the beginning of Covid and assisting to make it a reality for me. Thank you for the guidance and assistance whenever available. Also, to Sam Mc Carlie, for lighting a way out of multiple problems and just guiding to a different direction to think. Thank you also to the UFS students and staff who went out of their way to assist me in this abnormal long-distance arrangement. I would like to thank Carlo Visser in particular for practical arrangements and assistance. Also, to Mart-Louise and Bernadette for helpful tips and tricks during the most difficult week of this study.

A thank you is also appropriate to the people at Anca Foods who assisted in this. From the support and trust of the company in allowing a commercial trial at this scale, to the specific support of Devin Isemonger, who pushed and supported me to pursue this path. Thank you too Jacques, John and Julian who assisted on the farms, especially on early winter mornings. Thank you to also to Stefan Swart, Bruce Green, Stuart Vickers, and Rob Ross.

Thank you to my parents, Mike and Pam, and my siblings, Bianca, Simoné and Michael, the path that led me to this was paved by your time, effort, patience and sacrifice. Finally, to my wife, Jean-Dré, my love, my heart, my rest, my safety, my passion. Every success I will ever attain is yours too. I will never be able to thank you for the life I have because of you.

Chapter 1: Literature Review

1.1 Introduction to the study

Poultry meat is a significant contributor to the South African diet. It is the most widely consumed meat in the country and plays a vital role in food security (SAPA, 2017). Antibiotics are routinely used by the majority of poultry producers, and no viable alternatives to the use of antibiotics on a commercial scale have been identified. With the proliferation of antibiotic resistance, the poultry production environment must rapidly adapt, or suffer the consequences of reduced performance and output.

Alternatives to antibiotics have been identified, but this study proposes that an older set of techniques, known as biosecurity, may be the most promising solution. Some modernisations of decades old techniques will, however, be needed. The reduction of infectious material in the environment, along with the prevention of the introduction of new infectious material will result in reduced disease incidence and mortality among broilers. How this should be done is still up for debate. Very little work has been published on the effectiveness of cleaning and sanitation programs in animal agriculture (Gosling, 2018), likely due to the process seeming obvious, although this is not necessarily true.

This study aims to address the gap in the literature by investigating the link between a disinfection program, hygiene indicators and performance on a commercial scale. In addition, the aspect of virus eradication is also investigated and compared to bacterial reduction.

A notable concern is the recent evidence pertaining to the development of disinfectant resistance along with antibiotic resistance (Mc Carlie *et al*., 2020). It is known that disinfectant resistance genes can be transferred between organisms and may be transferred along with antibiotic resistance genes (Kim *et al*., 2018; Mc Carlie *et al*., 2020; Tong *et al*., 2021; Zhu *et al*., 2021). The possibility of the use of certain disinfectants and increased resistance should be considered as a possible contributing factor to antibiotic resistance, and possibly a global health threat on its own.

As previously mentioned, there is a distinct lack of field trials pertaining to animal production in the published literature. This has led to a situation where there is a disconnect between farmers who have on-the-ground experience and knowledge, and the scientists who study the phenomena they encounter. Conducting field trials in real-life agricultural settings is a tool that can be used to introduce this valuable information into the scientific community, even though the testing environment is semi-controlled. For this study a commercial farm will be used to conduct an on-the-ground field trial at a commercial scale to truly test the capabilities of the system in a South African context. The study aims to evaluate whether biosecurity, with a modernized continuous disinfection program, is a viable alternative to antibiotic use.

1.2 Aims and objectives

The main aim of the study is to investigate how a continuous disinfection program will affect broiler production, bacterial load and viral load. This is done by implementing a field trial where a continuous disinfection program is used on commercial broilers and collecting the associated data.

The first objective of the study is to determine the effectiveness of the continuous disinfection program by comparing the bacterial composition and reduction after cleaning as well as the viral reduction after cleaning, compared to a standard disinfection program. This includes bacteriological study and molecular methods, including investigation of bacteria that survive after disinfection.

The second objective of the study is to evaluate the effects of the continuous disinfection program on the performance parameters of commercial broilers. This includes measurement of mortalities, feed consumption, water consumption and weight of broilers to see if there is a production benefit or detriment to continuous disinfection.

1.3 Poultry production in South Africa

Poultry meat is the most popular meat in South Africa (Makgopa, 2020). It is consumed at a rate of 33kg per year per capita, as measured in 2019 (Makgopa, 2020). Additionally, it is the largest agricultural sector in the country, more significant than any other animal or plant production (Makgopa, 2020). Poultry production in South Africa has significantly been affected in recent years, with the introduction of stricter tariffs on imported poultry and the introduction of the poultry sector master plan by government, and industry to promote local production of poultry (Makgopa, 2020). The ability to produce poultry consistently and profitably in South Africa has a clear impact on the country's food security and economic opportunity.

High levels of pathogenic organisms in the poultry production environment lead to reduced performance, increased incidence of disease, and a greater likelihood of pathogens reaching the consumer in the end product (Payne *et al.*, 2005). Poultry is thought to be the leading cause of foodborne disease incidents in humans -s the majority of which are due to bacterial contamination by *Salmonella* species and *Clostridium* species (Chai *et al.*, 2017).

The poultry industry is currently facing several challenges regarding the traditional methods of disease control. These challenges include growing antibiotic resistance, a lack of alternatives to antibiotics, the use of biosecurity and questions regarding the best forms of implementation, the forms of measuring virus eradication and an emerging threat of disinfectant resistance.

1.4 Antibiotic resistance in poultry production

In poultry production, the use of antibiotics to control infectious diseases is commonplace. Additionally, antibiotics are used in poultry production to combat disease and at sub-therapeutic levels to increase production performance (Apata, 2009; Kirchhelle, 2018). The concern with the liberal use of antibiotics on animal production is the rapid increase in antibiotic resistance in both

pathogenic and commensal bacteria (Apata, 2009). This extends the threat of disease and production losses but raises the possibility of transmitting antimicrobial-resistant zoonotic disease.

Although the existence and awareness of antimicrobial resistance due to the process of selection by overuse of antibiotics was established in the 1960's (Kirchhelle, 2018); the bleak history of antibiotic use in agriculture has shown that regulatory guidance has been delayed for the majority of the 80-plus years antibiotics have been used in animal production. Instead, self-regulation by the industry has been the most effective at stewardship efforts (Kirchhelle, 2018). The threat of antimicrobial resistance is now regarded as a major threat to humans on an existential scale (Gilbert & McBain, 2003).

In 2017, the World Health Organisation (WHO) published the Guidelines on use of medically important antimicrobials in food-producing animals (World Health Organization, 2017). The guidelines support the urgency of antimicrobial control based on a general reduction. The thought is that overuse of antibiotics will lead to increased antibiotic resistance in bacteria in the agricultural environment. This will spread to the greater environment and eventually to humans who work with or consume the animals (World Health Organization, 2017). Therefore, there is a need to decrease antibiotic use to reduce the risk of antimicrobial-resistant infections in humans, and the human health consequences outweigh any negative consequences. In addition to these guidelines, which support general reduction and specific reduction of medically important antibiotics, the WHO also published the critically important antibiotic (CIA) list. This lists the most important antibiotics representing the last line of defence against a growing threat of resistant bacteria and therefore should be used only when absolutely necessary (World Health Organization, 2017).

In South Africa, the Department of Agriculture, Forestry and Fisheries (DAFF), along with the veterinary industry, has proposed strategic guidelines and goals to more effectively control and reduce the use of antibiotics in animal production, including measures such as restricting use to emergencies,

mandatory withdrawal periods, and prohibiting the use of antibiotics for growth promotion (Eagar & Naidoo, 2017).

1.5 Alternative approaches to antibiotics

There are many proposed solutions to the looming antibiotic resistance crisis, these include changes in practice, antibiotic stewardship, reduction of use in agriculture and avoiding use for viral infections. (Bartlett *et al.*, 2013). Alternatives to antibiotics have also been proposed such as essential oils (Barbour *et al.*, 2015), bacterial vaccines, targeted bacteriophages and phage enzymes have been identified as possible alternative compounds/approaches to replace antibiotics as reviewed by Bragg *et al.* (2018). A growing concern with these alternative solutions is their increased cost by companies exploiting the urgent need to replace routine antibiotics. In addition, there is a need for increased knowledge and study, which may be years away, if not unattainable.

In agriculture, improved biosecurity may be the most effective form of disease control. Biosecurity is an established beneficial set of principles that should form part of any good farming operation. Biosecurity is generally seen to consist of three layers, namely conceptual, structural and procedural biosecurity (Torremorell, 2021). Conceptual biosecurity refers to the location of the farm and physical separation from possible hazards that may be introduced. This includes distance to other farms, distance to public roads and amenities, access control and pest control (Torremorell, 2021). Structural biosecurity refers to the composition of the farm, including physical barriers like fencing, but extending to shower-in-shower-out facilities, farm layout and feed storage. Procedural biosecurity are documented procedures that are employed to reduce or remove introduction of pathogens, this includes the implementation of showering requirements, cleaning and disinfection programs, and routine checks to ensure policies are updated and relevant (Torremorell, 2021). There are cleaning steps in a sound biosecurity system, using detergents to remove organic material, followed by disinfection with one of many available disinfectants to sanitise the area where production is to take

249 place (Burbarelli *et al.*, 2017). This is then accompanied by regular disinfection of all water supplies
250 before, and sometimes during, the production period. The addition of good agricultural practices like
251 showering before coming onto farms, disinfection of feed trucks, distance between farms, foot baths,
252 pest control, prevention of access to wild birds, and fencing contributes to hygienic conditions that
253 reduces the introduction and spread of pathogenic organisms in the poultry houses (Tablante *et al.*,
254 2002).

255 ## 1.6 Biosecurity
256

257 Biosecurity is a term that includes all practices and measures to inhibit or prevent living organisms
258 from harming a poultry flock. Tablante and co-workers (2002) found that improved biosecurity
259 measures positively correlated to the performance of broilers, with a noted emphasis on regular
260 disinfection of water supply lines.

261 In practice, the most crucial measurement of biosecurity is the mortality rate. Although zero mortality
262 is not achievable on a commercial scale, there are pathogens that could result in as high as 100%
263 mortality (Yassin *et al*., 2009). Mortality rates vary significantly between different growing conditions,
264 however Yassin and co-workers (2009) propose that the daily cumulative mortality rate in seven
265 consecutive flocks should be below 1% + (0.6% x slaughter age in days) of the flock. Heier and co-
266 workers (2002) found an average cumulative mortality of 1.54% in the first week, followed by a weekly
267 cumulative mortality rate of 0.48% per week for the rest of the growth period. The South African
268 Poultry Association (SAPA) reported an average mortality rate of 6.7% per cycle in 2017 (SAPA, 2017).
269 It has been found that at low levels of contamination and pathogenicity, with low probabilities of
270 disease outbreaks, and taking into account the effects on mortality, the economic benefits of
271 adequate biosecurity are obvious (Gifford *et al*., 1987).

1.6.1 Cleaning and disinfection as a biosecurity tool

In poultry production, after removing litter from the previous cycle, it is common practice to clean and disinfect the poultry house (Payne *et al*., 2005). The cleaning and disinfection process aims to remove pathogenic organisms from the environment that could affect the health of the poultry and those pathogenic organisms that may persist into the abattoir and onto the store shelves, where they can result in foodborne diseases in consumers (Payne *et al.*, 2005). Evidence suggests that using a cleaning and disinfection program improves broiler performance (Burbarelli *et al*., 2015).

In the common disinfection programs for poultry production there is a cleaning and disinfection step, followed by the use of footbaths as best practice. In some cases, drinking water is regularly treated with an antimicrobial substance. Some investigation has been aimed at adding a step by using disinfectants during the production step directly onto the birds in a spray or fog in a step analogous to food production and the regular handwashing and sanitising of hands and surfaces (Bragg & Plumstead, 2003). The efficacy of this procedure has little large-scale experimental evidence, but aldehyde aerosol treatment would be unsuitable due to its harmful effects on the respiratory tracts of broiler chicks (Zulki *et al*., 1999).

1.6.2 Disinfectants used in poultry production

Various disinfectants are used in the poultry industry. These include free chlorine compounds, oxidising agents, alcohols, iodophors, phenolic compounds, quaternary ammonium-containing compounds, and aldehydes, most commonly glutaraldehyde and formaldehyde (Burbarelli *et al*., 2017; Fate *et al*., 1985; Payne *et al*., 2005). Although sparse, there have been studies into which disinfectants are optimal for use in broiler production. The differences between farms and even individual houses make selecting a suitable compound a complicated process (Payne *et al*., 2005). Fate and co-workers (1985) found that glutaraldehyde was the most effective disinfectant for reducing bacterial counts in poultry houses and cresylic acid was the most effective when considering mould reduction. However, they did stress that all disinfectants have their drawbacks; for example, cresylic

297 acid has a pungent odour that lingers, making it unsuitable for most applications. When comparing
298 four disinfectants, Payne and co-workers (2005) found that four different classes of disinfectants
299 effectively reduced bacterial content, but that quaternary ammonium compounds became less
300 effective in environments with a high organic load.

301 It is logical that for the cleaning and sanitation step to be successful, careful consideration needs to
302 be given to the chemical used and the mode of application.

303 ### 1.6.4 Continuous disinfection

304 A continuous disinfection program has previously been attempted in South Africa. Bragg and
305 Plumstead (2003) performed a small-scale and commercial-scale trial of continuous disinfection using
306 a commercial disinfectant, Virukill® . Virukill® is a quaternary ammonium compound (QAC) based
307 disinfectant with a modified didecyldimetylammonium chloride (DDAC) as its active ingredient. It has
308 been proposed as a compound suitable for a continuous disinfection program due to reduced toxicity
309 and its legal registration through Act 36 to be used in drinking water and as a spray on birds in South
310 Africa. In a continuous disinfection program, cleaning and sanitation is not the final step where an
311 antimicrobial chemical is applied. In the program proposed by Bragg and Plumstead (2003), the
312 chemical is used to clean and disinfect the poultry house after standard cleanout of organic matter.
313 The water lines are also sanitised with the same chemical. During growth of the chickens, the chemical
314 is directly applied utilising a spray at regular intervals and continually in the drinking water. In the
315 commercial trial, the continuous disinfection program yielded reduced mortalities over the first 16
316 days. However, due to outside interference and a Newcastle Disease (ND) outbreak, the producer
317 halted the trial on day 20. Up to this point, it should be noted that the houses on the continuous
318 disinfection program had reduced mortalities compared to controls (Bragg & Plumstead, 2003), in the
319 face of a significant ND challenge in the other houses on the farm. Given these results showing a
320 reduced mortality in the smaller-scale trial and at the beginning of the commercial trial, the use of a
321 continuous disinfection program on a commercial scale warrants further investigation.

1.7 Virus eradication in poultry production

1.7.1 Impact of viruses

Due to their ubiquity, viruses affect poultry production significantly. Some avian viral diseases are notorious for their immediate rapid death rates. The Avian Influenza virus and Avian Paramyxovirus-1, the causative agent of Newcastle Disease (ND), can have devastating mortality rates of up to 100% (Capua & Marangon, 2006; Cattoli *et al*., 2011). Other avian viruses cause less immediate effects but can be equally harmful to the producer. The Infectious Bronchitis (IB) virus, which causes respiratory symptoms, commonly causes a morbidity rate of 100% but varies significantly in mortality rate between flocks (Jackwood & de Wit, 2020).

Along with respiratory distress, the IB virus results in a reduction in weight gain in broilers, which causes major economic impacts and creates the opportunity for secondary infections of bacteria (Jackwood & de Wit, 2020). Considering the efforts to reduce the use of antibiotics in poultry production, the secondary bacterial infections caused by various viral infections will have a very serious consequence on poultry production. The enteric viruses of poultry have recently gained attention as a major causative agent of enteric disease, which is characterised by a decrease in nutrition utilisation, resulting in abnormally slow growth, wet litter (due to diarrhoea), and reduced uniformity and lethargy (Mettifogo *et al*., 2014). In the past, most enteric diseases had been attributed to bacterial causes alone, but now various enteric viruses are thought to contribute to the runting and stunting symptom development (Mettifogo *et al*., 2014). It is a concern as it mainly affects younger birds, which are more susceptible to adverse environments and challenges that will affect the entire production cycle. The described enteric viruses include the chicken astrovirus, rotavirus, reovirus, chicken parvovirus, fowl adenovirus and avian nephritis virus (Mettifogo *et al*., 2014). Interestingly, these are all non-enveloped viruses. A non-enveloped virus also causes infectious Bursal Disease (IBD). The IBD virus attacks the Bursa of Fabricius, an organ involved with the immune response in chickens,

resulting in a clear immunosuppressive effect, which leads to secondary infections (Müller *et al*., 2003; Winterfield *et al*., 1978).

1.7.2 Cleaning and disinfection and viruses

The standard method for verifying the effectiveness of cleaning and disinfection of poultry houses is bacterial determination, either by direct swab and spectrophotometric load determination (Bragg & Plumstead, 2003), swab and streaking on agar (Burbarelli *et al*., 2017; Meroz & Samberg, 1995), sponges (Payne *et al*., 2005), Rodac plates (Fate *et al*., 1985) or by biosampler (Jiang *et al*., 2018). However, the pathogenic pressure in the chicken house is not only of a bacterial nature. As discussed in the previous section, avian viruses are significant contributors to disease incidence in poultry.

The common feature of enveloped viruses is their lipid bilayer required for cell entry which can be interfered with using disinfectants with a lipophilic character. However, it is much more challenging to inactivate non-enveloped viruses, such as the aforementioned enteric viruses, and IBD, as denaturation of the capsid proteins is required to prevent entry into the host cells (Lin *et al*., 2020).

The enteric viruses are hardy and resistant to removal; therefore, they are the "golden standard" for disinfection. Benton and co-workers (1967) found that the IBD virus was not affected by phenol and merthiolate but was inactivated by 0.5% formalin after 6 hours of exposure in a bursal homogenate. They also found that exposure to a high pH (12) for 1 hour at 30°C could inactivate the virus (Benton *et al*., 1967) Some success has been found, with Gay & Mundt (2010) successfully inactivating the virus in chicken litter using metam-sodium. Due to the ubiquity of the virus, inactivation combined with adequate protection with maternal antibodies through vaccination may serve as adequate protection to reduce the viral load to a level that can reduce disease development. Finally, precedent for quaternary ammonium compounds being effective against the IBD virus has been established. The work of Shirai and co-workers (1994) showed that didecyldimethylammonium chloride (DDAC) with 0.05% NaOH effectively inactivates the virus.

Considering the above, the bacterial methods are inadequate to address the effectiveness of cleaning and disinfection as they have no means of determining whether viruses were removed or reduced in the environment. To this author's knowledge, no studies have evaluated the effectiveness of different chemicals in cleaning and disinfection of poultry houses by viral methods.

1.7.3 The Vir-Check system

To evaluate whether the cleaning and disinfection of poultry houses effectively removed viruses, GD Animal Health in the Netherlands developed a system to detect non-enveloped viruses (GD Animal Health, n.d.). The VIR-Check system is a multiplex-PCR to detect rotaviruses A and D, reovirus, Avian Nephritis Virus 3 and Astrovirus, all non-enveloped enteric viruses. The test is done by examining swabs of live birds at 6-7 days of age to determine the viral load in the house at placement, and scoring these with a formula that corrects for maternal transfer (GD Animal Health, n.d.). The logic of the test is based on the aforementioned hardy nature of non-enveloped viruses. If these are in low prevalence in the actual chickens, cleaning and disinfection would have addressed the viral load in the environment, and by extension, should have affected the bacterial loads.

There is a clear advantage to a more profound knowledge of the pressures inside the poultry house. Undoubtedly improvements in our knowledge regarding viral loads after disinfection will more accurately guide decisions on future biosecurity measures.

1.8 *Escherichia coli* in poultry and emerging disinfectant resistance

1.8.1 Colibacillosis

Colibacillosis is any infection in poultry with Avian Pathogenic *Escherichia coli* (APEC) as the causative organism (Nolan *et al.*, 2020). The systemic or localised infections can lead to a wide array of symptoms that resemble other opportunistic pathogens; hence lesions alone are not enough to confirm colibacillosis (Nolan *et al*., 2020). Positive identification of APEC must be done by molecular or culture methods.

Although it is difficult to determine precisely, there is a general agreement that colibacillosis is the leading avian disease caused by bacteria (Nolan *et al*., 2020). This supports the idea that colibacillosis infection is a significant contributor to disease-related losses in the industry through direct mortality, condemnation at ante-mortem and post-mortem, and impaired growth (Nolan *et al*., 2020).

1.8.2 APEC virulence

Avian Pathogenic *Escherichia coli* (APEC) has historically been viewed as an opportunistic pathogen associated with co-infections (Nolan *et al*., 2020). However, there is evidence to suggest that the APEC strains are adapted explicitly for an extra-intestinal environment, which could indicate that in some instances, they may be strictly pathogenic (Johnson, *et al*., 2008).

No clear APEC pathotype has been described, however Rodriguez-Siek and co-workers (2005) provided provisional information by identifying more prevalent genes in APEC isolates than isolates from "healthy" chickens. They proposed the value of a genetic typing method after the identification of common APEC virulence genes. Research by Ewers and co-workers (2007) and Rodriguez-Siek and co-workers (2005) indicate that the most common genes associated with the APEC pathotype are found on plasmids. In particular, genes associated with the pTJ100 plasmid were prevalent in APEC isolated in both studies(Ewers *et al*., 2007; Rodriguez-Siek *et al*., 2005). This may be concerning as this plasmid and similar plasmids have been identified to carry genes associated with antimicrobial and disinfectant resistance (Johnson *et al*., 2004). Therefore, it may be proposed that using antimicrobial agents or disinfectants could exert selective pressure on the population and increase the proportion and virulence of APEC.

Although there is no complete description of one or a set of genes that definitively describe the APEC pathotype, several genes have been proposed as possible candidates. Avian Pathogenic *E. coli* (APEC) has historically been classified as a subtype of extra-intestinal pathogenic *E. coli* (ExPEC), which includes uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) (Nolan *et al*., 2020). Due to this classification, APEC may share similar mechanisms for surviving the extracellular environment.

