Assessment of the effect of cleaning agents on *Campylobacter* with focus on the ability to survive in a viable but nonculturable state (VBNC)

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Preface and acknowledgement

This master thesis of 30 ECTS points has been conducted from September 2023 to February 2024 at the National Food Institute at DTU, under the supervision of Annette Nygaard Jensen, Nao Takeuchi-Storm, and Brian Lassen.

Part of the thesis is done together with Agnes Sigrid Bjørnstad (s183469), meaning some of the results are found in both theses. The following sections are: The comparison of DNA methods(section 6.1), Evaluation of PMA-qPCR discrimination of viable and heat-inactivated *C. jejuni* cell (section 6.2), Standard curve over the viable PMAtreated cell (section 6.3) and LIVE/DEAD *Bac*Light (section 6.4). The written part of results are done individual.

I want to thank Annette Nygaard Jensen, Nao Takeuchi-Storm, and Brian Lassen for this amazing opportunity and their guidance and help during this project. A special thanks to the three Laboratory Technicians, Pia Engelsmann, Resadije Idrizi, and Margrethe Carlsen, for always helping in the laboratory.

Abstract

Campylobacter is Denmark's leading cause of food-borne diseases. Despite national and international efforts to reduce the number of campylobacteriosis cases, there is no indication that the number of incidents is decreasing. One of the issues regarding *Campylobacter* research is that the traditional culturing methods may underestimate the cells in a viable but nonculturable state (VBNC). A more sensitive detection method is needed to detect and quantify the viable *Campylobacter* to prevent infections. Therefore, this thesis aimed to establish and optimize a method to distinguish the different states (live, dead, and VBNC) of *Campylobacter* using propidium monoazide quantitative PCR (PMA-qPCR) and to investigate how cleaning products affect the state of *Campylobacter*using traditional plating and the PMA-qPCR method. Additionally, *Bac*Light Bacterial Viability (LIVE/DEAD *Bac*Light) two color fluorescence assays were tested to determine the different states of *Camylobacter*.

PMA-qPCR uses propidium monazite, which can inhibit DNA amplification from cells with a compromised membrane. Together with qPCR, it allows PMA to detect only viable cells. LIVE/DEAD *Bac*Light can distinguish between live and dead cells based on membrane integrity. LIVE/DEAD *Bac*Light distinguishes between live and dead cells by using stains to color live (green) and dead (red) cells. The PMA-qPCR and LIVE/DEAD *Bac*Light were tested on a 10-folded dilution series of *C. jejuni* (10^1 to 10^7 CFU/ml) of both viable and heat-inactivated cells (15 minutes at 90℃). Following proof of concept, the PMA-qPCR was used to detect *C. jejuni* exposed to cleaning products used on a poultry farm in different concentrations. The effect was also tested with*C. jejuni* combined with 5% chicken juice to stimulate the dirty environment on a farm.

The results show that PMA-qPCR can discriminate between viable and heat-inactivated cells, but the concentration of PMA (20µM) used in this study may not be optimal. The experiment with the cleaning agent (Kombinon Special) indicated that *C. jejuni* can form colonies after being exposed to a concentration of $\leq 0.5\%$. At a concentration of 2%, all of the *C. jejuni* went into the VBNC state. When the bacteria solution was added 5% chicken juice, it seemed to prolong the survival of *C. jejuni* when exposed to Kombinon Special in different concentrations. *C. jejuni* was capable of being cultured for up to 24 hours after exposure to a 0.1% concentration of Kombinon Special when 5% chicken juice was added, while colonies were not detected after 1 hour of exposure time without the 5% chicken juice.

The results from LIVE/DEAD *Bac*Light did not give a clear result when trying to detect *C. jejuni* in the different states. Future testing is needed to specify the methods when using *C. jejuni* for detecting

This thesis showed that the PMA-qPCR method could detect *C. jejuni* in it VBNC state. The PMA-qPCR methods can be used to assess the effectiveness of cleaning agents to inactive

C. jejuni, because it has the ability to detect the bacteria in its VBCN state. This can be a major advantage in the action towards assessing the cleaning agents' actual biocide properties towards *C. jejuni*.