Gene studies have been used to screen for multiple virulence factors in APEC isolates to attempt to describe the APEC pathotype. Genes that have been targeted are those involved in iron acquisition (Bäumler *et al*., 1998; Johnson *et al*., 2008; Kronstad & Caza, 2013; Runyen-Janecky *et al*., 2003; Sabri *et al*., 2008), toxin production (Morales *et al*., 2004; Parreira & Gyles, 2003), adhesion (Li *et al*., 2010; Mellata *et al*., 2003; Tivendale *et al*., 2004), and protectin encoding (Johnson *et al.*, 2008; Pavelka *et al*., 1991). Additionally genes that play roles in surviving low oxygen conditions (van der Westhuizen & Bragg, 2012) and regulation of gene expression (Herren *et al*., 2006) have been targets.

Due to the strong linkage of APEC virulence to plasmids (Johnson *et al*., 2008; Rodriguez-Siek *et al*., 2005), certain plasmid associated genes may also be indicators of APEC. Rodriguez-Siek and co-workers (2005) found that *ompT* which encodes a colicin cleaving gene, is highly prevalent in APEC isolates. In addition, *cvaC*, the structural gene of the ColV plasmid, has been found to contribute to virulence APEC (Skyberg *et al*., 2006) but was recently found to be less widespread than previously thought (Newman *et al*., 2021).

Newman and co-workers (2021) screened 61 APEC isolates for the presence of 44 virulence-associated genes and 24 antimicrobial genes. The ColV plasmid-associated genes were the most prevalent, with the *iroN, ompTp* and *hlyF* genes the most common. From this study and others, the notion of a single APEC pathotype determined by a specific combination of genes has been placed under doubt. Many combinations of genes may confer the necessary attributes to survive the extracellular environment, resulting in pathogenesis.

The current knowledge supports a more dynamic genetic environment. The genetic composition of bacteria changes with mobile genetic elements through transfer within and between cells through well-described mechanisms (Mc Carlie *et al*., 2020). Therefore, the virulence-related genes in APEC populations can move within the population to confer evolutionary advantages in survival. This results in a diverse gene pool with multiple combinations of genes responsible for the pathogenic lifestyle.

1.8.3 APEC resistance

In a recent study, Newman and co-workers (2021) found supporting evidence for the established notion that APEC virulence is most commonly associated with the ColV plasmids. In their study, up to 90% of isolates had ColV associated genes present. A concern is the detection of only 34% of all isolates being susceptible to all antimicrobials tested. Similarly, Barbieri and co-workers (2013) reported resistance to tetracycline (69.4%) and sulphonamides (59.7%) in high frequency. However, for all other antimicrobials, they found resistance in less than 30% of isolates. Johnson and co-workers (2012) also found significant increases in resistance to several antimicrobials between commensal and APEC. They found a widespread distribution of multidrug resistance (MDR) in APEC isolates and evidence of widespread distribution of mobile genetic elements. The study also showed a wide prevalence of ColV and R plasmids encoding MDR, suggesting co-transfer of these elements being common in APEC. They concluded that there is clear support for a correlation between virulence factors and resistance (Johnson *et al*., 2012). The concern is that there may be selective pressures in poultry production, promoting the development and spread of APEC virulence factors and resistance genes due to conjugative plasmids. Even more concerning is the possibility that the similarities of human pathogenic ExPEC and APEC could transmit these mobile genetic elements giving rise to new zoonotic pathogens (Johnson *et al.*, 2008).

1.8.4 Resistance to disinfectants

With the established correlation between APEC virulence and resistance, there exists the possibility that disinfectant use could also lead to the increased dissemination of resistance and zoonotic pathogen emergence. Recently, much more attention has been given to the development and spread of disinfectant resistance (Bragg *et al*., 2018; Mc Carlie *et al*., 2020). In particular, Kim and co-workers (2018) found that MDR resistance arose alongside resistance to Benzalkonium Chloride (BC), a commonly used first-generation QAC.

The APEC classification study by Newman and co-workers (2021) also determined the most common plasmid replicon types for APEC to be FIB and I1. They found significant correlations between the replicon type and resistance to antimicrobial genes (Newman et al., 2021). In addition, the FIB replicon was also associated with genes linked to heavy metal resistance (*pco* and *sil* for copper and silver) and QAC resistance (*qac* genes) (Newman et al., 2021). This suggests that the plasmids that carry APEC virulence and MDR genes will likely be carrying disinfectant resistance genes. Finally, the FIB replicon was also significantly linked with *int* integron genes (Newman *et al.*, 2021), which supports that these factors and resistance genes may form part of genetically mobile arrays.

Resistance to QAC's in *E. coli* is linked to chromosomal and extrachromosomal genetic determinants (Zou *et al.*, 2014). The chromosomal genes include those that encode transporters that belong to the major facilitator superfamily (MFS) like *mdfA*, and small multidrug resistance (SMR) family like *sugE, emrE,* and the co-expressed *ydgE/ydgF* (Zou *et al*., 2014), which all function to transport the QAC from the interior of the cell. The extrachromosomal genes include plasmid-encoded genes and genes on integrons that also encode transporters of the SMR family including *qacE, qacEΔ1, qacF, qacG,* and *sugE* (Zou *et al*., 2014).

The presence of resistance genes in APEC isolates has not been studied extensively but may offer valuable insight into the persistence and survival of these organisms after disinfectant exposure.

1.9 Summary and Conclusions

In this first theoretical chapter, the literature regarding the current state of poultry production was presented. It is evident that poultry production in South Africa is of major economic and food security importance. This production is placed in direct jeopardy with the rise of antibiotic resistance microorganisms that may reduce performance and could become harmful as food-borne human pathogens. Biosecurity as an alternative was presented, including adequate cleaning and disinfection measures to remove persistent microbes and particularly viruses. To this effect continuous

494 disinfection using a modified QAC disinfectant was discussed as a promising avenue of study. Finally,
495 the possibility of emerging disinfectant resistance and its possible link to antibiotic resistance was
496 discussed as a major concern over the use of disinfectants in animal production. In the next chapter
497 the application of the modified DDAC disinfectant, Virukill®, in a commercial poultry production
498 cleaning and disinfection plan will be investigated.

Chapter 2: Pre-placement disinfection of a continuous disinfection program

2.1 Introduction

Poultry houses serve as a growth environment for broilers. The organisms present in the environment influences the health of the poultry and can lead to reduced performance due to disease outbreaks (Burbarelli *et al.*, 2015; Collet, 2020; Payne *et al.*, 2005). Pathogenic organisms, like *Salmonella* species and *Campylobacter* species may also be present in the environment and can be spread to humans by consumption of the meat (Payne *et al*., 2005). To reduce the negative effects of these pathogenic organisms, poultry houses are routinely cleaned, washed and disinfected to prevent the persistence of harmful organisms into the subsequent growth cycles (Burbarelli *et al.*, 2015; Meroz & Samberg, 1995; Payne *et al.*, 2005). There is an agreement that the logic and benefits of cleaning and disinfection programs seem obvious, but there remains little experimental evidence on the practice (Gosling, 2018).

Cleaning and disinfection forms an integral part of the biosecurity program (Meroz & Samberg, 1995; Tablante *et al.*, 2002). Cleaning and disinfection is the removal of contaminants by physical and chemical steps to reduce or eliminate pathogenic organisms (Meroz & Samberg, 1995). Cleaning is essential since organic material that remains in the poultry house will reduce the effectivity of certain disinfectants (Meroz & Samberg, 1995). Cleaning starts with dry cleaning, where all equipment and litter is removed and all debris from the previous growth cycle is removed to prevent persistence of infectious material (Meroz & Samberg, 1995). Wet cleaning follows, with wetting and application of detergents to remove accumulated dirt, this is rinsed out at high pressure systematically from the back of the house to the front (Meroz & Samberg, 1995). Finally, houses are disinfected using one of an array of commercially available chemicals (Jiang *et al.*, 2018; Meroz & Samberg, 1995). The efficacy

of a disinfectant can be influenced by an array of factors including: specificity of the disinfectant towards a particular pathogenic organism, dilution rate at which disinfectant is effective against each organism, the rate at which the disinfectant is applied (i.e. is there enough disinfectant used), the contact time of the disinfectant, the temperature of the area which should be above 20^{o}C as a general rule, organic load of the area that can deplete available disinfectant, and water quality which includes hard water and contaminated water (Meroz & Samberg, 1995). Even if all of the previous steps are followed, it should again be noted that it is impossible to remove all bacterial and viral materials from a poultry farm in any practical setting, however the reduction of disease challenge remains worthwhile (Meroz & Samberg, 1995).

Payne and co-workers (2005) provide evidence to show cleaning and disinfection reduces the presence of bacteria that are present in poultry houses. Burbarelli and co-workers (2017) also found increased performance with a complete removal of organic matter, followed by cleaning and disinfection stages, and found some ability to remove *Campylobacter* species from floors and drinkers, but ultimately no difference in the occurrence of *Campylobacter* species was seen. Payne and co-workers (2005) notes that the removal of organic material is especially critical as chemical disinfectant efficacy reduces with an increased organic load. The concern is raised that ineffective cleaning in this high organic load environment will result in increased disease, which in turn leads to increased mortality and decreased performance (Payne *et al.*, 2005).

Poultry houses are most commonly cleaned using chlorine, ozone, QAC's and aldehydes (Jiang *et al.*, 2018). The relative effectiveness of different cleaning chemicals are debated, but not widely studied in field trials (Battersby *et al.*, 2017; Burbarelli *et al.*, 2015; Burbarelli *et al.*, 2017; Fate *et al.*, 1985; Jiang *et al.*, 2018; Payne *et al.*, 2005). Fate and co-workers (1985) compared a glutaraldehyde-disinfectant, a combined QAC-formaldehyde disinfectant, a cresylic acid, and an iodophor. They found the glutaraldehyde based disinfectant to be most effective at reducing bacteria in poultry houses (Fate *et al.*, 1985). Payne and co-workers (2005) showed that four disinfectants with active ingredients of,

549 a phenolic compound, a QAC, a nascent oxygen compound, and potassium peroxymonosulfate and
550 sodium chloride, were all capable of significantly reducing total aerobic bacterial counts in poultry
551 houses after disinfection. Battersby and co-workers (2017) compared the bacterial reduction of
552 different compounds used on farms and found a glutaraldehyde-QAC complex applied as a fog to be
553 more effective than hydrogen peroxide, sodium hydroxide, or a potassium peroxymonosulfate-
554 sulfamic acid-sodium chloride compound. Jiang (2018) compared ozone, available chlorine, a QAC salt,
555 glutaraldehyde, and a glutaraldehyde-QAC mixed disinfectant in reduction of bacteria in the air of
556 poultry houses. They found that the order of effectiveness (from least to most) was available chlorine,
557 ozone, quaternary ammonium salt, glutaraldehyde, and finally, the mixed disinfectant was most
558 effective (Jiang *et al.*, 2018). None of these studies measured the effects of the cleaning programs on
559 viral loads in the poultry houses studied, this is identified as a major gap. The assumption is that viruses
560 are hardier than bacteria, so if the program fails to remove bacteria, it will surely fail to remove the
561 viruses. Although this argument may have merit, face-value assumption ignores the fact that
562 incomplete removal is an accepted part of cleaning and disinfection, however the specific removal of
563 certain targeted pathogenic organisms is essential, therefore evaluating specific antiviral activity of
564 disinfectants may be more important if one considers the devastating impacts of certain viruses on
565 poultry populations (Gay & Mundt, 2010).

566 In the modern age, biosecurity is unfortunately still widely misunderstood and not applied well within
567 the poultry sector as reviewed by Bragg *et al.* (2014). Cleaning and disinfection are applied regularly,
568 but by no means uniformly in the South African poultry value chain. A major concern is the frequent
569 misuse of antimicrobial agents, including detergents and disinfectants, (Bragg *et al*., 2014; Roca *et al.*,
570 2015). If disinfectants are used improperly, bacteria that are more resilient to the disinfectant will
571 survive and be selected for within the population (Roca *et al.*, 2015). Specifically, recent evidence has
572 showed that disinfectant resistance has a complex molecular basis (as reviewed by Mc Carlie *et al.*,
573 2020) and may be transferred within a population. Specifically, the genetic transfer of QAC resistance
574 has been observed (Kim *et al.*, 2018), which raises concerns over the use of QACs in cleaning and

disinfection. In addition, Newman and co-workers (2021) found disinfectant and antimicrobial resistance genes on a single plasmid isolated from Avian Pathogenic *E. coli* (APEC), indicating that the use of disinfectants may co-select for antibacterial and disinfectant resistance.

The goal of this chapter is to investigate the use of a novel cleaning alternative, using a modified QAC based disinfectant, in cleaning and disinfection of a commercial poultry farm. This will be done by measuring the bacterial levels and viral levels in the houses. The chapter will also seek to identify whether there is a potential for disinfectant resistance by evaluating the effectiveness of different disinfectants on organisms that survive the cleaning process. Finally, the organisms that do survive will be identified using molecular methods to gain insight into which organisms survive the disinfection process.

2.2 Materials and methods

2.2.1 Strains used in this study

For this study *Escherichia coli* ATCC 11775 was used.

2.2.2 Area of study

Four commercial poultry production houses on a farm in Stutterheim in the Eastern Cape, South Africa were subjected to an experimental cleaning and disinfection program. The houses are all 1800 m^2 and can house up to 42000 chicks for a standard cycle. All houses are environmentally controlled units. The farm is fenced, pest control is maintained, and showering facilities are available and utilised.

2.2.3 Cleaning and disinfection

2.2.3.1 Aldehyde versus continuous disinfection program

Two non-consecutive growth cycles of broilers were assessed for cleaning and disinfection. These are termed Experiment 1 and Experiment 2.

Two control houses underwent a standard cleaning and disinfection program as part of their biosecurity program with the following steps: all organic matter from the previous growth cycle was

599 removed, the houses were blown out to remove dust, the houses were rinsed with water under
600 pressure, the houses were foamed with a standard surfactant (cleaning), the houses were rinsed
601 again, finally an aldehyde disinfectant was applied at 1% to the houses. Footbaths were placed at the
602 entrance to the houses containing the same aldehyde disinfectant at 2% and replaced when dirty or
603 at a minimum every Monday, Wednesday and Friday. Houses were empty and closed for 9 days prior
604 to house preparation including laying of shavings. Birds were placed on day 10 after disinfection.
605 Growth periods were between 31 and 34 days to catch.

606 Two houses underwent the Virukill®® continuous disinfection programme described by (Bragg &
607 Plumstead, 2003) with the following modifications. The same basic sequence of steps as the standard
608 program were followed. The cleaning step was done with a 0.5% solution of Virukill® and disinfection
609 was done with a 1% solution of Virukill®.

610 #### 2.2.3.2 Peracetic acid versus continuous disinfection program

611 Two poultry houses underwent cleaning and disinfection. One house was cleaned as per the cleaning
612 and disinfection steps of the continuous disinfection program (described above) and the other as per
613 the standard cleaning program, apart from exchanging the disinfectant to a peracetic-acid based
614 disinfectant.

615 ### 2.2.4 Sampling

616 #### 2.2.4.1 Experiments 1 and 2

617 For Experiment 1 Tryptic Soy Agar (TSA) contact plates were used to sample 10 points in each house
618 before cleaning (after blowout step)(n=10), after cleaning (n=10), and after disinfection (n=10) the
619 areas sampled included the floor, feeder lines, and nipple lines at the front and back of the house, side
620 walls (left and right), side curtains (left and right). Cloacal swabs were taken as described by (GD
621 Animal Health, n.d.) by taking 6 pooled samples of randomly selected chicks at 7 days and streaking
622 them on an FTA card. These were sent for Vir-Check analysis of naked virus by G.D. Animal Health. The
623 Vir-Check tests for 5 non-enveloped viruses and a patented formula is used to convert the viral load

detected to a score. These scores are Green 0-30, Yellow 30-60, Orange 60-90, Red 90+, with green being above average, yellow being average, orange being below average, and red being very poor cleaning and disinfection. This was repeated for Experiment 2, with the following modification: TSA plates were taken from one house on the standard program and one house on the continuous disinfection program. Samples were taken only on flat surfaces before cleaning and after disinfection.

2.2.4.2 Peracetic acid versus continuous disinfection

Tryptic Soy Agar (TSA) contact plates were used to sample 20 points from flat surfaces (n=20), of these 10 were taken from the floor and 10 were taken from walls, this was performed after cleaning (before rinsing) and after disinfection.

2.2.5 Resistance screening

Samples from contact plates with growth post-disinfection were exposed to 0.5% and 1% concentrations of Virukill® for 5 minutes. After 5 minutes samples were re-streaked onto Tryptic Soy Agar and incubated at 37 ^{o}C for 24 hours. Colonies that grew after disinfection were used for minimum inhibitory concentration (MIC) study and 16S rRNA identification.

2.2.6 Minimum inhibitory concentration (MIC)

Overnight cultures of the three samples, as well as the type of strain were made by inoculating BHI broth with single colonies of each strain. The overnight cultures were standardised to OD_{600} of 0.8. stock solutions of 0.2% of three disinfectants, Benzalkonium Chloride (BC), Didecyldimethylammoniumchloride (DDAC), and Virukill® were made and serially diluted two-fold with sterile water in 96-well microtiter plates. Bacteria were inoculated at 10% inoculum and left for a contact time of 15 minutes. After contact time was completed, 5 µL of the culture-disinfectant mixture was used to inoculate 95 µL of fresh BHI in a new plate. These were incubated overnight, and the MIC was determined to be the lowest concentration where no growth was observed. Four (4) technical repeats for each organism were performed.

2.2.7 16S Molecular bacterial identification

The three organisms isolated after disinfection and the *E. coli* ATCC type strain were subjected to identification experiments to determine the identity of the isolated organisms.

2.2.7.1 DNA extraction

Overnight bacterial cultures were created by inoculating the bacteria in BHI broth. Cells were harvested by centrifuging 1.5 mL of each culture at 20 000 x *g* for 10 minutes at 4 °C. The pellets were re-suspended in 500 µL lysis buffer (100 mM Tris-HCl pH 8, 50 mM EDTA pH 8, 1% SDS) and approximately 200 µL of glass beads were added. This was vortexed and placed on ice 1 minute at a time for a total of 5 minutes. Thereafter, 250 µL of ammonium acetate (7M, pH7) was added and the mixture was vortexed to mix. The mixture was then incubated at 65 °C for 5 minutes followed by 5 minutes on ice, before 500 µL of chloroform was added, followed by vortexing and centrifugation at 20 000 x *g* at 4°C. The hydrophilic top layer was transferred to a clean 1.5 mL tube and 750 µL isopropanol was added. The tube was left at room temperature for 5 minutes, followed by centrifugation at 20 000 x *g* for 2 minutes at 4°C. The supernatant was discarded, and the pellet was washed with ice-cold 70% ethanol. This was centrifuged again at 20 000 x *g* for 2 minutes at 4°C. The supernatant was aspirated off and the pellet was airdried in a SpeedyVac. The pellet was dissolved in 100 µL TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and 5 µL RNase (0.5 mg/L). This was incubated at 37 °C for 60 minutes and stored at -20 °C.

2.2.7.2 Agarose gel preparation and visualisation

DNA extraction was visualised by preparing a 1% agarose gel in TAE buffer, whereafter 1 µL Ethidium Bromide (0.01 mg) was added to the gel. Samples were prepared by adding 1 µL 6X Loading dye to 5 µL of each sample. 3 µL of DNA Ladder Mix (ready-to-use 0.1 µg/µL, 50 µg) was added to the first well, followed by the samples. The samples were run for 34 minutes at 90V. Gels were visualised using the Bio-Rad Gel Doc™ EZ Imager and Image Lab 6.0 software.

2.2.7.3 DNA quantification

To determine the concentration of the DNA extracts, samples were analysed using a NanoDrop ND-1000 Spectrophotometer on 2 µL of each sample. Wavelengths at 260 nm, concentration (ng/µL) and 260/280 nm ratios were determined for all samples.

2.2.7.4 PCR amplification of 16S

Extracted DNA of the post-disinfection samples and the Type strain were used for 16rRNA DNA amplification. PCR reactions were setup using 1.5 µL template DNA, 0.4 µL dNTPs, 2 µL NEB 10X Thermopol reaction buffer, 0.12 µL NEB Taq DNA Polymerase, 0.4 µL forward primer (one set using 16S 8F universal primer and one set using 27F universal primer), 0.4 µL reverse primer (one set using 16S 1525R primer and one set using 1492R Primer), and 15.18 µL nuclease free water. Reactions were performed with initial denaturation at 95 °C for 1 cycle for 1 minute, before going to 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 49 °C for 45 seconds, and extension at 68 °C for 82 seconds. Final extension was performed at 68 °C for 5 minutes for 1 cycle.

2.2.7.5 PCR Visualization and quantification

PCR products were visualised by running a 1% agarose gel as previously described. The 27F and 1492R primers successfully amplified all of the samples and were used for the sequencing preparation.

2.2.7.6 Sequencing

The 16S PCR reactions were cleaned up using the Promega Wizard SV Gel and PCR clean-up kit according to the manufacturer's instructions.

Samples were prepared for sequencing using the BigDye™ Terminator v3.1 sequencing kit. Reaction mixtures were set up with each primer and each PCR product. Reactions consisted of 1 µL BigDye Premix, 1 µL primer (for forward – 27F, for reverse – 1492R), 5 µL nuclease free water, 2 µL BigDye Buffer, and 1 µL template DNA. Reactions were performed with initial denaturation at 96 °C for 1 cycle for 1 minute. 25 cycles of denaturation at 96 °C for 10 seconds, annealing at 50 °C for 5 seconds, and extension at 60 °C for 4 minutes. Samples were stored at -20 °C.

Sequencing samples were cleaned using the EDTA/Ethanol protocol. The reaction volume was adjusted to 20 µL by adding 10 µL nuclease free water. This was transferred to a 1.5 mL tube containing 5 µL 125 mM EDTA and 60 µL absolute ethanol. This was briefly vortexed and left at room temperature for 15 minutes. The mixture was then centrifuged at 20 000 x *g* for 15 minutes at 4 °C. The supernatant was aspirated. 200 µL ice-cold 70% ethanol was added to the samples followed by centrifugation at 20 000 x *g* for 15 minutes at 4 °C. The supernatant was aspirated again, and the pellet was dried in the SpeedyVac for 5 minutes. The samples were stored at 4 °C in the dark and sent for sequencing.

The sequences for each organism were trimmed for primers and aligned with the corresponding forward and reverse primers using Geneious Prime® 2022.1.1 software and the Clustal Omega alignment with default parameters. The sequences were identified using the NCBI BLAST tool (Altschul, 1997). Where reverse sequences were not readable, forward sequences alone were trimmed and analysed.

2.3 Results

2.3.1 Evaluation of microbial load

The standard disinfection plan showed a clear reduction in total viable counts (TVC) for all areas tested (Table 1). Post disinfection all areas had a countable (less than 200) number of colonies. In Standard House 1 the front floor, left side curtain, right wall, right curtain, feeder back, nipple line back, and floor back all had 0 colony counts after disinfection. For Standard House 2 the left side wall and left curtain only had 0 counts after disinfection.

Table 1: Total viable counts (CFU/Contact plate) for two poultry houses that underwent standard cleaning and disinfection program (Experiment 1)

	Standard House 1 - Aldehyde			Standard House 2 - Aldehyde		
	Before	Post Cleaning	Post Disinfection	Before	Post Cleaning	Post Disinfection
Floor Front	>200	>200	0	>200	>200	107
Feeder Front	>200	>200	3	>200	>200	15
Nipple Line Front	>200	>200	48	>200	>200	5
Side Wall Left	>200	>200	2	>200	>200	0
Side Curtain Left	142	57	0	78	>200	0

Side Wall Right	104	155	0	>200	>200	3
Side Curtain Right	>200	11	0	68	>200	15
Feeder Back	>200	>200	0	>200	>200	53
Nipple Line Back	>200	>200	0	>200	>200	103
Floor Back	>200	>200	0	>200	>200	>200
Mean	184.6	162.3	5.3	174.6	200	50.1

718

719 **Table 2*:*** Total viable counts (CFU/Contact plate) for two poultry houses that underwent the Virukill®
720 continuous cleaning and disinfection program (Experiment 1)

	Test House 1-Virukill®			Test House 2-Virukill®		
	Before	Post Cleaning	Post Disinfection	Before	Post Cleaning	Post Disinfection
Floor Front	>200	>200	>200	>200	>200	>200
Feeder Front	>200	>200	69	>200	>200	28
Nipple Line Front	>200	>200	>200	>200	>200	45
Side Wall Left	>200	0	19	>200	2	1
Side Curtain Left	>200	0	12	>200	8	2
Side Wall Right	>200	0	32	>200	64	0
Side Curtain Right	>200	0	9	>200	>200	2
Feeder Back	>200	>200	>200	>200	>200	10
Nipple Line Back	100	83	>200	>200	>200	>200
Floor Back	>200	>200	>200	>200	>200	>200
Mean	190	108.3	114.1	200	147.4	68.8

721

722 The cleaning and disinfection using the modified QAC compound (Table 2) was not as successful as
723 the standard cleaning program (Table 1). Test House 1 had five post disinfection counts higher than
724 200 CFUs and no 0 counts. Interestingly, Test House 1 (Table 2) had four zero counts, for both side
725 curtains and side walls. Test House 2 only had 3 counts over 200 CFU and had one zero count for the
726 side wall.

727 The results were unexpected, and troubleshooting was performed to identify any possible shortfalls.
728 A second round was performed, termed Experiment 2 and results for this study is presented in Table
729 3. Surfaces sampled were optimised to smooth, flat surfaces to accommodate the sampling technique.

730

Table 3: Total viable counts (CFU/Contact plate) for two poultry houses that underwent the Virukill® continuous cleaning and disinfection program with sampling from flat surfaces (Experiment 2)

	Test House 1 -Virukill®		Standard House 1-Aldehyde	
	Before Cleaning	**Post Disinfection**	**Before Cleaning**	**Post Disinfection**
Back Left Wall	>200	0	>200	0
Back Right Wall	>200	0	0	0
Back Floor Middle	>200	>200	>200	0
Middle Left Wall	>200	14	0	0
Middle Left Curtain	>200	67	16	0
Middle Right Wall	>200	0	>200	0
Middle Right Curtain	>200	0	0	0
Front Left Wall	>200	0	0	0
Front Right Wall	>200	0	>200	0
Front Floor Middle	>200	>200	>200	0
Mean	200	48.1	101.6	0
	Before Cleaning	**Post Disinfection**	**Before Cleaning**	**Post Disinfection**
Back Left Wall (b)	>200	0	>200	0
Back Right Wall (b)	>200	0	>200	0
Back Floor Middle (b)	>200	>200	>200	0
Middle Left Wall (b)	>200	0	>200	0
Middle Left Curtain (b)	>200	0	>200	0
Middle Right Wall (b)	>200	0	>200	0
Middle Right Curtain (b)	>200	0	>200	0
Front Left Wall (b)	>200	0	>200	0
Front Right Wall (b)	>200	0	>200	0
Front Floor Middle (b)	>200	>200	>200	1
Mean	200	40	200	0.1

Taking the results into account, it had been established that confluent growth would always be observed before cleaning. The Virukill® cleaning program was followed as described, without the aspects applied during the growth cycle to compare the Virukill® cleaning and disinfection against a peracetic acid based disinfectant as well. In addition, the sampling method was changed to consist of 10 floor plates and 10 wall plates for each house after cleaning and after disinfection and the results are presented in Table 4.

741 **Table 4:** Total viable counts (CFU/Contact plate) for two poultry houses that underwent the Virukill®
742 continuous cleaning and disinfection program and a peracetic acid standard cleaning program with
743 sampling from flat surfaces

	Virukill® Cleaning Program		Standard House 1- Peracetic acid	
	After Cleaning – Pre-Rinse	**Post Disinfection**	**After Cleaning – Pre-Rinse**	**Post Disinfection**
Floor 1	>200	42	>200	96
Floor 2	>200	>200	>200	>200
Floor 3	>200	>200	>200	>200
Floor 4	>200	98	>200	156
Floor 5	>200	36	>200	300
Floor 6	>200	>200	>200	192
Floor 7	>200	>200	>200	>200
Floor 8	>200	>200	>200	152
Floor 9	>200	>200	>200	>200
Floor 10	>200	>200	>200	>200
Wall 1	10	13	>200	6
Wall 2	>200	15	>200	11
Wall 3	48	48	>200	44
Wall 4	11	8	>200	4
Wall 5	22	48	>200	100
Wall 6	0	0	>200	10
Wall 7	6	4	>200	21
Wall 8	9	6	>200	32
Wall 9	2	7	>200	13
Wall 10	18	2	>200	24
Mean	116.3	86.35	200	108.05

744

745 The results indicate that Virukill® and the peracetic acid based disinfectants were fairly equal in
746 effectivity on floors based on this technique, while Virukill®l was more effective when applied to
747 smooth, impermeable walls. Interestingly, Virukill® use resulted in a lower mean bacterial CFU counts
748 at the cleaning stage and not only at disinfection.

749

2.3.2 Evaluation of viral load

Table 5: Vir-Check results for two experiments where a standard aldehyde or Virukill® continuous disinfection program was implemented

Experiment 1

House	**Vir-Check Score***	**Relative Score**	**Conclusion**
Trial House 1 - Virukill®	13	Green	C&D was successful
Std. House 1 - Aldehyde	4	Green	C&D was successful
Trial House 2 - Virukill®	38	Yellow	C&D was average
Std. House 2 - Aldehyde	16	Green	C&D was successful

Experiment 2

House	**Vir-Check Score***	**Relative Score**	**Conclusion**
Trial House 1 - Virukill®	4	Green	C&D was successful
Std. House 1 - Aldehyde	51	Yellow	C&D was average
Trial House 2 - Virukill®	22	Green	C&D was successful
Std. House 2 - Aldehyde	21	Green	C&D was successful

* The Vir-Check score is based on a patented formula written by GD Animal Health. A low VIR- check score represents a low exposure to the viruses tested, which is suggestive to a successful C&D procedure, including for the enveloped viruses, gram-positive and gram-negative bacteria as they are more sensitive to disinfection than the tested viruses

The Vir-Check analysis further provided detail on the exact organisms detected.

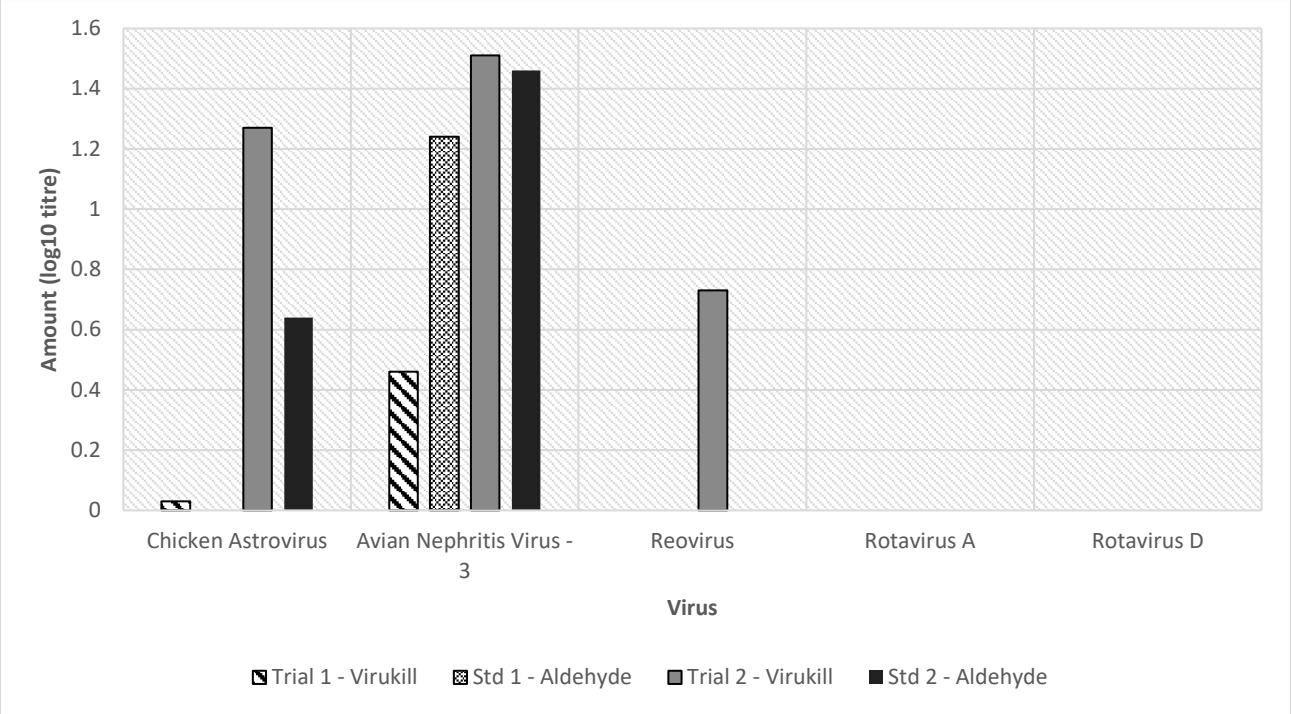


Figure 1: Viruses detected (log10 titre) for houses in Experiment 1 undergoing standard aldehyde disinfection and Virukill® continuous disinfection program

In the first experiment it is evident that the second trial house had the least effective cleaning, regarding virus eradication. The first standard house, conversely, had the most effective cleaning and disinfection schedule. This agrees with the bacterial data obtained (Table 1) as standard house 1 also had the lowest overall bacterial presence after disinfection. In this round the Avian nephritis virus – 3 had the highest load present after disinfection, being found in all four houses post disinfection. The Vir-Check scoring is not influenced by vertical transmission from parent stock birds (GD Animal Health, n.d.) The second trial house is of notable concern as Chicken Astrovirus, Avian Nephritis Virus -3, and Reovirus were all detected in this house.

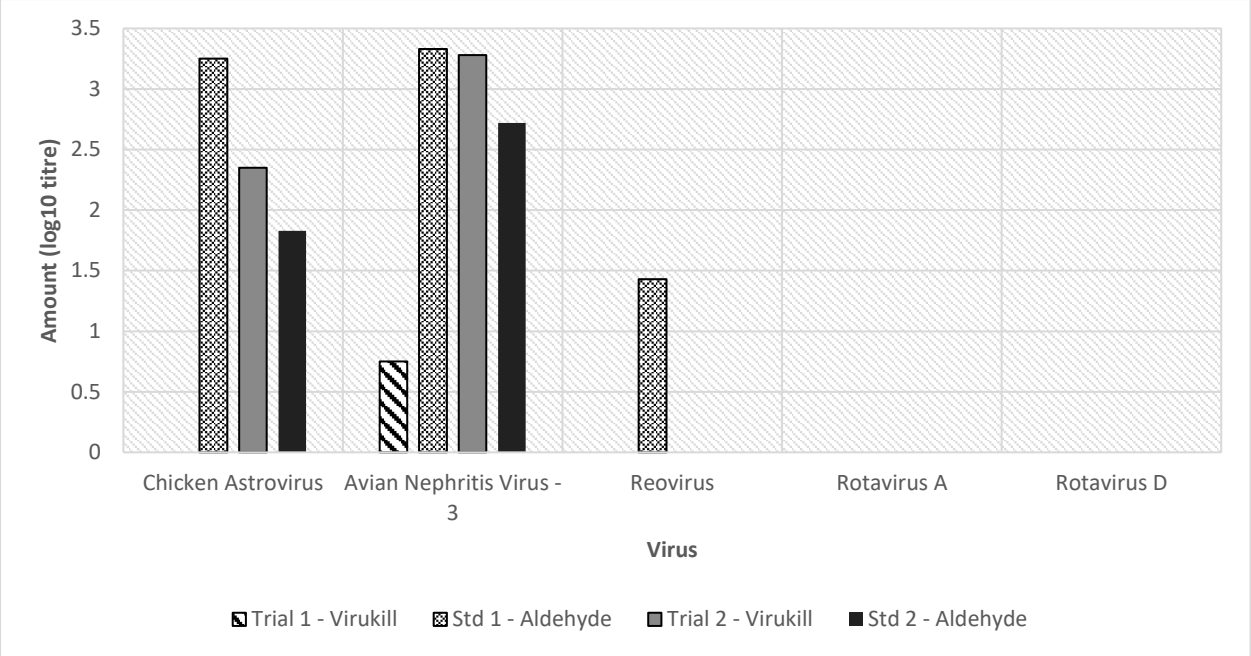


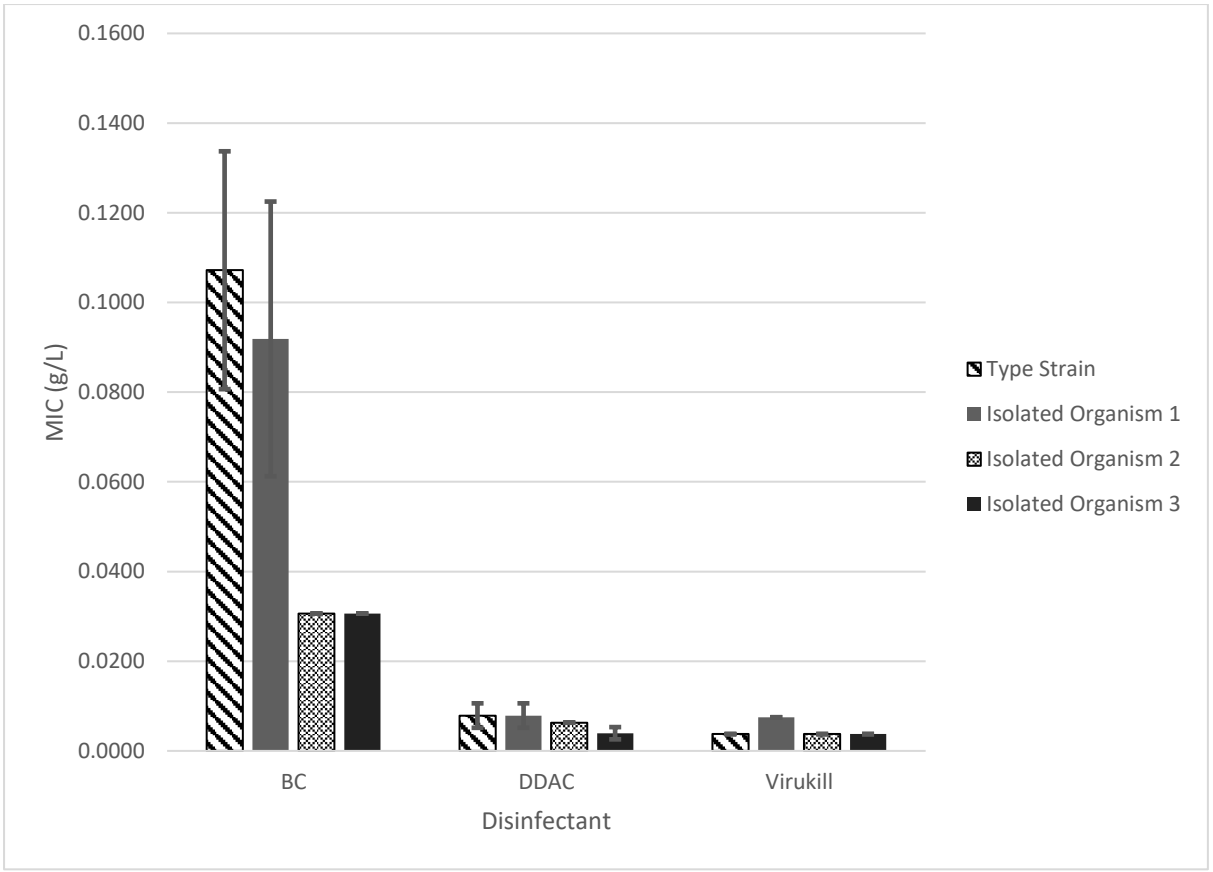
Figure 2: Viruses detected (log10 titre) for houses in Experiment 2 undergoing standard aldehyde disinfection and Virukill® continuous disinfection program

In the second experiment the first trial house had the most effective outcome with regards to virus eradication and the first standard house had the least effective results. This did not agree with the bacterial results in Table 4 as the trial house had the worst bacterial performance of all four houses, while the first standard house showed optimal post-disinfection results. These results strongly suggest that the contact plate method as the sole technique to evaluate cleaning and disinfection efficacy may be flawed.

In experiment two Avian Nephritis Virus -3 was again found in all four houses. Chicken Astrovirus was detected in all but the first trial house and reovirus was only found in the first standard house. The levels of detected virus were also higher overall in the second experiment, compared to the first, suggesting that the cleaning and disinfection in the first experiment was more successful in removing viruses, which is the opposite found in terms of bacteria.

2.3.3 MIC determination

The minimum inhibitory concentration (MIC) of three disinfectants were determined for the *E. coli* ATCC type strain as well as three isolated organisms that survived Virukill® screening at 0.5% exposure.



786

787 **Figure 3:** Minimum inhibitory concentration of four organisms against three QAC disinfectants (n=4),
788 p<=0.05.

789

790 The results indicated that DDAC and Virukill® were significantly more effective than BC at inhibiting
791 the growth of all the organisms. Notably Isolated Organism 2 and Isolated Organism 3 had significantly
792 lower MIC values for BC than the type of strain and Isolated Organism 2. The Virukill® disinfectant had
793 lower MIC values for all organisms apart from Isolated Organism 1, which had the same MIC for DDAC
794 and Virukill®.

795

2.3.4 Isolated organism identification

DNA extraction was performed on the *E.coli* type strain and three isolated organisms that were isolated from plates after Virukill application, in duplicate.

Table 6: NanoDrop DNA measurements after extraction for type strain and three isolated organisms

Sample	Concentration (ng/µL)	A260	A280	260/280
Type Strain (1)	109.53	2.191	1.164	1.88
Isolated Org. 1 (1)	57.22	1.144	0.556	2.06
Isolated Org. 2 (1)	106.84	2.137	1.068	2.00
Isolated Org. 3 (1)	322.28	6.466	3.238	1.99
Type Strain (2)	838.92	16.778	10.010	1.68
Isolated Org. 1 (2)	228.28	4.566	2.289	1.99
Isolated Org. 2 (2)	332.13	6.643	3.377	1.97
Isolated Org. 3 (2)	323.6	6.472	3.257	1.99

The second set of extractions had higher yields and were used in the remainder of the study. The extractions were used for 16S amplification for identification purposes.

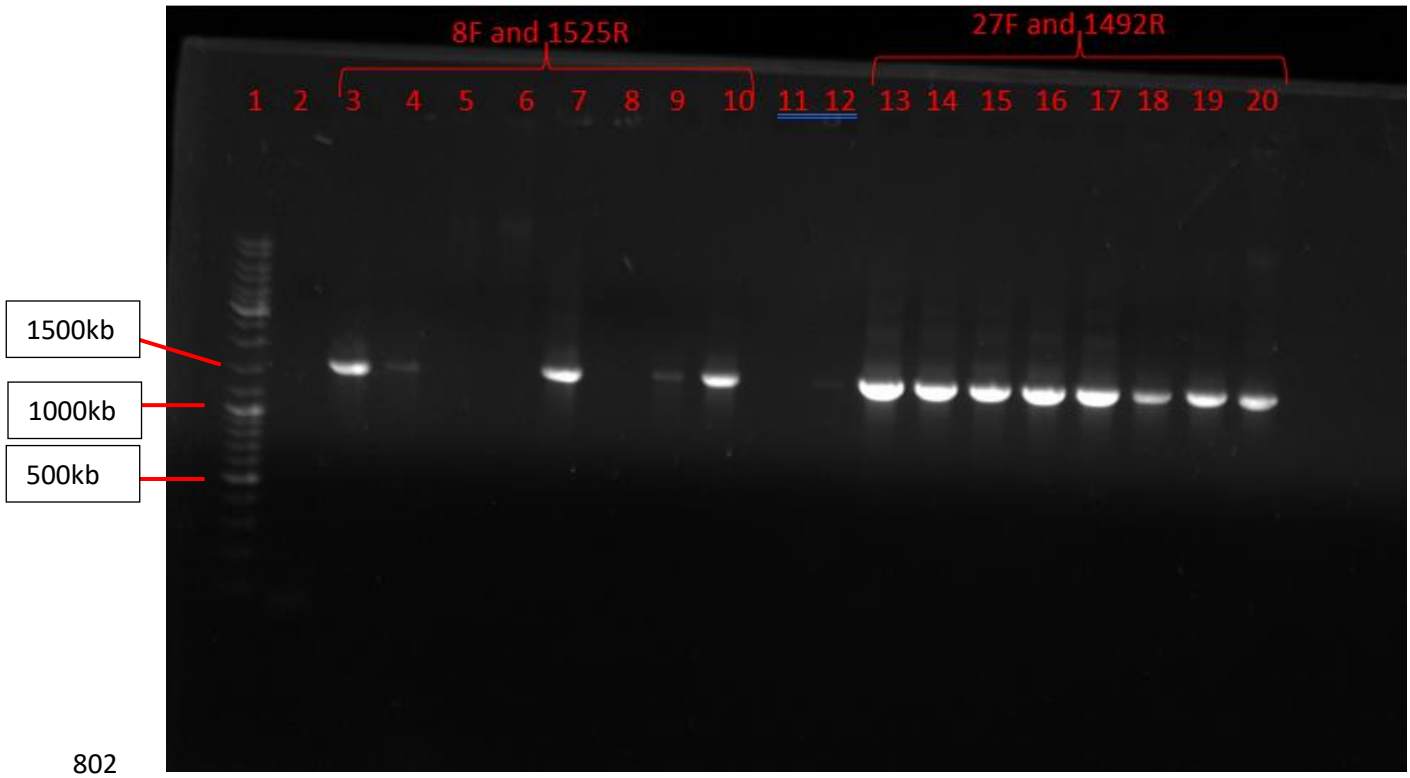


Figure 4: 16S rRNA DNA Amplification with two sets of primers. Lane 1 contains O'GeneRuler DNA Ladder Mix. Lane 3 to 4 contain DNA extracted from Type Strain, Isolated Organism 1-3 in two sets in this order, with 8F and 1525R primers for PCR reaction. Lanes 13-20 contain DNA extracted from Type Strain, Isolated Organism 1-3 in two sets in this order, with 27F and 1429R primers for the PCR reaction.