Resume

Campylobacter er den fødevarebårne sygdomme i Danmark, der er årsag til flest sygdomstilfølde. På trods af nationale og internationale bestræbelser på at reducere antallet af campylobacteriosis-tilfælde er der intet, der tyder på, at antallet af hændelser er faldende. Et af spørgsmålene vedrørende *Campylobacter*-forskning er, at de traditionelle dyrkningsmetoder kan undervurdere de levedytige celler ved ikke at vise celler i den såkaldte levedygtige, men ikke-dyrkningabre tilstand (VBNC). En mere følsom detektionsmetode er nødvendig for at detektere og kvantificere den levedygtige *Campylobacter* for at forhindre infektioner. Derfor var formålet med denne afhandling at etablere og optimere en metode til at skelne mellem de forskellige tilstande (levende, død og VBNC) af *Campylobacter* ved brug af propidiummonoazid kvantitativ PCR (PMA-qPCR) og til at undersøge, hvordan rengøringsmidler påvirker tilstanden af *Campylobacter* ved brug af traditionel plettering og PMA-qPCR-metoden. Derudover blev *Bac*Light Bacterial Levedygtighed (LIVE/DEAD *Bac*Light) tofarvet fluorescensassay testet for at bestemme de forskellige tilstande af *Camylobacter*.

PMA-qPCR bruger propidium monoazide, som kan hæmme DNA-amplifikation af celler med en ødelagt membran. Sammen med qPCR tillader det PMA kun at detektere levedygtige celler. LIVE/DEAD *Bac*Lys kan skelne mellem levende og døde celler baseret på membranens integritet. Metoden bruger farvning til at farve levende (grønne) og døde (røde) celler og derved visuelt skelne mellem levende og døde celler. De to metoder blev testet på en 10-foldet fortyndingsserie af *C. jejuni* (10^1 til 10^7 CFU/ml) af både levedygtige og varmeinaktiverede celler (15 minutter ved 90℃). PMA-qPCR blev efterfølgende brugt til at detektere *C. jejuni* påvirker af rengøringsmidler i forskellige koncentrationer. *C. jejuni* blev kombineret med 5% kylling saft for at stimulere et beskidt miljø på en gård.

Resultaterne viser, at PMA-qPCR kan skelne mellem levedygtige og varmeinaktiverede celler, men koncentrationen af PMA (20 µM) anvendt i denne undersøgelse er muligvis ikke optimal. Forsøget med rengøringsmidlet (Kombinon Special) viste, at *C. jejuni* kan danne kolonier efter at være blevet udsat for en koncentration på $\leq 0,5\%$. Ved en koncentration på 2% var alle *C. jejuni* i VBNC-tilstanden. Når bakterieopløsningen blev tilsat 5% kyllingesaft så det ud til at forlænge overlevelsen af *C. jejuni* når de blev udsat for Kombinon Special i forskellige koncentrationer. *C. jejuni* var i stand til at danne colonier i op til 24 timer efter eksponering af en koncentration på 0,1% Kombinon Special, når 5% kyllingesaft blev tilsat, mens kolonier ikke blev påvist efter 1 times eksponeringstid uden 5% kyllingesaft . Resultaterne fra LIVE/DEAD *Bac*Light gav ikke et klart resultat, da man forsøgte at detektere *C. jejuni* i de forskellige stater. Fremtidig test er nødvendig for at kunne bruge den til at undersøge *C. jejuni*

Denne afhandling viste, at PMA-qPCR metoden kunne detektere *C. jejuni* i den VBNC-tilstand. PMA-qPCR-metoderne kan bruges til at vurdere effektiviteten af rengøringsmidler til inaktive *C. jejuni*, fordi den har evnen til at detektere bakterierne i dens VBCN-tilstand. Dette

kan være en stor fordel i indsatsen for at vurdere rengøringsmidlernes faktiske biocidegenskaber mod *C. jejuni*.

Contents

1 Abbreviations

BA	Blood agar
BPR	Biocidal Products Regulation
Campy LNA	Campylobacter Locked Nucleic Acid
C. jejuni	Campylobacter jejuni
CFU	Colony Forming Units
ECHA	European Chemicals Agency
E. coli	Escherichia coli
EU	European Union
FAM	6-carboxyfluorescein
IAC	Internal Amplification Control
LIVE/DEAD *Bac*Light	LIVE/DEAD Bacterial Viability Kit
LOQ	Limit of quantification
mCCDA	Modified Charcoal Cefoperazone Deoxycholate Agar
NC	Negative control
NB	Neutralization buffer
NTC	Non-template control
ON	Overnight
PC	Positive control
PCR	Polymerase Chain Reaction
PI	Propidium iodide
PMA	Propidium Monoazide
PC	Positive control
qPCR	Qquantitative Real-Time PCR
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
saline	saline (0.9% w/v NaCl)
VBNC	Viable but Non-Culturable