The samples in lanes 13-16 were selected to process for sequencing. These represent DNA samples for the 16SrRNA genes of *E. coli* ATCC (Lane 13), Isolated Organism 1 (Lane 14), Isolated Organism 2 (Lane 15), and Isolated Organism 3 (Lane 16). These four samples were cleaned up and prepared for sequencing.

Table 7: 16SrRNA genetic identification of three organisms isolated after Virukill® disinfection and screened against 0.5% Virukill® exposure

Query Sequence	Top Hit ID	Pairwise Identity (%)	Identical Sites (%)
Type Strain	*Escherichia coli* strain NBRC 102203	96.1%	95.7%
Isolated Org. 1	*Pantoea agglomerans* strain NBRC 102470	74.5%	69.0%
Isolated Org. 2	*Pantoea agglomerans* strain DSM 3493	73.5%	64.4%
Isolated Org. 3	*Pantoea ananatis* strain LMG 2665 16S	69.4%	62.1%

2.4 Discussion

The trial results of the first experiment suggest that the continuous disinfection plan as it was implemented is not as effective as a standard aldehyde-based cleaning and disinfection program in the eradication of bacteria from the environment. In the first trial the Standard Houses both had lower TVC counts post-disinfection (Table 1 and Table 2). The use of the modified QAC disinfectant was also successful in reducing the bacterial load in the poultry houses. This may indicate that either a higher concentration, flow rate or increased contact time may be necessary to achieve the same effects with a continued disinfection program in comparison with the standard disinfection program. What is evident is that the Virukill® disinfection program may need more fine-tuning and validation prior to implementation to ensure success in a commercial environment. After evaluation of the testing methodology, focus was placed on testing flat surfaces to ensure even distribution and equal comparison. A second round of testing was performed ensuring surfaces were sampled within an hour of disinfectant application. The Virukill® continuous disinfection program performed better in this trial, with significant reduction of aerobic bacteria in all surfaces tested, except for the floors (Table 3). The aldehyde-based disinfectant also reduced aerobic bacterial counts, including the heavily soiled surfaces of the poultry house floor. Differences in cleaning methods will inevitably be introduced as it is a human process. In a third cleaning trial, the Virukill® cleaning program was compared to the standard cleaning program using a peracetic acid-based disinfectant (Table 4). In this trial similar results were obtained with the Virukill® cleaning program resulting in a decrease in the aerobic bacterial counts after cleaning and after disinfection. Comparatively the peracetic acid-based disinfectant performed slightly worse. Both disinfectants resulted in a high level of growth on the contact plates sampled on the floors of the house after disinfection. Therefore, the trials in this study suggest that the aldehyde disinfectant was the most effective at reducing aerobic bacterial load, followed by Virukill®, and lastly the peracetic acid-based disinfectant.

Fate and co-workers (1985) evaluated the bacterial colony reduction of four types of disinfectants in poultry houses. They found the highest reduction from glutaraldehyde, followed, in order, by cresylic acid, iodophors, and a QAC-formaldehyde mixture. It is interesting to note that in this study there was above 200 colonies (too many to count) for all floor samples taken for all compounds. There were also areas where no counts (0 CFU) were found in the house. Burbarelli (2015) applied a program of cleaning and disinfection with a glutaraldehyde and formaldehyde mixture, followed by para-chlor-meta-cresol on walls and floors after complete organic material cleanout. They found that all surfaces tested with exception of the smooth curtains had some bacteria present after disinfection. Even with the higher mean bacterial colonies present, an increased performance was seen with disinfection compared to no disinfection step. Each experimental group for this study was only 30 birds, raising the question on applicability on a commercial scale. A later study by Burberelli (2017) used the same disinfection program with aldehydes and para-chlor-meta-cresol and found a more pronounced decrease in total microorganisms after disinfection compared to just cleaning with a neutral detergent. Burberelli (2017 also did not find complete absence of organisms after disinfection, and repeated the improved performance findings

The current study further provides evidence that complete sterilization of poultry houses is not possible in a real-life environment, corresponding to the previous findings in literature. In this study the Virukill® and peracetic acid disinfectants resulted in over 200 CFU per contact plate on floor samples, which also agrees with previous literature (Burberelli, 2015 & 2017), but is not an ideal situation as potential pathogenic bacteria may survive on floors. Interestingly, this study indicates that the aldehyde-based disinfectant can effectively reduce bacterial levels below detection limit, even on floors. This would make the aldehyde-based disinfectant the most effective of the compounds tested under the conditions of the trial. Several factors may contribute to reduced efficacy of disinfectants, including contact time, application rate, concentration of disinfectant, and the organism being targeted, amongst others (Lin et al., 2020). The effectiveness of QACs can be negatively influenced by the presence of organic material and/or water hardness (Araújo et al., 2013). In this study, the heavily

soiled floors may have impacted the effectiveness of the Virukill®, which would have resulted in the high floor bacterial counts obtained. In addition, the trial conditions called for the use of Virukill® as a detergent and as a disinfectant, therefore there may be discrepancies between the use of a commercial detergent and Virukill® in the removal of organic matter. In addition, the removal of organic matter is a very labour-intensive process, so variation is likely to occur between different houses, however the repetition of trials and the fact that all floor samples were similar per disinfectant, indicates that this was not an issue in this case. Although the manufacturer's specifications were followed, it may be necessary to increase the application rate or concentration for these specific poultry houses to ensure better coverage on the floors during disinfection under specific farm conditions. Finally, the concentration of the disinfectant cannot be determined rapidly on site, this means that if the venturi-based dilution systems, that come standard in the agricultural sector, are inaccurate there is no rapid means of determining the concentration and what needs to be done to remedy the situation. This can result in applying lower than expected concentrations of the disinfectant, which would also reduce the effectiveness of the disinfection process.

When the viral detection method was used, a different picture of cleaning effectiveness emerges. In both experiments the cleaning was at a satisfactory level. In Experiment 1 (Table 5) cleaning and disinfection was successful in three of the four houses, with a slightly lower effectiveness in Experimental House 2. In the second experiment three of the four houses were successfully cleaned and disinfected again, with Standard House 1 using aldehyde-based disinfectants having the poorest results, although it still maintained an average rating. When using bacterial plating methods, the standard aldehyde disinfectant unequivocally performed better than the Virukill® disinfection, however when using viral detection methods, the two methods are equivalent. The Vir-Check score is a score derived from a patented formula by GD animal health based on the detection and viral load determination of 5 non-enveloped viruses in young chicks (GD Animal Health, n.d.). A lower score indicates birds were not exposed to the viruses, therefore the area was cleaned and sanitised well. This a notable result as historically, bacterial methods have been the industry standard for evaluating

892 cleaning effectivity, these results suggest that using viral evaluation methods, such as the Vir-Check
893 system, may be more relevant to the poultry industry. In Chapter 4 this will be discussed further in the
894 context of overall performance.

895 The viruses detected in the Vir-Check system are indicative of overall cleaning as they are non-
896 enveloped and therefore difficult to eliminate completely. However, it must be noted that the
897 organisms being tested for are not major pathogens, but are rather seen as indicator organisms.
898 Infectious Bursal Disease Virus (IBDV) is the causative organism of "Gumboro" disease, or Infectious
899 Bursal Disease (IBD) (Müller e*t al.*, 2003). Infectious Bursal Disease is characterized by lymphoid
900 depletion which ultimately leads to complete destruction of the Bursa of Fabricius, a major immune-
901 related organ in chickens (Müller e*t al.*, 2003). Infectious Bursal Disease IBD results in reduced immune
902 function and increased infections, with increased mortality and reduced performance as the result
903 (Müller e*t al.*, 2003). The IBDV is also a naked virus, with a clearer direct impact on production. One
904 of the shortfalls of the Vir-Check system is that there is no detection on the effective removal of IBDV,
905 or any other major pathogens (such as NCDV or IBV). Work is currently being done to develop a similar
906 molecular method at the University of the Free State. A detection protocol that can evaluate
907 effectiveness similarly to Vir-Check, but with the added benefit of detection of IBDV would be highly
908 beneficial to the poultry industry.

909 Bacterial methods have frequently shown that the complete removal of bacteria from poultry houses
910 is not possible (Burbarelli *et al*., 2015; Burbarelli *et al*., 2017; Fate *et al.*, 1985). One reason for this is
911 the fact that, even when surfaces are completely sanitised, the air in poultry houses still contain
912 diverse bacterial populations (Jiang *et al*., 2018). Moelling and Broecker (2020) evaluated the air
913 microbiome and found that airborne viruses do not constitute a significant portion of the air
914 microbiome, with most of this minority being bacteriophages associated with bacterial populations. It
915 may be possible that bacterial loads in the air within and from around the poultry house may rapidly
916 repopulate surfaces in the poultry house, and that the same risk is not as high for viruses as they do

not occur in as high frequency in the air microbiome. This logically leads to an argument that the effectiveness of cleaning and disinfection of poultry houses should be measured by the effectiveness of removal of viruses from surfaces as primary indicator as this is the highest risk factor to poultry health, instead of the transient removal of bacteria. This is supported by the adoption of break periods wherein no poultry is placed in poultry houses, which results in loss of effectivity of some infectious agents as they will not remain infectious for extended periods (Butcher & Miles, 2012).

The improved performance seen with disinfection may be as a result of a reduction in environmental infection pressure (Burbarelli *et al.*, 2017; Payne *et al.*, 2005). Burbarelli and co-workers (2017) also propose that the reduction in infection pressure may lead to improved balance in the intestinal microbiota of the broilers which ultimately improves performance through improved nutrient uptake. Other authors, such as Collet (2020) have recently emphasised the shift in thinking from a traditional approach of disease being caused by a specific organism which needs to be eradicated, to one of an environment that impacts the health status of the flock in a more holistic sense. Jiang and co-workers (2018) compared five disinfectants' (ozone, available chlorine, QAC-Salt, Glutaraldehyde and a mixture of aldehydes-QAC-alcohol) effects on microbial communities in poultry houses and found when disinfectants are used there is a significant decrease in airborne aerobic bacteria compared to houses where disinfectant is not used. The mixed disinfectant had the largest reduction in bacterial concentration, followed by glutaraldehyde, QAC-Salt, ozone, and finally available chlorine. This would suggest a benefit to diverse disinfectant types in reduction of bacteria in poultry houses. Jiang and co-workers (2018) also found that the use of disinfectants reduced the number of detected phyla with a total of 32 phyla detected with no disinfectant and 21 for ozone, 27 for available chlorine, 28 for QAC-Salt, 17 for glutaraldehyde, and only 6 for the mixed disinfectant. The distribution of bacterial genera was also affected by disinfection with a reduction in *Escherichia-Shigella, Bacillus,* and *Pseudomonas* genera known for opportunistic pathogens (Jiang *et al.*, 2018). A benefit to disinfection therefore arises in the reduction of bacterial diversity and specifically, pathogen reduction in the growth environment.

Current evidence suggests that some poultry pathogens have the potential to harbour multidrug resistance (MDR), including antibiotic and disinfectant resistance genes on plasmids (Johnson *et al*., 2012; Newman *et al*., 2021). It is also known that the use of Benzalkonium Chloride exposure co-selects for antibiotic and disinfectant resistance (Kim *et al*., 2018), especially when organisms are exposed to these disinfectants in non-optimal conditions. The use of QAC's in poultry house environments with high organic matter content may reduce its efficacy (Lin *et al*., 2020), which could result in selection of antibiotic and disinfectant resistant strains. In this study no clear evidence was observed for disinfectant resistance development, although the study was limited in its scope and duration and this needs further research. The results suggest that longer chain QACs are less conducive to disinfectant resistance development, or that disinfectant resistance may be more effective against the first generation QACs. The use of QACs must be carefully considered and further research is needed into the development and causes of disinfectant resistance in production environments.

The organisms isolated in this experiment were identified as *Pantoea agglomerans* and *Pantoea ananatis* species. However, the pairwise identity was not high enough to guarantee the identity of the organisms. In addition, the 16SrRNA of *Pantoea* is highly conserved and therefore differing between *Pantoea agglomerans* and *Pantoea ananatis* species is unreliable (Coutinho & Venter, 2009). *Pantoea* species are ubiquitous and are most widely known as a plant pathogen, but *P. ananatis* has also been identified as being able to occupy many diverse ecological niches, including as a human pathogen (Coutinho & Venter, 2009). This study therefore indicates that there is a possibility of resistance developing in organisms in the environment, and that these organisms could evade disinfectants applied during the disinfection process. Selective pressures will inevitably result in resistant organisms becoming the dominant organism in the area and could result in spread of resistance through horizontal gene transfer (Apata, 2009; Hedman et al., 2020).

2.5 Conclusions

In this first experimental chapter, an alternative cleaning method namely a continued disinfection program using Virukill was implemented on an commercial broiler farm. The effectiveness of cleaning was evaluated by contact plate method and using the Vir-Check viral evaluation method. During comparison using bacterial methods, aldehyde-based disinfectants were most successful and had the highest reduction in bacterial counts after disinfection. Interestingly, the Virukill® cleaning and disinfection only had bacterial counts on the heavily soiled floor surfaces, indicating flow rates or concentrations may need to be adjusted to achieve the desired effects. When comparing by viral methods, both cleaning methods had similar results and equal efficacy. This indicates that Virukill® is a reasonable alternative for broiler house disinfection. The use thereof should be trialled and troubleshot to ensure correct disinfection is achieved. In addition, there is a growing concern of disinfectant resistanceso the use of disinfectants must be used cautiously and warrants further investigation. With cleaning and disinfection efficacy established the full continuous disinfection program needed to be evaluated to determine the influence on production performance.

Chapter 3: Performance of commercial broilers undergoing a continuous disinfection program

3.1 Introduction

The ultimate goal of any poultry producer is to produce the largest amount of usable end product at the lowest cost. In poultry production, feed alone accounts for up to 70% of costs (Yi *et al.*, 2018). Therefore, the ability to convert feed into body weight is most likely the biggest determinant of profitability. This metric is called the feed conversion ratio (FCR) and is widely applied to assess the growth performance of poultry (Astral, 2019). However, the FCR does not account for the dynamic array of influences in the production environment. In addition to FCR, the mortality metric, *i.e.* how many birds have died as a proportion of the flock, is widely used as a general indicator of health and performance. To account for the loss of birds through mortality and different ages at slaughter in a single comparable value the performance efficiency factor (PEF) is commonly used (Astral, 2019).

. Historically poultry performance and health were treated as separate matters, where diseases were only treated when clear morbidity or mortality was observed. Collet (2020) points out that there has been a shift from treating individual animals for specific known diseases, to a recognition and constant management of diseases whether they are pronounced or subclinical. There is an increased emphasis on flock health instead of only focusing on the individual causative organisms of disease through complete eradication (Blas & Fairhurst, 2021; Collet, 2020). In addition, focus is being placed on the interrelatedness of flock health management and economic performance as well as welfare, quality, and even wider global implications like antimicrobial resistance (Collet, 2020).

Collet (2020) states that modern flock health management focuses on avoiding the inadequate immune response, while also preventing inappropriate immune responses that increase stress and

reduce performance. This includes a focus on allostasis, or the ability to adapt to changing circumstances with an appropriate response by balancing energy to certain physiological processes (Blas & Fairhurst, 2021). The ability to resist disease while also not exceeding the limit where the immune response reduces productivity is known as resilience (Collet, 2020). The resilience will entirely depend on the baseline stress levels experienced by the flock, as increased stress lowers the threshold of resiliency (Collet, 2020). The performance outcome of this is known as uniformity. Where health is directly related to the level of variation within a particular flock.

A potential useful tool in poultry production would be a compound that can ease the stress in the growing environment, partly by reducing pathogen load during the growth cycle. A known risk becomes apparent with a lifecycle approach. In the intensive growth conditions of broilers, with shared space and large populations, more opportunities for spread of these pathogens will become available. Importantly, Collet (2020) notes that modern management programs are focused on obtaining a tolerable level of disease instead of complete eradication, with the exception of the most damaging, and often notifiable pathogens, such as Avian Influenza.

Literature on cleaning and disinfection in field trials for commercial broiler production systems are sparse (Gosling, 2018). Even rarer are the cleaning and disinfection studies that pertain specifically to broiler production and the performance of the broilers (Bragg & Plumstead, 2003; Burbarelli *et al*., 2015; Burbarelli *et al*., 2017; Luyckx *et al*., 2014; Mituniewicz *et al*., 2008). Mituniewicz and co-workers (2008) compared three different litter treatments aimed at reducing bacterial load before and during growth and found improved performance with two of the three compounds tested. Burbarelli and co-workers (2015,2017) found improved performance in broilers after application of a cleaning and disinfection program. Bragg and Plumstead (2003) performed a continuous disinfection program where houses were cleaned and disinfected with the same non-toxic compound was used to treat the drinking water and was used as a spray during growth. They reported an improved FCR, increased weight, and reduced mortality with the continuous disinfection program under experimental

conditions (Bragg & Plumstead, 2003). A field trial was started and had promising results but was halted due to outside influences when a NCD outbreak occurred (Bragg & Plumstead, 2003).

The aim of this experimental chapter is to evaluate the effects of the continuous disinfection program described in Chapter 2 on the performance of broilers during growth on a commercial scale. The performance data in terms of body weight, mortality, and feed consumption were recorded over the growth period of 31-34 days to assess the difference between a standard program and the continuous disinfection program. Thereafter, the performance metrics of FCR and PEF will be calculated and compared to assess whether the use of a continuous disinfection program improves the performance of a commercial broiler operation.

3.2 Material and methods

3.2.1 Area of study

A commercial trial was set up as described in Chapter 2. In short, four 1800 m^2 poultry houses were subjected to a field trial whereby two were cleaned and disinfected according to the Virukill® continuous disinfection program as described by Bragg and Plumstead (2003) and two were subjected only to a standard cleaning and disinfection program.

3.2.2 Continuous disinfection

After placement of birds the continuous disinfection was implemented as follows: the birds in the trial houses were sprayed with a 1% Virukill® solution using a Stihl mist blower at certain intervals. In Experiment 1 the following was done: Trial House 1 was sprayed on day 1 after placement, 5, 8, 11 ,20 and 24 at an average rate of 5.92 mL per m^2 or 0.26 mL per bird. Spray days were chosen to accommodate at least two sprays per week, except for during the vaccination window of day 12-18, and where any house was vaccinated no sprays would be done on the same day as the same equipment is used for both processes. Trial house 2 was sprayed on day 1, 4, 7, 16, 22 at an average

rate of 5 mL per m^2 or 0.21 mL per bird. In Experiment 2 the following was done: Trial House 1 was sprayed on day 3, 7, 11, 16, 20, 25, and 27. Trial House 2 was sprayed on day 3, 7, 11, 16, 21, and 23. All sprays were done at a rate of 5.56 mL per m^2 or 0.24 mL per bird.

In addition, in both experiments, daily dilutions of Virukill® stock solution of 100 mL in 20 L were made and dosed at 2% proportionally into the water supply to apply at 100 ppm as per manufacturers' instructions. Foot baths at trial house entrances were filled with a 2% Virukill® solution and replaced as they became dirty and at a minimum every Monday, Wednesday, and Friday

To evaluate the effects of the continuous disinfection on production parameters, measurements were taken of weight and feeding rates. Daily mortality runs were performed to remove mortalities and perform necessary culling. Mortalities were also recorded.

3.2.3 Performance parameters

FCR was calculated by using the formula: $FCR = \frac{Feed\ consumed(kg)}{Body\ Weight\ gain\ \ (kg)\ at\ end\ of\ study}$

PEF was calculated using the formula: $PEF = \frac{\%\ Liveability\ x\ Ave\ Body\ Weight\ (kg)\ x\ 100}{FCR\ x\ trial\ duration\ (days)}$

3.2.4 Statistical analysis

Performance parameters were compared using the t-Test: paired sample for means with an alpha value of 0.05 using Microsoft Excel Data Analysis ToolPak. The trials were sorted into repetitions of the house that they represent, e.g. House 1. These were compared between Standard and Trial houses.

3.3 Results

3.3.1 House grouping

The study was done over two separate, non-consecutive growth cycles. The two growth cycles are denoted as Experiment 1 and Experiment 2.

The first pair of houses were denoted Trial House 1 and Standard House 1 and will be treated as a pair for the entirety of the study as they had a common feed supply and similar placement and slaughter periods.

3.3.2 Cumulative mortality

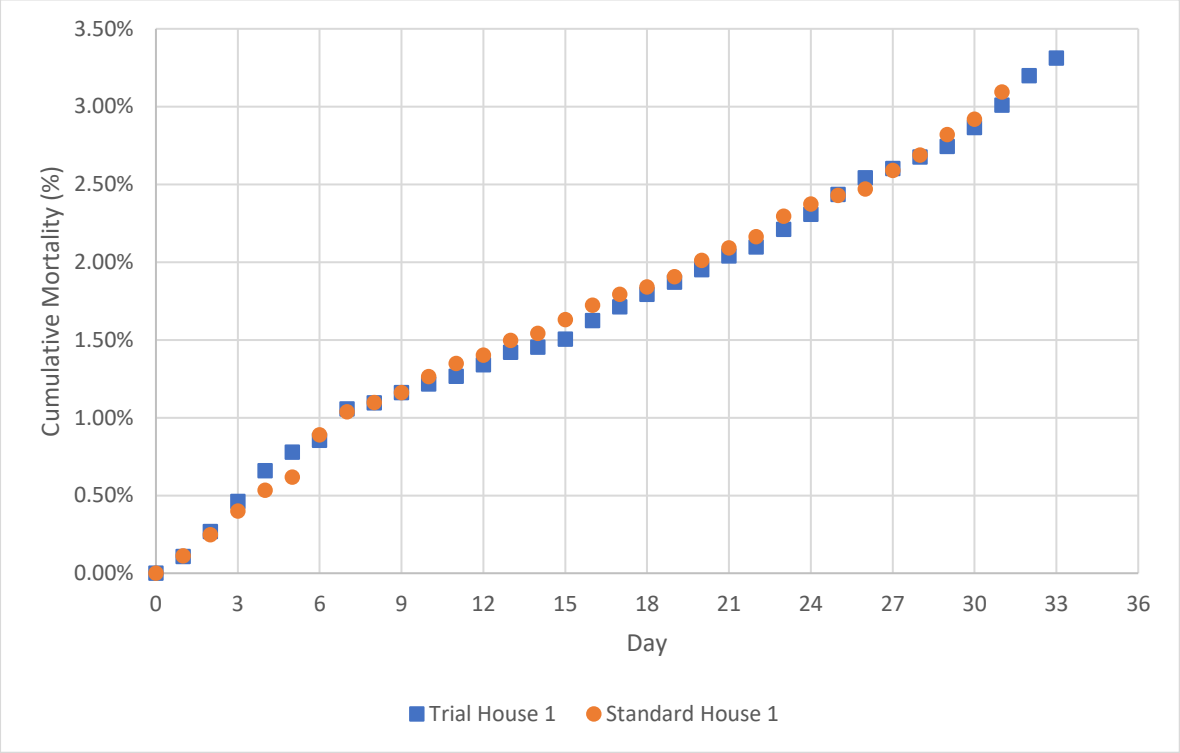


Figure 5: Cumulative mortality over the growth period for Trial House 1 and Standard House 1 in Experiment 1

In the first experiment the cumulative mortality for Standard House 1 was higher at day 31, the final day of direct comparison with 3.10% compared to the Trial House cumulative mortality of 3.01%. For the growth cycle, however, Trial House 1 had a higher cumulative mortality at 3.31% compared to 3.10% for Standard House 1. In the second set, Trial House 2 had a lower cumulative mortality at 33 days with 5.78% compared to 8.51%. The end of growth cycle cumulative mortality for Trial House 2 was notably smaller, at only 6.03% compared to the 8.51% of Standard House 2.

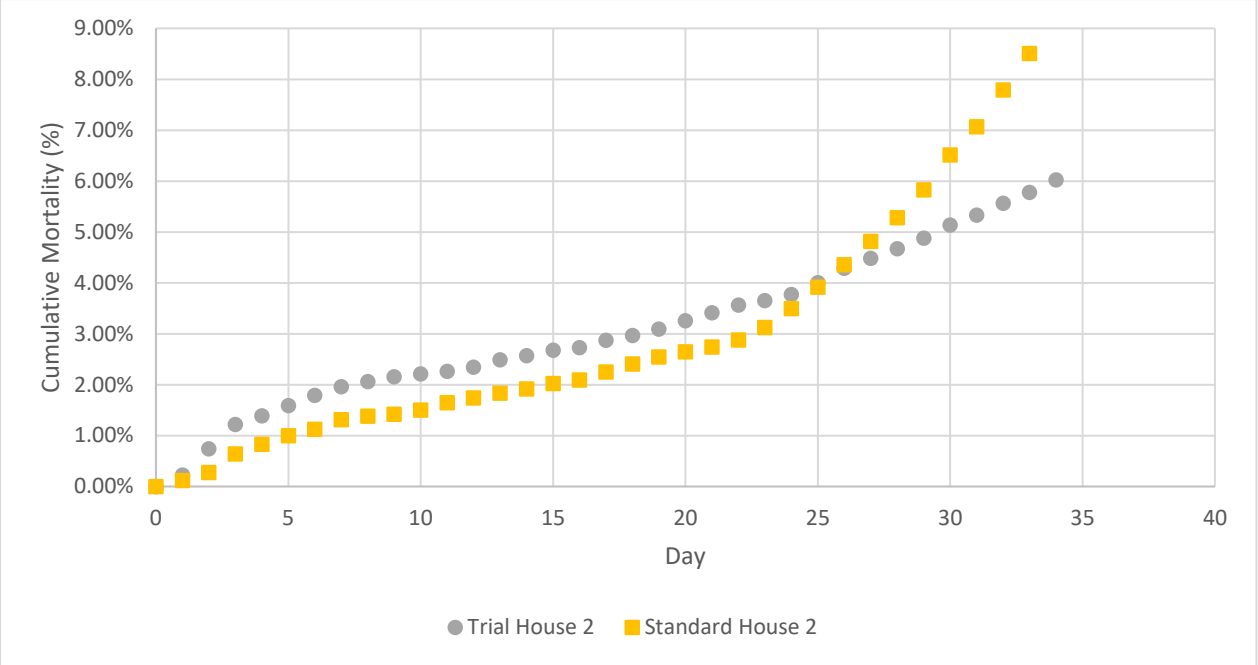


Figure 6: Cumulative mortality over the growth period for Trial House 2 and Standard House 2 in Experiment 1

The same pairing convention was used in the second Experiment.

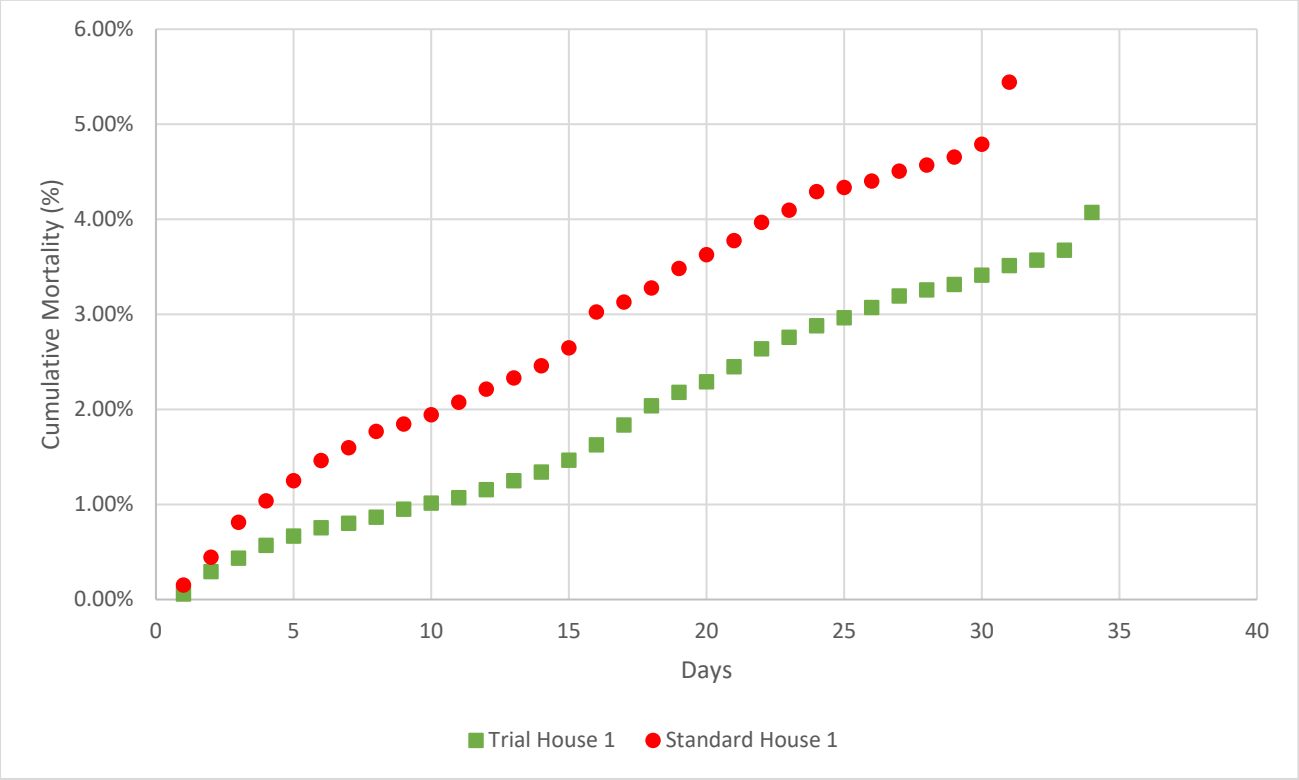


Figure 7: Cumulative mortality over the growth period for Trial House 1 and Standard House 1 in Experiment 2

In the second cycle tested, Trial House 1 had the lowest cumulative mortality, with an end mortality rate of 4.07%. Standard house 1 and Standard House 2 had similar results with 5.44% and 5.81% mortality, respectively. The second Trial House had the highest cumulative mortality of this cycle at 8.3%. It should be noted that this house had particularly high mortality between day 2 and 5 of the growth cycle. The mortality profiles in this cycle were more gradual than the those found in Experiment 1, suggesting that there was not as much of a late pressure.

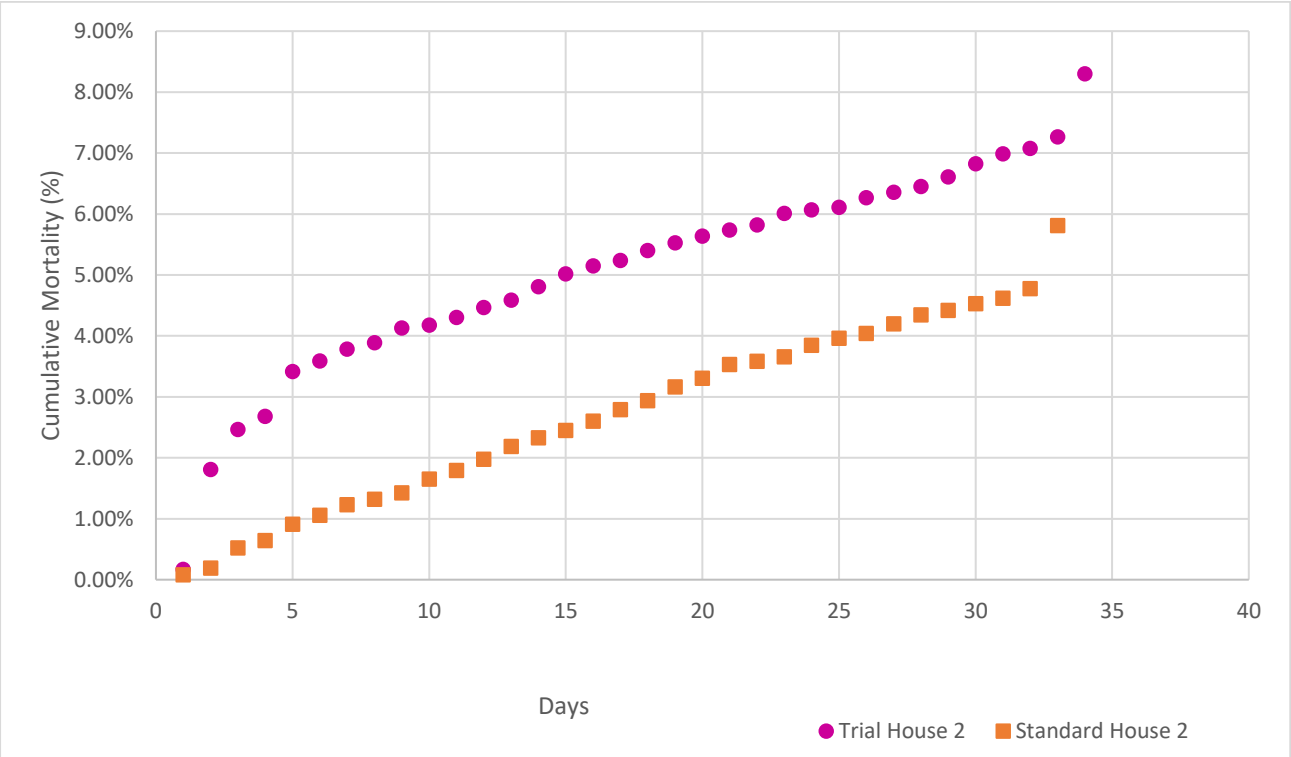


Figure 8: Cumulative mortality over the growth period for Trial House 2 and Standard House 2 in Experiment 2

Due to the notable high early mortality, the daily mortality profiles may be better to illustrate the different mortality profiles in the two experiments.

3.3.3 Daily mortality profiles

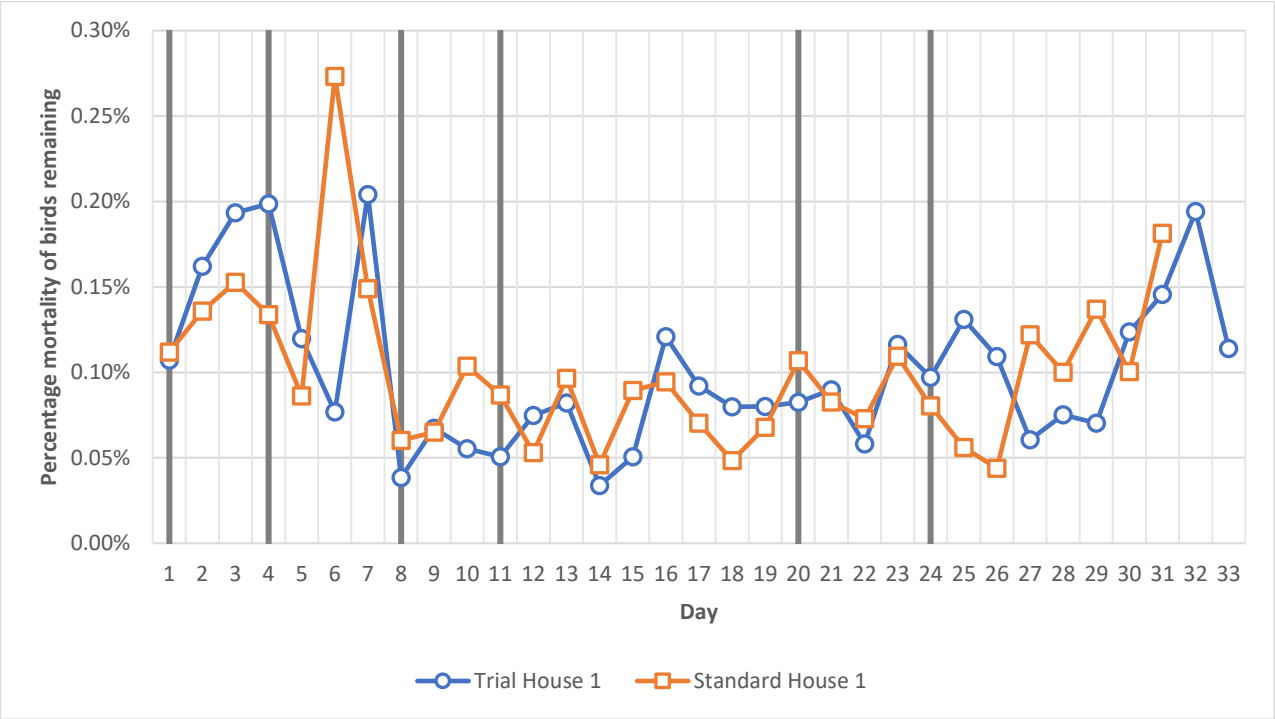


Figure 9: Daily mortality profile over the growth period for Trial House 1 and Standard House 1 in Experiment 1 – lines denote days when Virukill® sprays were performed

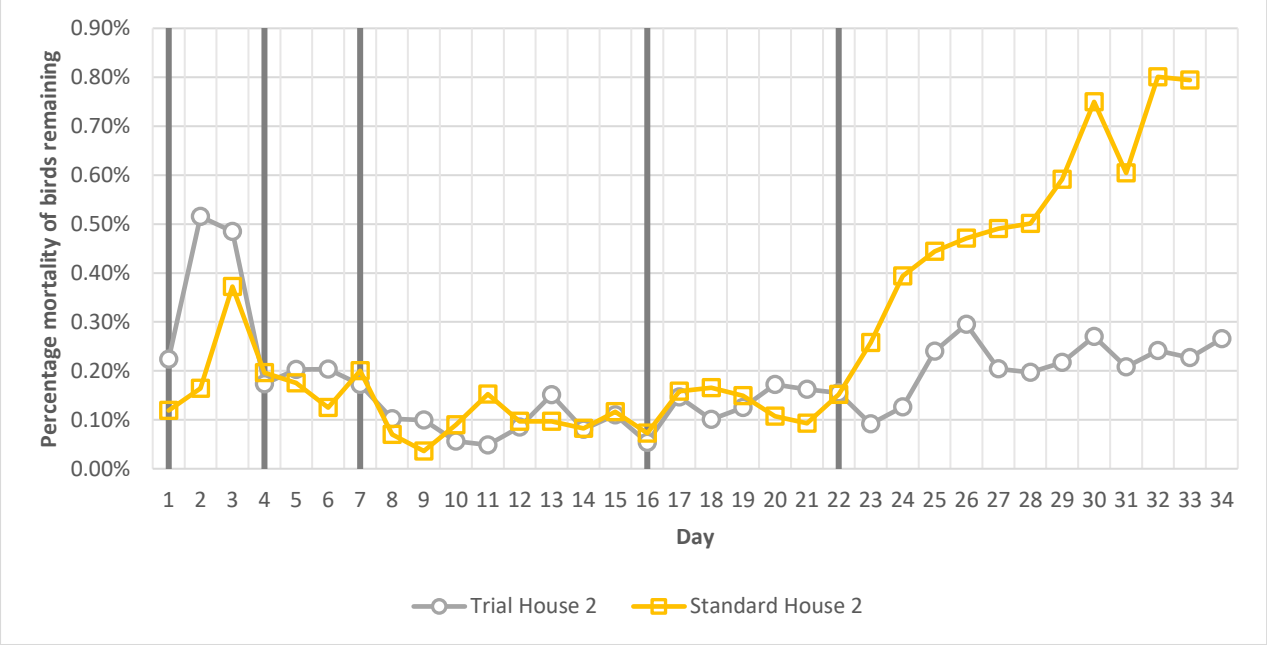


Figure 10: Daily mortality profile over the growth period for Trial House 2 and Standard House 2 in Experiment 1 – lines denote days when Virukill® sprays were performed

In experiment 1 Trial House 1 and Standard House 1 had similar percentage mortality profiles per day for the growth period. Interestingly, at day 4, 8, 20, and 24 a decrease in mortality was seen within

two days of the spray application. In the second set of houses there is a stark difference between the late mortalities from day 23 onwards, with the standard house increasing rapidly compared to Trial House 2.

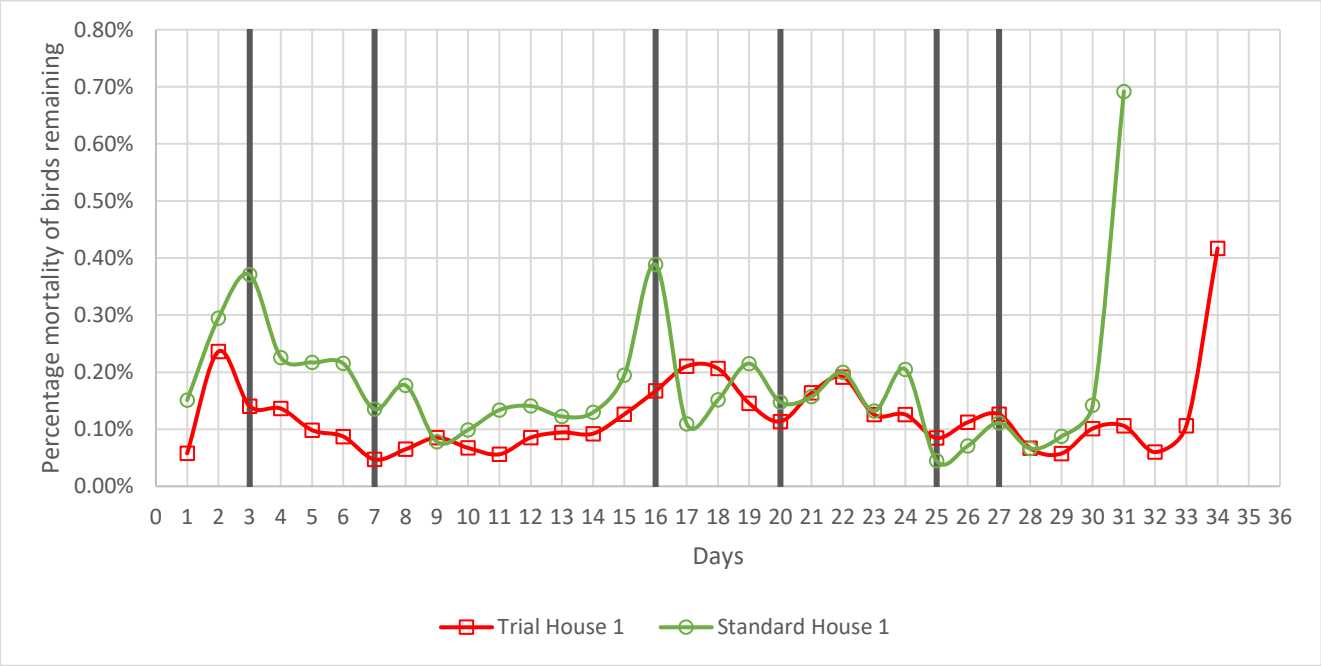


Figure 11: Daily mortality profile over the growth period for Trial House 1 and Standard House 1 in Experiment 2 – lines denote days when Virukill® sprays were performed

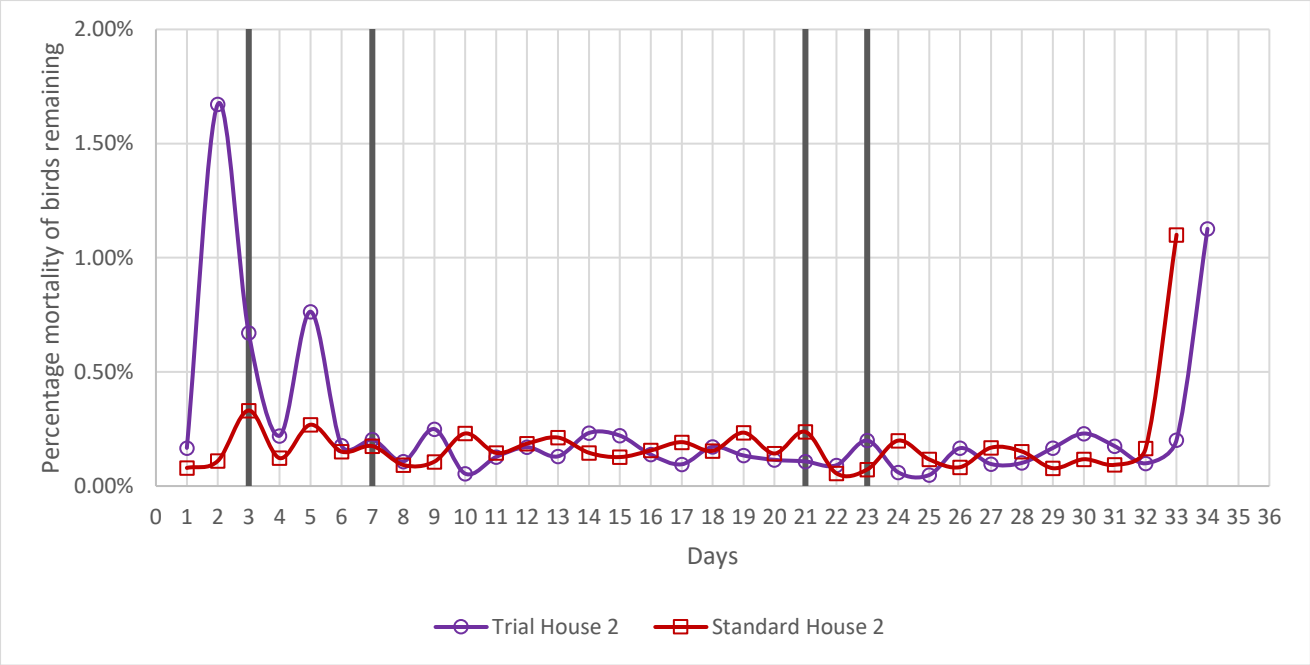


Figure 12: Daily mortality profile over the growth period for Trial House 2 and Standard House 2 in Experiment 2 – lines denote days when Virukill® sprays were performed

In the second experiment the first set of houses had a similar mortality profile to the first experiment. The percentage mortality of living birds remained below 0.4% of the population (Figure 12) up to the slaughter stage, which introduces intense stress and results in culling of the last birds that are not suited for slaughter. The second set of houses in experiment two had a much different profile. In this group there is a very large spike in early mortality (before day 7) followed by mortality rates below 0.4% throughout the growth period. Interestingly, in this experiment the gradual increase after day 20 was not seen in any of the houses.

3.3.4 Growth curves

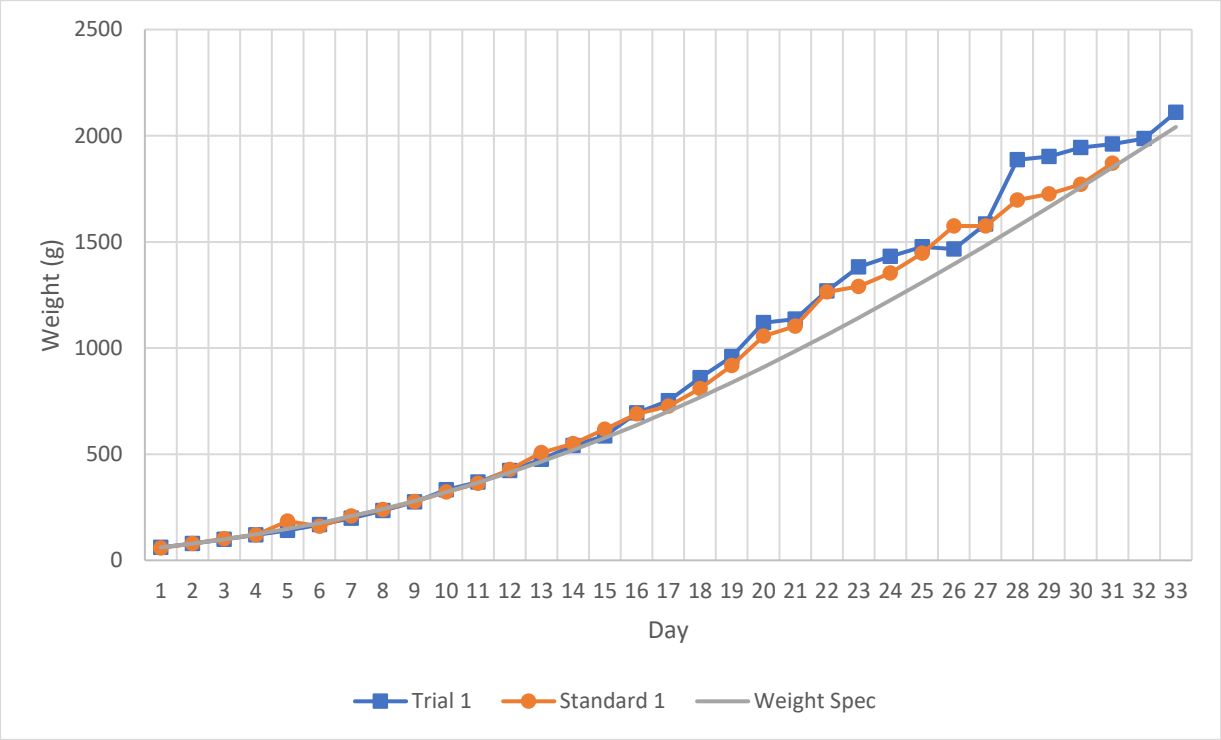
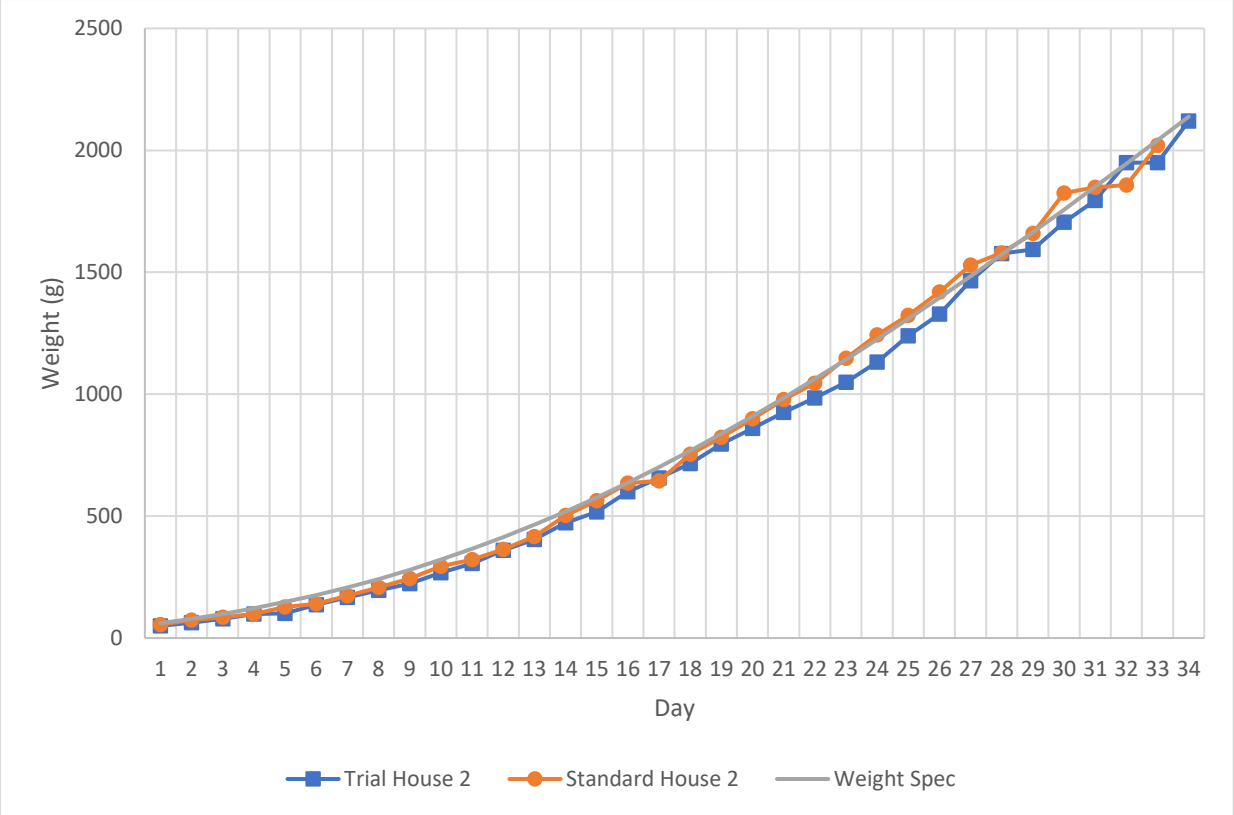


Figure 13: Growth curve of birds in Trial House 1 and Standard House 1 in grams for the growth cycle in Experiment 1

The pair had similar starting weights, and Standard House 1 had a higher live body weight at day 7 and day 14. However, daily gain increased for Trial House 1 and it had higher end weight at 30 days and therefore end of growth. Both houses achieved higher final body weights than the Aviagen Ross standard.



1152

1153 **Figure 14:** Growth curve of birds in Trial House 2 and Standard House 2 in grams for the growth cycle
1154 in Experiment 1

1155 Trial House 2 had a lower average body weight on day 33 than the standard house, however

1156 performance at end of cycle for Experiment 2 was closer to the Aviagen Ross Standard as it was 18

1157 grams below the standard compared to 21 grams below for Standard House 2.

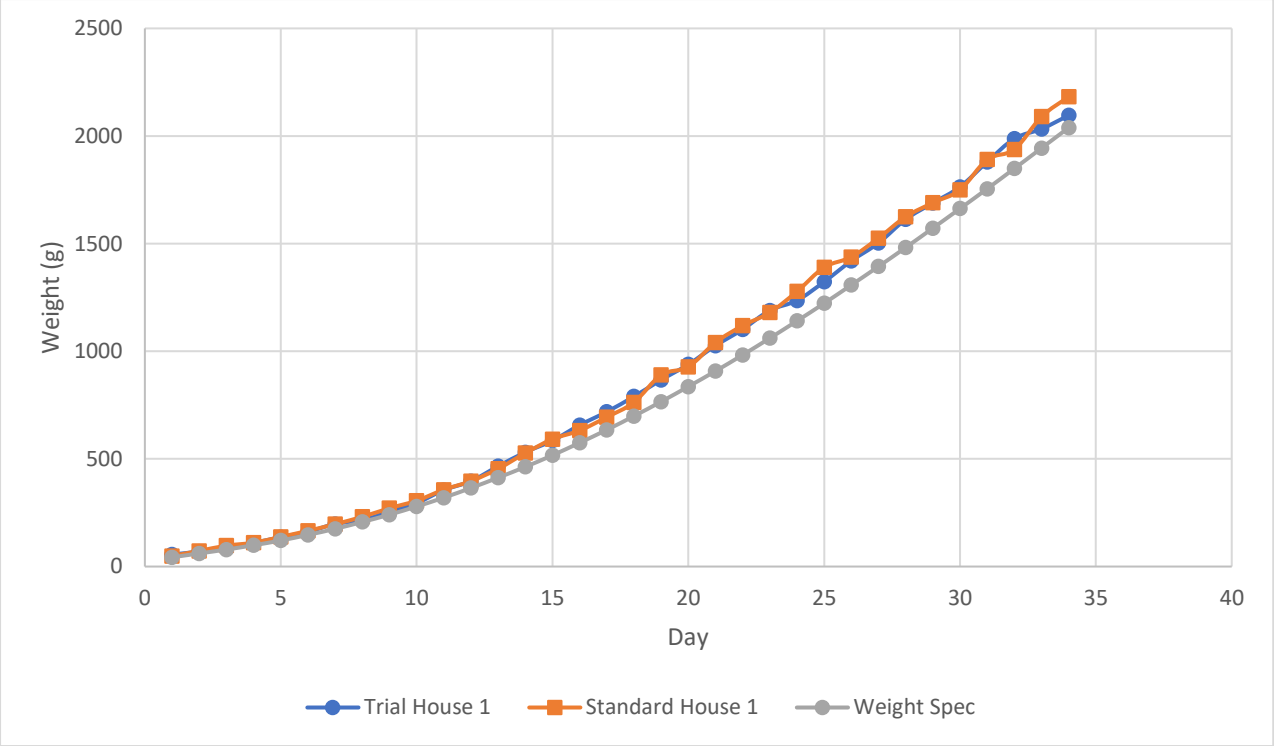


Figure 15: Growth curve of birds in Trial House 1 and Standard House 1 in grams for the growth cycle in Experiment 2

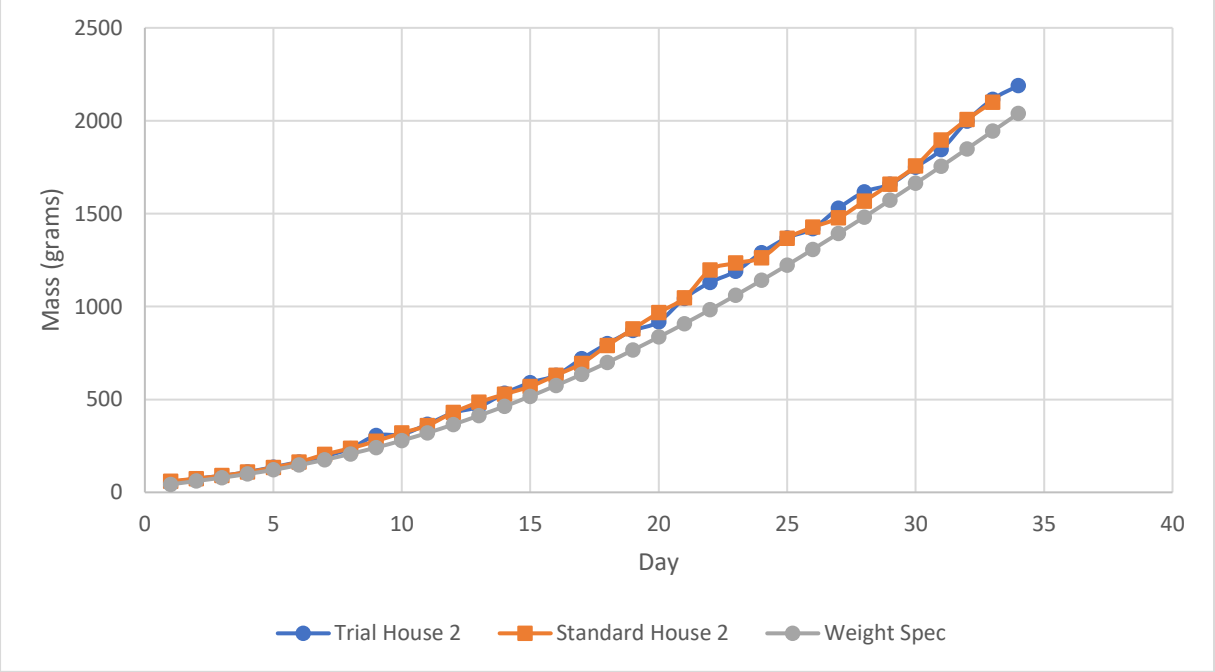


Figure 16: Growth curve of birds in Trial House 2 and Standard House 2 in grams for the growth cycle in Experiment 2

In Experiment 2, Trial House 1 and Standard House 1 both had higher end weights than the Aviagen Ross standard. Trial House 2 and Standard House 2 also outperformed the industry standard.

3.3.5 Performance parameters

FCR was calculated by using the formula: $FCR = \frac{Feed\ consumed\ (kg)}{Weight\ gained\ (kg) at\ end\ of\ study}$

PEF was calculated using the formula: $PEF = \frac{\%\ Liveability\ x\ Ave\ Body\ Weight\ (kg)\ x\ 100}{FCR\ x\ trial\ duration\ (days)}$

Table 8: Performance parameters of poultry houses undergoing standard and continuous disinfection programs

	Experiment 1			
	Trial House 1	Standard House 1	Trial House 2	Standard House 2
Mortality (%) (at end)	3.48%	3.19%	6.12%	9.10%
FCR	1.48	1.44	1.54	1.58
PEF	406.97	408.75	359.41	352.21
	Experiment 2			
	Trial House 1	Standard House 1	Trial House 2	Standard House 2
Mortality (%) (at end)	3.69%	6.45%	8.40%	5.86%
FCR	1.40	1.57	1.49	1.53
PEF	426.08	362.35	394.81	388.89

In the first cycle tested, Experiment 1, Standard House 1 has a lower end mortality, however this house was slaughtered younger than Trial House 1. At day 31, the slaughter age of Standard House 1, Trial House 1 had a lower mortality of 3.01%. Trial House 2 had a much lower end mortality than Trial House 2, even though they were slaughtered later. The FCR for Standard House 1 was the best of all four houses during experiment 1. Trial House 2 outperformed Standard House 2 in FCR and PEF as seen from findings in Table 8. This phenomenon was not observed with the first set of houses, and many other factors could have contributed to these specific findings

In Experiment 2, the first trial house had markedly lower end mortality than the standard house. However, the second trial house had a much higher end mortality than the second standard house. The feed conversion ratios of both Trial Houses were better.

Table 9: Mortality comparison of 7 day and 14 days for both experiments

Experiment 1				
	Trial 1	Std 1	Trial 2	Std 2
First Week Mortality	1.06%	1.04%	1.96%	1.31%
Target (Heier *et al*., 2002)	1.54%	1.54%	1.54%	1.54%
Cumulative Mortality	3.48%	3.19%	6.12%	9.10%
Target (Heier *et al*., 2002)	3.31%	3.21%	3.36%	3.33%
Experiment 2				
	Trial 1	Std 1	Trial 2	Std 2
First Week Mortality	0.80%	1.59%	3.78%	1.23%
Target (Heier *et al*., 2002)	1.54%	1.54%	1.54%	1.54%
Cumulative mortality	3.69%	6.45%	8.40%	5.86%
Target (Heier *et al*., 2002)	3.39%	3.39%	3.39%	3.34%

3.4 Discussion

In this study two pairs of broiler production houses were subjected to a field trial to compare a novel disinfection programme against a standard disinfection programme as applied in industry. Gosling, (2018) is of the opinion that studies of this nature are usually avoided due to the vast array of variables that are uncontrolled in the environment. Nevertheless, this is the closest to the actual environment in which the information is applied, therefore these field trials are highly valuable to the farmer. It should be noted at this point that all houses in this trial had no antibiotic administration and are grown by a producer that uses no routine antibiotics. The only medication administered are routine chemically synthesised coccidiostats in the diet, and Newcastle Disease Virus, Infectious Bursal Disease Virus and Infectious Bronchitis Virus vaccines.

First week mortality is an essential measure to indicate quality of chicks and sets the tone for the production cycle (Yassin *et al*., 2009). The normal seven day mortality has been shown to be around 1.4%, and for raised without antibiotics (RWA) or no antibiotics ever (NWA) broilers this may increase by 0.5 - 1% (Smith *et al.*, 2020). Other estimates have indicated that 7 day mortality should be around 1.54% and 0.48% per week for the remainder of the growth cycle (Heier *et al*., 2002). Yassin and co-

workers (2009) found an average first week mortality of 1.5%. In Experiment 1 all houses, except Trial House 2 had first week mortality lower than the target, however Trial House 2 had much lower end mortality than Standard House 2 in this experiment (Table 9). The cumulative mortality in Standard House 2 was higher than Trial House 2, and the increase in this set was due to late mortality, after 20 days. The house on continuous disinfection in this case had a lower end mortality. In the Experiment 2 the same circumstances were observed, where Trial House 2 had the highest first week mortality. This was especially high at 3.78%, indicating some major event may have contributed to the increase in mortalities. This could be house related, or may be due to flock issues, hatchery issues, or transport related (Heier *et al*., 2002; Yassin *et al*., 2009). Early mortality is a difficult barrier to overcome, and it is generally assumed that early mortality is not primarily caused by infection pressures within a chicken house, but rather more significantly flock and hatchery related (Yassin *et al*., 2009). Trial House 1, in the second experiment, had a much lower end mortality compared to Standard House 1. It is notable that all houses represented in this work have no routine antibiotics and are being compared against standards where antibiotic use is not specified. In addition, these results suggest that the use of a continuous disinfection program may be beneficial in reducing late mortality in broiler houses, consequently reducing cumulative mortality. When testing the Virukill® continuous disinfection program in a controlled trial, Bragg and Plumstead (2003) found reduced cumulative mortality compared to pre-disinfection and pens with no disinfection treatment, the decrease in mortality also being after seven days.

In the poultry house, broilers are subjected to various stressors, such as high or low temperatures, handling, lack of feed, competition, and pathogens (Blas & Fairhurst, 2021; Siegel, 1995). Recent attention has been focused on how stress affects the overall performance, instead of focusing on specific etiological agents alone. The house itself can be seen as a disease determinant due to the host of conditions and factors it may introduce to the population (Collet, 2020).

Stress in poultry is stimulated by environmental factors which can be sensed by the Hypothalamus-Pituitary-Adrenal (HPA), Sympathetic-Adrenergic (SA), or Microbiota-Gut-Brain (MGB) systems, which in turn induce physiological responses to attempt to return the bird to an allostatic state (Nazar & Estevez, 2022; Oakley *et al*., 2014). The characteristic stress response in poultry is activation of the HPA axis and release of corticosteroids (CS) (Siegel, 1995). Corticosteroids (CS) exert a wide range of effects on birds associated with long term stress, these include modification of nutrient uptake through gastrointestinal lesions and alteration of the normal immune response. Metabolic shifting attributed to the stress response includes depletion of muscle weight and increased urine flow and subsequent increased water uptake, these together lead to reduced feed intake (Siegel, 1995). Damage to the intestinal lining also contributes to weight loss. The stress response also alters the immune response of poultry. CS performs an anti-inflammatory role and is associated with a reduction in circulating antibodies. Stress can therefore increase disease susceptibility in broilers (Siegel, 1995). Stress also reduces inflammation, which may be beneficial in non-specific inflammatory responses, but would be highly dangerous when localised inflammation is needed to halt disease spread (Siegel, 1995). Stress is therefore a major determinant in the growth of broilers and will greatly influence their performance. Newer research indicates that the MGB axis also plays a major role in resilience and the manifestation of stress (Nazar & Estevez, 2022; Oakley *et al*., 2014). The host microbiome plays a role in competitive exclusion, nutrient uptake, inflammation among other physiological responses, (Oakley *et al*., 2014). Kogut (2021) adds that the immune system's reliance on protection and balance from the gut-microbiota adds a significant risk in that changes in the microbiota's composition can lead to the disease state. For example, it is known that some opportunistic pathogens form part of the microbiota, and can "bloom" in certain stress conditions, or when the microbiota is disturbed (Kogut, 2021; Oakley *et al*., 2014). The use of disinfectants will surely affect the birds, but the exact nature of this effect is uncertain. It is possible, that the effects of Virukill® may be through reducing infection pressure from the environment, however it may also be possible that Virukill® affects the stress response, directly or indirectly. Even more likely are the effects of Virukill® on the microbiome of

broilers, as the effects on the environment, the sprays and direct ingestion will surely alter the internal bacterial composition, however whether this is a positive or negative outcome needs further study. The application of a spray, for example, can be a stressful interruption due to noise, movement, and the spray itself disturbing the birds, the effect of such a spray should be considered in conjunction with the potential additional stress imposed.

In a previous study Bragg and Plumstead (2003) performed a continuous disinfection program with Virukill® on two production houses with 3500 broilers per house. On day 16 of the experiment Newcastle Disease (NCD) was diagnosed on the farm. At this point the control house mortality rate rapidly increased. Notably, the two trial houses mortality levels remained low, even with the disease challenge. However, on day 20 the trial was abandoned by the farmer, a rapid increase in mortalities was observed in the trial houses from day 22 onwards. In the first experiment in this study the differences between the standard and trial house 1 were miniscule. However, in trial house 2 and standard house 2 a different picture emerged. From week 4 onwards in this set of houses there was a notable increase in mortality. In post-mortem dissections infectious material in the form of septicaemia, ascites, pericarditis and perihepatitis were commonly found. These birds seemed to have had a similar stress or disease response during their growth cycle which was characterised by the increased mortality. Like the previous commercial trial, the birds on the Virukill® continuous disinfection program performed better, with a clear reduction in the severity of the negative effects with reduced mortality and improved performance under stressful conditions.

In this study performance advantages were seen in some trials where the continuous disinfection program was administered. In Experiment 1, the first set of houses had similar performance., however Trial House 2 did perform better that the Standard house 2, with a lower FCR and a higher PEF. In Experiment 2, the differences were much more pronounced, with Trial House 1 outperforming Standard House 1 by a in terms of FCR (1.4 against 1.57) and PEF (426 against 362). Similarly, Trial House 2 outperformed Standard House 2 in FCR and PEF, even with a much higher cumulative

mortality. Therefore, in three of four trials, the use of a continuous disinfection program resulted in increased performance. Bragg and Plumstead (2003) found increased average body weight when implementing a continuous disinfection program compared to pre-disinfection and no treatment. In addition, a lower FCR rate was observed in for the continuous disinfection than only pre-disinfection and no treatment (Bragg & Plumstead, 2003). This study shows strong evidence to support improved performance of broilers undergoing the Virukill® continuous disinfection program.

There is a wide variety of factors that can influence the performance of broilers, including heat, disease, feed, parent flocks, biosecurity, genetics, water quality and many others (Meroz & Samberg, 1995). In this study it was shown that in some circumstances the benefits of a continuous disinfection program are not immediately clear. However, when challenges are introduced, the value of the continuous disinfection program could not be argued. The clearest benefit is in the overall decrease in mortality, which was seen in two out of the four trials, with one trial with no clear difference and the other being especially affected by very early mortality in the first 3 days post placement.

Colibacillosis has been shown to especially affect late mortality rates in broilers (Seneviratna, 1969). In addition, colibacillosis may affect broilers at any age if their resistance is reduced (Seneviratna, 1969). In this study a notable increase in late mortality was seen in trial house 2 and standard house 2. A variety of factors may have contributed to this increase in mortality. It is possible and likely that this was due to APEC that affected these houses, but this cannot be determined for certain due to the difficult nature in diagnosing and characterising APEC (Nolan *et al*., 2020). Future research should investigate the link between APEC and a continuous disinfection programme more specifically, with a focus on professional diagnosis and characterisation of APEC found.

3.5 Conclusions

.

In this experimental chapter the effects of a continuous disinfection program compared to standard disinfection were compared with regards to production performance. Two experiments consisting of

two sets of trials (a standard and a trial house for each), were conducted. The results indicated that a Virukill® continuous program is a viable solution to reduce mortality, especially mortality after 20 days, in commercial broiler production. The trials also provided evidence that the continuous disinfection program resulted in improved broiler performance, with better FCR and PEF in three out of four trials conducted. The mechanism of action of the Virukill® continuous disinfection program in improving performance and reducing mortalities may be due to reduction of pathogenic pressure, (Nazar & Estevez, 2022), altering the internal microbiota (Burbarelli *et al*., 2017; Oakley *et al*., 2014), effecting stress responses (Siegel, 1995), or a host of interactions, such as reduction of inflammation, that affect the allostatic state, and reduce the energy requirements expended (Blas & Fairhurst, 2021). All these effects provide exciting opportunities for further investigation.

Chapter 4: General discussion and conclusions

With a drought of new antibiotic discoveries and the rapid rise of antibiotic resistant bacteria, urgent solutions are required to prevent an era where antibiotics are ineffective (Bragg *et al*., 2018). In addition, the uncontrolled use of antibiotics in the animal production sector (Samuels *et al.* , 2021) may lead to the development of zoonoses as antibiotic resistance genes transfer between bacterial populations in the environment, especially when adequate hygiene measures are not in place (Hedman *et al*., 2020). Biosecurity is a term for a set of measures that controls and prevents disease spread through various means, including cleaning and hygiene, and physical means of prevention such as barriers to wild birds and ring-fencing farms (Bragg *et al*., 2018; Gifford *et al*., 1987; Gosling, 2018). A major concern for industry, however is that stopping the use of antibiotics will lead to a loss in production performance, which ultimately will harm food security and can prevent the development of emerging economies (Hedman *et al*., 2020). A viable, working, cost-effective alternative to antibiotics needs to be found for sustainable poultry production.

This study aimed to evaluate the viability of one possible biosecurity measure in a commercial broiler production environment, this being the use of a modified-QAC based disinfectant in a novel continuous disinfection program throughout the growth period. The first aim of the study was to investigate how pre-placement disinfection using Virukill® compared to a standard formal based cleaning and disinfection program, using bacterial and viral evaluation methods. The second aim was to determine the effects of the continuous disinfection program on two non-consecutive growth cycles of broilers on a commercial scale. In this chapter, these will be discussed, along with a synthesis of cleaning and disinfection and the following performance results achieved to evaluate the effect of adequate or inadequate biosecurity application on performance.

A poultry farm in Stutterheim in the Eastern Cape province of South Africa was used to perform two non-consecutive growth cycle studies. In both studies two of the houses underwent standard cleaning and disinfection only with an aldehyde-based disinfectant and the other two houses were subjected to the Virukill® continuous disinfection program. The continuous disinfection program consisted of cleaning and disinfection using Virukill® at 0.5% and 1% concentration, using Virukill® for footbaths, direct spray of a 1% Virukill® at 21 – 26 mL per bird, and Virukill® dosing in drinking water at 100 ppm.

In the second chapter the cleaning and disinfection of the experiments were compared. This work is the first to this author's knowledge to compare a cleaning and disinfection program by bacterial and viral evaluation methods, especially at a commercial scale and in a field trial. This study showed that when using bacterial evaluation methods aldehyde disinfectants appear to be the most effective, followed by Virukill®, with a peracetic acid disinfectant performing the worst. However interestingly, viral cleaning and disinfection evaluation methods did not correspond to the bacterial evaluation results. In one instance in this study, complete eradication was suggested by the bacterial methods, but the Vir-Check score for enteric virus eradication was the worst of all the experiments. Other results showed most of the uncountable bacterial growth but had satisfactory Vir-Check results. This may be a shocking result, and undoubtedly deserves further investigation. Recently, advances in identification methods have shown that classical microbiological culture techniques present only a minority of organisms present (Oakley *et al*., 2014). In addition, the air in poultry houses still contain diverse bacterial populations after cleaning and disinfection (Jiang *et al*., 2018). This result may indicate that traditional bacterial-focused cleaning and disinfection methods are not only a partial picture as previously suspected but may be completely useless as indicators of cleaning and hygiene. Especially considering that cleaning and disinfection surely does not remove all bacteria from poultry houses (Burbarelli *et al*., 2015; Burbarelli *et al*., 2017; Jiang *et al*., 2018; Payne *et al*., 2005). Viral methods, such as the Vir-Check System (GD Animal Health, n.d.), or environmental sampling and sequencing methods, or specific pathogen detection and evaluation methods may prove to be more accurate means to measure cleaning and disinfectant efficacy. The bacterial results, however, did show a clear

reduction in bacteria before cleaning to after disinfection as expected. The Vir-Check results indicated that the standard program and Virukill® were equally effective at reducing viral loads. The results indicate that Virukill® applied in this manner is a viable cleaning and disinfection agent.

In chapter three of this study the performance of broilers undergoing the experiment were presented. Two growth cycles of four houses with 42000 birds per house were used in this study. This work is the first time a continuous disinfection program has been tested at this scale, thus serves as a proof of concept and field trial. In this study improved performance was seen in three of the four house pairs, providing strong evidence that the Virukill® continuous disinfection program can improve broiler performance at a commercial scale. This work supports and adds to the growing body of literature that shows the clear benefits of biosecurity as an approach to reduce antibiotic dependence and offers a viable alternative in the poultry sector.

Up to this point, cleaning effectiveness and its relationship to performance have not been discussed. This study consisted of eight poultry houses in total, therefore the study did not provide a vast data set. It should be noted that the field trial was performed over two cycles with bird counts of 42000 per house. The robustness of the data should be taken into account with the scale of the trials. The data are not significant due to repetition, but each house in itself is a notable data point.

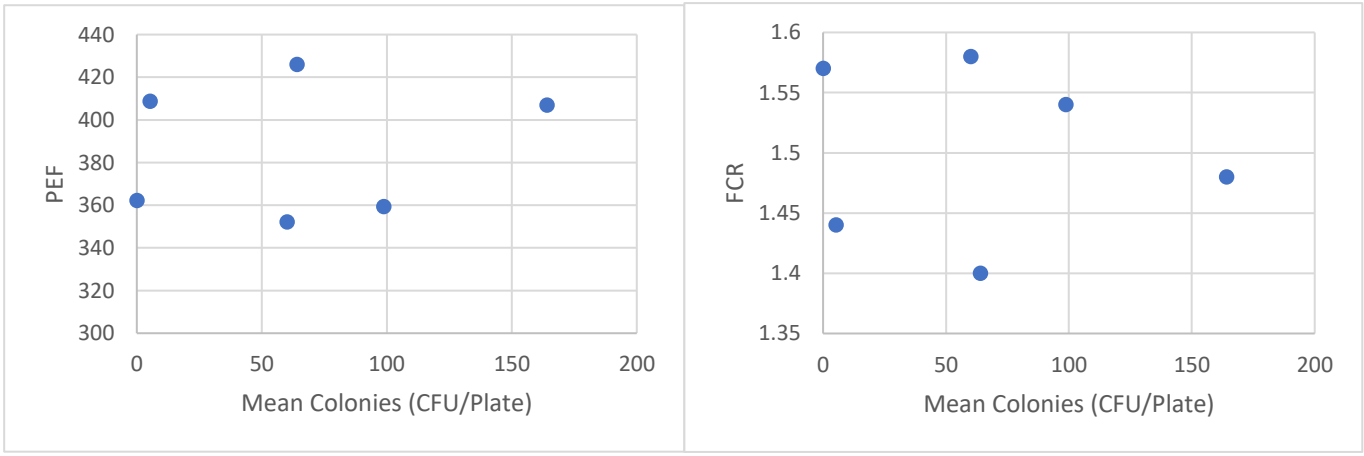
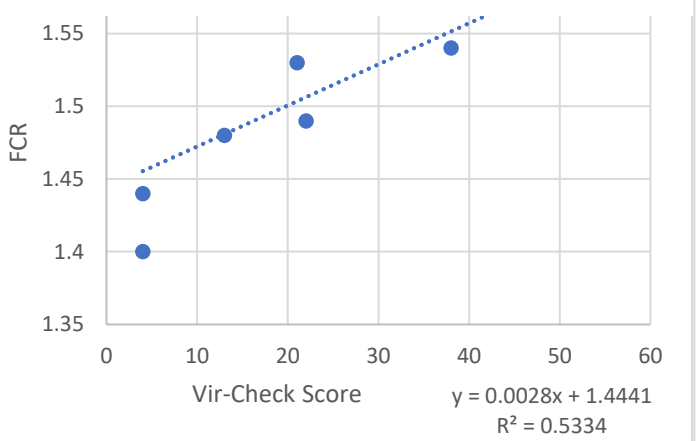
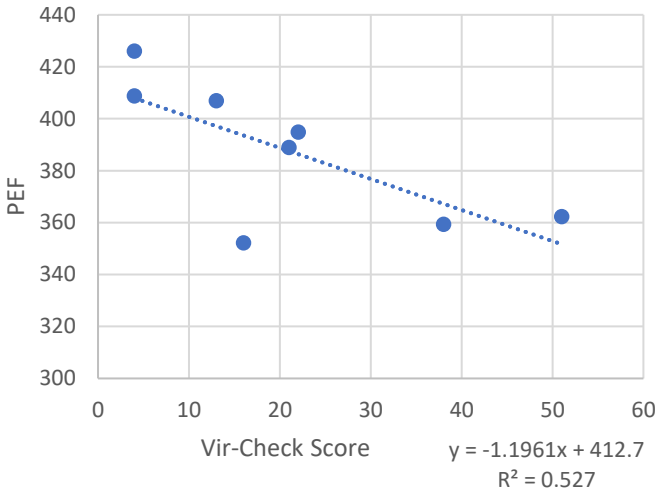


Figure 17: Relationship between mean colonies (CFU/plate) and performance parameters for eight poultry houses

There is no apparent relationship between the bacterial evaluation of cleaning and disinfection and performance (Figure 17). This suggests that either cleaning and disinfection has no effect on performance or that bacterial methods are not true reflections of cleaning and disinfection efficacy.

Figure 18: Relationship between the viral evaluation of c





The viral methods used in this study to evaluate cleaning and disinfection show a slightly positive correlation to performance parameters. This suggests that the level of cleaning and disinfection and consequent viral load affects the performance of commercial broilers in that better cleaning results in lower viral load and improved performance (lower FCR and higher PEF). Interestingly, the continuous disinfection program resulted in lower mean Vir-Check scores, lower mean FCR, and higher mean PEF, although these differences were not statistically significant when using the t-Test: Paired sample for means with alpha value of 0.05. This strongly suggests that the use of a continuous disinfection program and use of Virukill® results in improved cleaning, lower viral presence, and ultimately improved performance

Table 10: Differences in cleaning efficacy and performance between continuous disinfection and standard cleaning and disinfection

	Mean Vir-Check Score	Std Dev.	Mean FCR	Std Dev.	Mean PEF	Std Dev.
Trials (Virukill®)	19.25	12.55	1.48	0.05	396.82	24.30
Standard (Aldehyde)	23	17.30	1.53	0.05	378.05	22.22

In chapter two of this study, organisms that survived Virukill® disinfection were isolated after screening. These organisms were subjected to MIC evaluation against three QAC disinfectants along with a type-strain. These results showed that DDAC and Virukill® were significantly more effective than BC at inhibiting the growth of all organisms tested. The organisms isolated were identified to be *Pantoea* species, therefore the direct comparison to an *E. coli* type strain does not provide a true reflection of the degree of resistance to disinfectants observed. From the results of this study alone there is no indication that disinfectant resistance is present in the poultry growth environment. The possibility of disinfectant resistance developing, however remains a clear and real threat (Bragg *et al*., 2014; Kim *et al*., 2018; Mc Carlie *et al*., 2020) and care should be taken to ensure improper disinfectant use does not support development of disinfectant resistance. At this point, however the degree of efficacy loss associated with antibiotic use (complete inefficacy with resistance) indicates that antibiotic resistance development is a greater risk at present (Apata, 2009). Evidence has been presented that disinfectant and antibiotic resistance are co-carried on resistance plasmids, at least in avian pathogenic *E. coli* (APEC) (Newman *et al*., 2021; Nolan *et al*., 2020), therefore the use of certain disinfectants may also assist in the development of antibiotic and disinfectant resistance. This link warrants further investigation.

From this research, a wealth of new avenues is open for further research. The effects of the continuous disinfection program on birds with respect to their intestinal microbiota and stress levels merit further attention. In addition, the study suggested that Virukill® assisted in late mortality, the mechanism of action for this phenomenon warrants investigation, including the effects on APEC. The genetic composition of organisms that survive disinfectant application over time also merit attention to monitor the development of resistance.

This work has contributed to the growing body of knowledge of biosecurity measures to address a post-antibiotic world. As a practical proof of concept this work can be immediately transferred to commercial application and provides insights and alternatives to the industrial use of antibiotics and

associated losses with the cessation of their use. This work provides strong evidence that viral methods of evaluation cleaning and disinfection of poultry houses are more effective than bacterial enumeration methods. This work also shows that Virukill®, a modified QAC disinfectant, can successfully be used as a disinfectant and is equally or more effective than aldehyde-based disinfectants. This study also showed for the first time that the Virukill® continuous disinfection program improves broiler performance, and therefore that a biosecurity-based method can be used as an alternative to antibiotic use in South African agriculture. A weak positive correlation was also established for the first time between cleaning and disinfection and performance of broilers. The work also showed that the Virukill® continuous disinfection program resulted in lower viral loads and subsequent improved performance, thus for the first time establishing a quantifiable throughline between cleaning and disinfection and performance in broilers. The Vir-Check system was established as having a potentially predictive value for broiler performance. The work did not show evidence of any disinfectant resistance developing.

References

Altschul, S. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, *25*(17), 3389–3402. https://doi.org/10.1093/nar/25.17.3389

Apata, D. F. (2009). Antibiotic resistance in poultry. *International Journal of Poultry Science*, *8*(4), 404–408. https://doi.org/10.3923/ijps.2009.404.408

Araújo, P. A., Lemos, M., Mergulhão, F., Melo, L., & Simões, M. (2013). The Influence of Interfering Substances on the Antimicrobial Activity of Selected Quaternary Ammonium Compounds. *International Journal of Food Science*, *2013*, 1–9. https://doi.org/10.1155/2013/237581

Astral. (2019). *Performance Efficiency Factor*.

Barbieri, N. L., De Oliveira, A. L., Tejkowski, T. M., Pavanelo, D. B., Rocha, D. A., Matter, L. B., Callegari-Jacques, S. M., De Brito, B. G., & Horn, F. (2013). Genotypes and pathogenicity of cellulitis isolates reveal traits that modulate APEC virulence. *PLoS ONE*, *8*(8). https://doi.org/10.1371/journal.pone.0072322

Barbour, E. K., Bragg, R. R., Karrouf, G., Iyer, A., Azhar, E., Harakeh, S., & Kumosani, T. (2015). Control of eight predominant Eimeria spp: Involved in economic coccidiosis of broiler chicken by a chemically characterized essential oil. *Journal of Applied Microbiology*, *118*(3), 583–591. https://doi.org/10.1111/jam.12731

Bartlett, J. G., Gilbert, D. N., & Spellberg, B. (2013). *Seven Ways to Preserve the Miracle of Antibiotics*. *56*, 1445–1450. https://doi.org/10.1093/cid/cit070

Battersby, T., Walsh, D., Whyte, P., & Bolton, D. (2017). Evaluating and improving terminal hygiene practices on broiler farms to prevent Campylobacter cross-contamination between flocks. *Food Microbiology*, *64*, 1–6. https://doi.org/10.1016/j.fm.2016.11.018

Bäumler, A. J., Norris, T. L., Lasco, T., Voigt, W., Reissbrodt, R., Rabsch, W., & Heffron, F. (1998). IroN,

a novel outer membrane siderophore receptor characteristic of Salmonella enterica. *Journal of Bacteriology*, *180*(6), 1446–1453. https://doi.org/10.1128/jb.180.6.1446-1453.1998

Benton, W. J., Cover, M. S., Rosenberger, J. K., & Lake, R. S. (1967). Physicochemical Properties of the Infectious Bursal Agent (IBA). *Avian Diseases*, *11*(3), 438. https://doi.org/10.2307/1588192

Blas, J., & Fairhurst, G. D. (2021). Stress ecophysiology. In C. G. Scanes & S. Dridi (Eds.), *Sturkie's Avian Physiology* (7th ed., pp. 1028–1078). Elsevier.

Bragg, R., Jansen, A., Coetzee, M., van der Westhuizen, W., & Boucher, C. (2014). Bacterial Resistance to Quaternary Ammonium Compounds (QAC) Disinfectants. *Advances in Experimental Medicine and Biology*, 1–13. https://doi.org/10.1007/978-81-322-1774-9_1

Bragg, R. R., Meyburgh, C. M., Lee, J., & Coetzee, M. (2018). Potential Treatment Options in a Post-antibiotic Era. In *Infectious Diseases and Nanomedicine III* (pp. 51–61). Springer Singapore. https://doi.org/10.1007/978-981-10-7572-8

Bragg, R. R., & Plumstead, P. (2003). Continuous disinfection as a means to control infectious diseases in poultry . Evaluation of a continuous disinfection programme for broilers. *Onderstepoort Journal of Veterinary Research*, 219–229.

Burbarelli, M. F. de C., do Valle Polycarpo, G., Deliberali Lelis, K., Granghelli, C. A., Carão de Pinho, A. C., Ribeiro Almeida Queiroz, S., Fernandes, A. M., Moro de Souza, R. L., Gaglianone Moro, M. E., de Andrade Bordin, R., & de Albuquerque, R. (2017). Cleaning and disinfection programs against Campylobacter jejuni for broiler chickens: productive performance, microbiological assessment and characterization. *Poultry Science*, *96*(9), 3188–3198. https://doi.org/10.3382/ps/pex153

Burbarelli, M., Merseguel, C., Ribeiro, P., Lelis, K., Polycarpo, G., Carão, A., Bordin, R., Fernandes, A., Souza, R., Moro, M., & Albuquerque, R. (2015). The Effects of Two Different Cleaning and Disinfection Programs on Broiler Performance and Microbiological Status of Broiler Houses. *Revista Brasileira de Ciência Avícola*, *17*(4), 575–580. https://doi.org/10.1590/1516-

635X1704575-580

Butcher, G. D., & Miles, R. D. (2012). *Disease Prevention in Commercial Poultry*. http://edis.ifas.ufl.edu/pdffiles/VM/VM01100.pdf

Capua, I., & Marangon, S. (2006). Control of avian influenza in poultry. *Emerging Infectious Diseases*, *12*(9), 1319–1324. https://doi.org/10.3201/eid1209.060430

Cattoli, G., Susta, L., Terregino, C., & Brown, C. (2011). Newcastle disease: A review of field recognition and current methods of laboratory detection. *Journal of Veterinary Diagnostic Investigation*, *23*(4), 637–656. https://doi.org/10.1177/1040638711407887

Chai, S. J., Cole, D., Nisler, A., & Mahon, B. E. (2017). Poultry: The most common food in outbreaks with known pathogens, United States, 1998-2012. *Epidemiology and Infection*, *145*(2), 316–325. https://doi.org/10.1017/S0950268816002375

Collet, S. (2020). Principles of disease prevention, diagnosis and control. In D. Swayne (Ed.), *Diseases of Poultry 14th Edition* (14th ed., pp. 3–78). Wiley Blackwell.

Coutinho, T. A., & Venter, S. N. (2009). Pantoea ananatis: An unconventional plant pathogen. *Molecular Plant Pathology*, *10*(3), 325–335. https://doi.org/10.1111/j.1364-3703.2009.00542.x

Eagar, H., & Naidoo, V. (2017). Veterinary Antimicrobial Stewardship in South Africa. *International Biology Review*, *1*(2).

Ewers, C., Li, G., Wilking, H., Kießling, S., Alt, K., Antáo, E. M., Laturnus, C., Diehl, I., Glodde, S., Homeier, T., Böhnke, U., Steinrück, H., Philipp, H. C., & Wieler, L. H. (2007). Avian pathogenic, uropathogenic, and newborn meningitis-causing Escherichia coli: How closely related are they? *International Journal of Medical Microbiology*, *297*(3), 163–176. https://doi.org/10.1016/j.ijmm.2007.01.003

Fate, M. A., Skeeles, J. K., Whitfill, C. E., & Russell, I. D. (1985). Evaluation of four disinfectants under

poultry grow-out conditions using contact agar sampling technique. *Poultry Science*, *64*(4), 629–633. https://doi.org/10.3382/ps.0640629

Gay, L., & Mundt, E. (2010). Testing of a new disinfectant process for poultry viruses. *Avian Diseases*, *54*(SUPPL. 1), 763–767. https://doi.org/10.1637/8777-033109-Reg.1

GD Animal Health. (n.d.). *VIR-check*. Retrieved May 17, 2021, from https://www.gdanimalhealth.com/en/Lab-services/vir-check/technical-information

Gifford, D. H., Shane, S. M., & Weigler, B. J. (1987). *Evaluation of Biosecurity in Broiler Breeders*. *31*(2), 339–344. https://doi.org/https://doi.org/10.2307/1590882

Gilbert, P., & McBain, A. J. (2003). Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. *Clinical Microbiology Reviews*, *16*(2), 189–208. https://doi.org/10.1128/cmr.16.2.189-208.2003

Gosling, R. (2018). A review of cleaning and disinfection studies in farming environments. *Livestock*, *23*(5), 232–237. https://doi.org/10.12968/live.2018.23.5.232

Hedman, H. D., Vasco, K. A., & Zhang, L. (2020). A review of antimicrobial resistance in poultry farming within low-resource settings. *Animals*, *10*(8), 1–39. https://doi.org/10.3390/ani10081264

Heier, B. ., Høgåsen, H. ., & Jarp, J. (2002). Factors associated with mortality in Norwegian broiler flocks. *Preventive Veterinary Medicine*, *53*(1–2), 147–158. https://doi.org/10.1016/S0167-5877(01)00266-5

Herren, C. D., Mitra, A., Palaniyandi, S. K., Coleman, A., Elankumaran, S., & Mukhopadhyay, S. (2006). The BarA-UvrY two-component system regulates virulence in avian pathogenic Eschenchia coli O78:K80:H9. *Infection and Immunity*, *74*(8), 4900–4909. https://doi.org/10.1128/IAI.00412-06

Jackwood, M. J., & de Wit, S. (2020). Infectious Bronchitis. In D. Swayne (Ed.), *Diseases of Poultry* (14th ed., pp. 167–188). Willey Blackwell.

1526 Jiang, L., Li, M., Tang, J., Zhao, X., Zhang, J., Zhu, H., Yu, X., Li, Y., Feng, T., & Zhang, X. (2018). Effect of
1527 different disinfectants on bacterial aerosol diversity in poultry houses. *Frontiers in Microbiology*,
1528 *9*(SEP), 1–10. https://doi.org/10.3389/fmicb.2018.02113

1529 Johnson, T. J., Logue, C. M., Johnson, J. R., Kuskowski, M. A., Sherwood, J. S., Barnes, H. J., Debroy, C.,
1530 Wannemuehler, Y. M., Obata-Yasuoka, M., Spanjaard, L., & Nolan, L. K. (2012). Associations
1531 between multidrug resistance, plasmid content, and virulence potential among extraintestinal
1532 pathogenic and commensal Escherichia coli from humans and poultry. *Foodborne Pathogens and*
1533 *Disease*, *9*(1), 37–46. https://doi.org/10.1089/fpd.2011.0961

1534 Johnson, T. J., Skyberg, J., & Nolan, L. K. (2004). Multiple antimicrobial resistance region of a putative
1535 virulence plasmid from an Escherichia coli isolate incriminated in avian colibacillosis. *Avian*
1536 *Diseases*, *48*(2), 351–360. https://doi.org/10.1637/7121

1537 Johnson, T. J., Wannemuehler, Y., Doetkott, C., Johnson, S. J., Rosenberger, S. C., & Nolan, L. K. (2008).
1538 Identification of minimal predictors of avian pathogenic Escherichia coli virulence for use as a
1539 rapid diagnostic tool. *Journal of Clinical Microbiology*, *46*(12), 3987–3996.
1540 https://doi.org/10.1128/JCM.00816-08

1541 Johnson, T. J., Wannemuehler, Y., Johnson, S. J., Stell, A. L., Doetkott, C., Johnson, J. R., Kim, K. S.,
1542 Spanjaard, L., & Nolan, L. K. (2008). Comparison of extraintestinal pathogenic Escherichia coli
1543 strains from human and avian sources reveals a mixed subset representing potential zoonotic
1544 pathogens. *Applied and Environmental Microbiology*, *74*(22), 7043–7050.
1545 https://doi.org/10.1128/AEM.01395-08

1546 Johnson, T. J., Wannemuehler, Y. M., & Nolan, L. K. (2008). Evolution of the iss gene in Escherichia coli.
1547 *Applied and Environmental Microbiology*, *74*(8), 2360–2369.
1548 https://doi.org/10.1128/AEM.02634-07

1549 Kim, M., Weigand, M. R., Oh, S., Hatt, J. K., Krishnan, R., Tezel, U., Pavlostathis, S. G., & Konstantinidis,

1550 K. T. (2018). Widely used benzalkonium chloride disinfectants can promote antibiotic resistance.
1551 *Applied and Environmental Microbiology*, *84*(17), 1–37. https://doi.org/10.1128/AEM.01201-18

1552 Kirchhelle, C. (2018). Pharming animals: a global history of antibiotics in food production (1935–2017).
1553 *Palgrave Communications*, *4*(1). https://doi.org/10.1057/s41599-018-0152-2

1554 Kogut, M. H. (2021). Immunophysiology. In C. G. Scanes & S. Dridi (Eds.), *Sturkie's Avian Physiology*
1555 (7th ed., pp. 571–590).

1556 Kronstad, J. W., & Caza, M. (2013). Shared and distinct mechanisms of iron acquisition by bacterial
1557 and fungal pathogens of humans. *Frontiers in Cellular and Infection Microbiology*, *4*(NOV), 1–23.
1558 https://doi.org/10.3389/fcimb.2013.00080

1559 Li, G., Feng, Y., Kariyawasam, S., Tivendale, K. A., Wannemuehler, Y., Zhou, F., Logue, C. M., Miller, C.
1560 L., & Nolan, L. K. (2010). AatA is a novel autotransporter and virulence factor of avian pathogenic
1561 Escherichia coli. *Infection and Immunity*, *78*(3), 898–906. https://doi.org/10.1128/IAI.00513-09

1562 Lin, Q., Lim, J. Y. C., Xue, K., Yew, P. Y. M., Owh, C., Chee, P. L., & Loh, X. J. (2020). Sanitizing agents for
1563 virus inactivation and disinfection. *View*, *1*(2). https://doi.org/10.1002/viw2.16

1564 Luyckx, K., Dewulf, J., Van Weyenberg, S., Herman, L., Zoons, J., Vervaet, E., Heyndrickx, M., & De Reu,
1565 K. (2014). Comparison of sampling procedures and microbiological and non-microbiological
1566 parameters to evaluate cleaning and disinfection in broiler houses. *Poultry Science*, *94*(4), 740–
1567 749. https://doi.org/10.3382/ps/pev019

1568 Makgopa, M. (2020). *Poultry and Products Annual - Republic of South Africa*.

1569 Mc Carlie, S., Boucher, C. E., & Bragg, R. R. (2020). Molecular basis of bacterial disinfectant resistance.
1570 *Drug Resistance Updates*, *48*, 100672. https://doi.org/10.1016/j.drup.2019.100672

1571 Mellata, M., Dho-Moulin, M., Dozois, C. M., Curtiss, R., Brown, P. K., Arné, P., Brée, A., Desautels, C.,
1572 & Fairbrother, J. M. (2003). Role of virulence factors in resistance of avian pathogenic Escherichia

coli to serum and in pathogenicity. *Infection and Immunity*, *71*(1), 536–540. https://doi.org/10.1128/IAI.71.1.536-540.2003

Meroz, M., & Samberg, Y. (1995). Disinfecting poultry production premises. *Revue Scientifique et Technique (International Office of Epizootics)*, *14*(2), 273–291. https://doi.org/10.20506/rst.14.2.839

Mettifogo, E., Nuñez, L. F. N., Chacón, J. L., Santander Parra, S. H., Astolfi-Ferreira, C. S., Jerez, J. A., Jones, R. C., & Piantino Ferreira, A. J. (2014). Emergence of enteric viruses in production chickens is a concern for avian health. *The Scientific World Journal*, *2014*. https://doi.org/10.1155/2014/450423

Mituniewicz, T., Sowińska, J., Wójcik, A., Iwańczuk-Czernik, K., Witkowska, D., & Banaś, J. (2008). Effect of disinfectants on physicochemical parameters of litter, microbiological quality of Poultry house Air, health status and performance of Broiler chickens. *Polish Journal of Environmental Studies*, *17*(5), 745–750.

Moelling, K., & Broecker, F. (2020). Air Microbiome and Pollution: Composition and Potential Effects on Human Health, Including SARS Coronavirus Infection. *Journal of Environmental and Public Health*, *2020*, 1–14. https://doi.org/10.1155/2020/1646943

Morales, C., Lee, M. D., Hofacre, C., & Maurer, J. J. (2004). Detection of a novel virulence gene and a Salmonella virulence homologue among Escherichia coli isolated from broiler chickens. *Foodborne Pathogens and Disease*, *1*(3), 160–165. https://doi.org/10.1089/fpd.2004.1.160

Müller, H., Islam, M. R., & Raue, R. (2003). Research on infectious bursal disease - The past, the present and the future. *Veterinary Microbiology*, *97*(1–2), 153–165. https://doi.org/10.1016/j.vetmic.2003.08.005

Nazar, F. N., & Estevez, I. (2022). The immune-neuroendocrine system, a key aspect of poultry welfare and resilience. *Poultry Science*, *101*(8), 101919. https://doi.org/10.1016/j.psj.2022.101919

Newman, D. M., Barbieri, N. L., de Oliveira, A. L., Willis, D., Nolan, L. K., & Logue, C. M. (2021). Characterizing avian pathogenic Escherichia coli (APEC) from colibacillosis cases, 2018. *PeerJ*, *9*, 1–24. https://doi.org/10.7717/peerj.11025

Nolan, L., Vaillancourt, J.-P., Barbierie, N., & Logue, C. (2020). Collibacillosis. In D. E. Swayne (Ed.), *Diseases of Poultry 14th Edition* (14th ed., pp. 770–830). Wiley Blackwell.

Oakley, B. B., Lillehoj, H. S., Kogut, M. H., Kim, W. K., Maurer, J. J., Pedroso, A., Lee, M. D., Collett, S. R., Johnson, T. J., & Cox, N. A. (2014). The chicken gastrointestinal microbiome. *FEMS Microbiology Letters*, *360*(2), 100–112. https://doi.org/10.1111/1574-6968.12608

Parreira, V. R., & Gyles, C. L. (2003). A novel pathogenicity island integrated adjacent to the thrW tRNA gene of avian pathogenic Escherichia coli encodes a vacuolating autotransporter toxin. *Infection and Immunity*, *71*(9), 5087–5096. https://doi.org/10.1128/IAI.71.9.5087-5096.2003

Pavelka, M. S., Wright, L. F., & Silver, R. P. (1991). Identification of two genes, kpsM and kpsT, in region 3 of the polysialic acid gene cluster of Escherichia coli K1. *Journal of Bacteriology*, *173*(15), 4603–4610. https://doi.org/10.1128/jb.173.15.4603-4610.1991

Payne, J. B., Kroger, E. C., & Watkins, S. E. (2005). Evaluation of disinfectant efficacy when applied to the floor of poultry grow-out facilities. *Journal of Applied Poultry Research*, *14*(2), 322–329. https://doi.org/10.1093/japr/14.2.322

Roca, I., Akova, M., Baquero, F., Carlet, J., Cavaleri, M., Coenen, S., Cohen, J., Findlay, D., Gyssens, I., Heure, O. E., Kahlmeter, G., Kruse, H., Laxminarayan, R., Liébana, E., López-Cerero, L., MacGowan, A., Martins, M., Rodríguez-Baño, J., Rolain, J. M., … Vila, J. (2015). The global threat of antimicrobial resistance: Science for intervention. *New Microbes and New Infections*, *6*(February 2015), 22–29. https://doi.org/10.1016/j.nmni.2015.02.007

Rodriguez-Siek, K. E., Giddings, C. W., Doetkott, C., Johnson, T. J., & Nolan, L. K. (2005). Characterizing the APEC pathotype. *Veterinary Research*, *36*(2), 241–256.

https://doi.org/10.1051/vetres:2004057

Runyen-Janecky, L. J., Reeves, S. A., Gonzales, E. G., & Payne, S. M. (2003). Contribution of the Shigella flexneri sit, iuc, and feo iron acquisition systems to iron acquisition in vitro and in cultured cells. *Infection and Immunity*, *71*(4), 1919–1928. https://doi.org/10.1128/IAI.71.4.1919-1928.2003

Sabri, M., Caza, M., Proulx, J., Lymberopoulos, M. H., Brée, A., Moulin-Schouleur, M., Curtiss, R., & Dozois, C. M. (2008). Contribution of the SitABCD, MntH, and FeoB metal transporters to the virulence of avian pathogenic Escherichia coli O78 strain χ7122. *Infection and Immunity*, *76*(2), 601–611. https://doi.org/10.1128/IAI.00789-07

Samuels R, Qekwana DN, Oguttu JW, Odoi A. Antibiotic prescription practices and attitudes towards the use of antimicrobials among veterinarians in the City of Tshwane, South Africa. PeerJ. 2021 Jan 13;9:e10144. doi: 10.7717/peerj.10144. PMID: 33520429; PMCID: PMC7811296.SAPA. (2017). *Broiler Industry Stats Summary for 2017*.

Seneviratna, P. (1969). Diseases of poultry (including cage birds). *Diseases of Poultry Including Cage Birds and Pigeons.*, 229.

Shirai, J., Seki, R., Kamimura, R., & Mitsubayashi, S. (1994). Effects of invert soap with 0.05% sodium hydroxide on infectious bursal disease virus. *Avian Diseases*, *38*(2), 240–243. https://doi.org/10.2307/1591943

Siegel, H. S. (1995). Stress, strains and resistance. *British Poultry Science*, *36*(1), 3–22. https://doi.org/10.1080/00071669508417748

Skyberg, J. A., Johnson, T. J., Johnson, J. R., Clabots, C., Logue, C. M., & Nolan, L. K. (2006). Acquisition of avian pathogenic Escherichia coli plasmids by a commensal E. coli isolate enhances its abilities to kill chicken embryos, grow in human urine, and colonize the murine kidney. *Infection and Immunity*, *74*(11), 6287–6292. https://doi.org/10.1128/IAI.00363-06

Smith, J. A., Boulianne, M., Owen, R. L., & Gingerich, E. (2020). Disease Prevention and Control in

Antibiotic-Free Production. In D. E. Swayne (Ed.), *Diseases of Poultry 14th Edition* (14th ed., pp. 40–51). Wiley Blackwell.

Tablante, N. L., Myint, M. S., Johnson, Y. J., Rhodes, K., & Colby, M. (2002). *A Survey of Biosecurity Practices as Risk Factors Affecting Broiler Performance on the Delmarva Peninsula*. *2086*(July). https://doi.org/10.1637/0005-2086(2002)046

Tivendale, K. A., Allen, J. L., Ginns, C. A., Crabb, B. S., & Browning, G. F. (2004). Association of iss and iucA, but not tsh, with plasmid-mediated virulence of avian pathogenic Escherichia coli. *Infection and Immunity*, *72*(11), 6554–6560. https://doi.org/10.1128/IAI.72.11.6554-6560.2004

Tong, C., Hu, H., Chen, G., Li, Z., Li, A., & Zhang, J. (2021). Disinfectant resistance in bacteria: Mechanisms, spread, and resolution strategies. *Environmental Research*, *195*, 110897. https://doi.org/10.1016/j.envres.2021.110897

Torremorell, M., (February, 2021). The Three Levels of Biosecurity of Animals - Management and Nutrition - MSD Veterinary Manual. *MSD Veterinary Manual. https://www.msdvetmanual.com/management-and-nutrition/biosecurity/the-three-levels-of-biosecurity-of-animals.* Accessed: 04 March 2023.

van der Westhuizen, W. A., & Bragg, R. R. (2012). Multiplex polymerase chain reaction for screening avian pathogenic Escherichia coli for virulence genes. *Avian Pathology*, *41*(1), 33–40. https://doi.org/10.1080/03079457.2011.631982

Winterfield, R. W., Hoerr, F. J., & Fadly, A. M. (1978). Vaccination against infectious bronchitis and the immunosuppressive effects of infectious bursal disease. *Poultry Science*, *57*(2), 386–391. https://doi.org/10.3382/ps.0570386

World Health Organization. (2017). WHO guidelines on use of medically important antimicrobials in food-producing animals. In *World Health Organization*. https://apps.who.int/iris/bitstream/handle/10665/258970/9789241550130-

eng.pdf?sequence=1%0Ahttp://apps.who.int/iris/bitstream/handle/10665/258970/9789241550130-eng.pdf?sequence=1

Yassin, H., Velthuis, A. G. J., Boerjan, M., & van Riel, J. (2009). Field study on broilers' first-week mortality. *Poultry Science*, *88*(4), 798–804. https://doi.org/10.3382/ps.2008-00292

Yi, Z., Li, X., Luo, W., Xu, Z., Ji, C., Zhang, Y., Nie, Q., Zhang, D., & Zhang, X. (2018). Feed conversion ratio, residual feed intake and cholecystokinin type A receptor gene polymorphisms are associated with feed intake and average daily gain in a Chinese local chicken population. *Journal of Animal Science and Biotechnology*, *9*(1), 1–9. https://doi.org/10.1186/s40104-018-0261-1

Zhu, Z., Shan, L., Zhang, X., Hu, F., Zhong, D., Yuan, Y., & Zhang, J. (2021). Effects of bacterial community composition and structure in drinking water distribution systems on biofilm formation and chlorine resistance. *Chemosphere*, *264*, 128410. https://doi.org/10.1016/j.chemosphere.2020.128410

Zou, L., Meng, J., McDermott, P. F., Wang, F., Yang, Q., Cao, G., Hoffmann, M., & Zhao, S. (2014). Presence of disinfectant resistance genes in Escherichia coli isolated from retail meats in the USA. *Journal of Antimicrobial Chemotherapy*, *69*(10), 2644–2649. https://doi.org/10.1093/jac/dku197

Zulki, I., Fauziah, O., Omar, A. R., Shaipullizan, S., & Selina, A. H. S. (1999). *Respiratory Epithelium , Production Performance and Behaviour of Formaldehyde-exposed Broiler Chicks*. *23*, 91–99.