2 Study background

One out of four cases of diarrhea diseases is caused by *Campylobacter* globally [1]. *Campylobacter* is the leading food-borne pathogen in Denmark. Even though the recovery of *Campylobacter* infection is short, the economic burden of the disease in the health sector was estimated to 124 million euro in Denmark back in 2019 [2]. The detection of *Campylobacter* is done using the traditional culturing methods. The detection is based on colonies form at selective agar plate, where the colony-forming units (CFU) is determinate [3]. The problem by using the traditional culturing methods is because it often underestimate the viable cell count. The reason is that many bacteria, including *Campylobacter*, are capable of using a survival mechanism called viable but nonculturable (VBNC). When entering the VBNC state the bacteria are unable to grow on culture media, but are still viable. They are capable of maintaining the metabolic activity and may resuscitate to a virulence state, when being under favorable conditions.

To overcome the problem with underestimated viable cell new detection methods for *Campylobacter* is needed. Methods such as PMA-qPCR or LIVE/DEAD *Bac*Light may be the new candidates as detection method for *Campylobacter* [4]. PMA-qPCR are a method, which combine propidium monoazide (PMA) and qPCR. PMA binds to DNA with compromised membranes, and when exposed to light it modifies the DNA making it unavailable for PCR amplification [5]. By treating the DNA with PMA before the qPCR reaction only the cells with intact membrane(viable cells) is amplified. This make it possible to detect both the culturable cells with traditional culturing method, and the VBNC cells using the PMA-qPCR method. LIVE/DEAD *Bac*Light is another method that in combination with traditional culturing method may detect both culturable and VBNC cells. LIVE/DEAD *Bac*Light are using two different stain (SYTO9 and propidium iodide(PI)) to distinguish between viable and dead cells. SYTO9 are capable of penetrating all bacteria membranes, and stain the cells green, whereas PI only penetrate cells with a compromised membrane. The combination of the two stain produce red fluorescence cells [6]. This mean the viable cells become green, whereas the dead is red. In the same ways as for PMA-qPCR, may LIVE/DEAD *Bac* be able to detect the VBNC cell, whereas the culturable cells is detected using the traditional culturing method.

When the detecting of *Campylobacter* has been overcomed the investigating of how to avoid getting infected by *Campylobacter* can begin. It is known that the primary source of *Campylobacter* infection comes from infected broilers [7]. It may therefor be necessary to validate the cleaning procedure on a broiler farm, and maybe more specific what kind of cleaning agents they are using. Different kind of cleaning agents are effecting the bacteria in different way [8]. Meaning to remove *Campylobacter* from the broiler farm the right cleaning product needs to be used .

3 Study objectives

The overall aim of this study was to establish and optimize the method propidium monoazide quantitative PCR (PMA-qPCR) to distinguish the different states (live, dead, VBNC) of *Campylobacter* and to investigate how cleaning products affect the states of *Campylobacter* using traditional plating and PMA-qPCR methods.

To achieve the overall aim, the following specific objectives were pursued:

- Determine the ability of PMA-qPCR and *Bac*Light to discriminate between viable and heat-inactivated *Campylobacter* cells.
- Produce a standard curve using the PMA-qPCR method
- Determine the appropriate washing protocol for experiments with a cleaning agent
- Determine the effect on *C. jejuni* after treatment with cleaning agents with varying concentration, exposure time and addition of organic material using plate counts and PMA-qPCR.
- If time asses the presence/absence of *Campylobacter* in different states in samples collected in the field.

4 Introduction

4.1 *Campylobacter* and campylobacteriosis

Campylobacteriosis has since 2007 been the most commonly reported foodborne gastrointestinal infection in humans in the European Union (EU) [9]. It has been estimated that over 1.5 million people annually get infected in the United States [10]. Due to the mild and self-limiting symptoms of campylobacteriosis, many infected people recover without help from healthcare, which results in many underreported cases. Even though the majority of the infected recover after a few days, the illness can lead to life-threatening complications for people with weakened immune systems. As illustrated in figure 1, humans can get infected through various routes. The three overall transmission routes are foodborne, environmental, and direct contact with infected humans or animals. The overall routes from the farm to the consumer have been evaluated to prevent humans from getting infected, and different actions have been considered to help prevent campylobacteriosis. European Food Safety Authority (EFSA) has four levels of control(farmer, manufacturing, distributor, and consumer), and developed an action plan for each level of step where the infection may occur [11]. At the farmer level, the plan revolves around stabilizing the biosecurity on-farm, whereas the three others are to optimize proper product handling and hygiene.

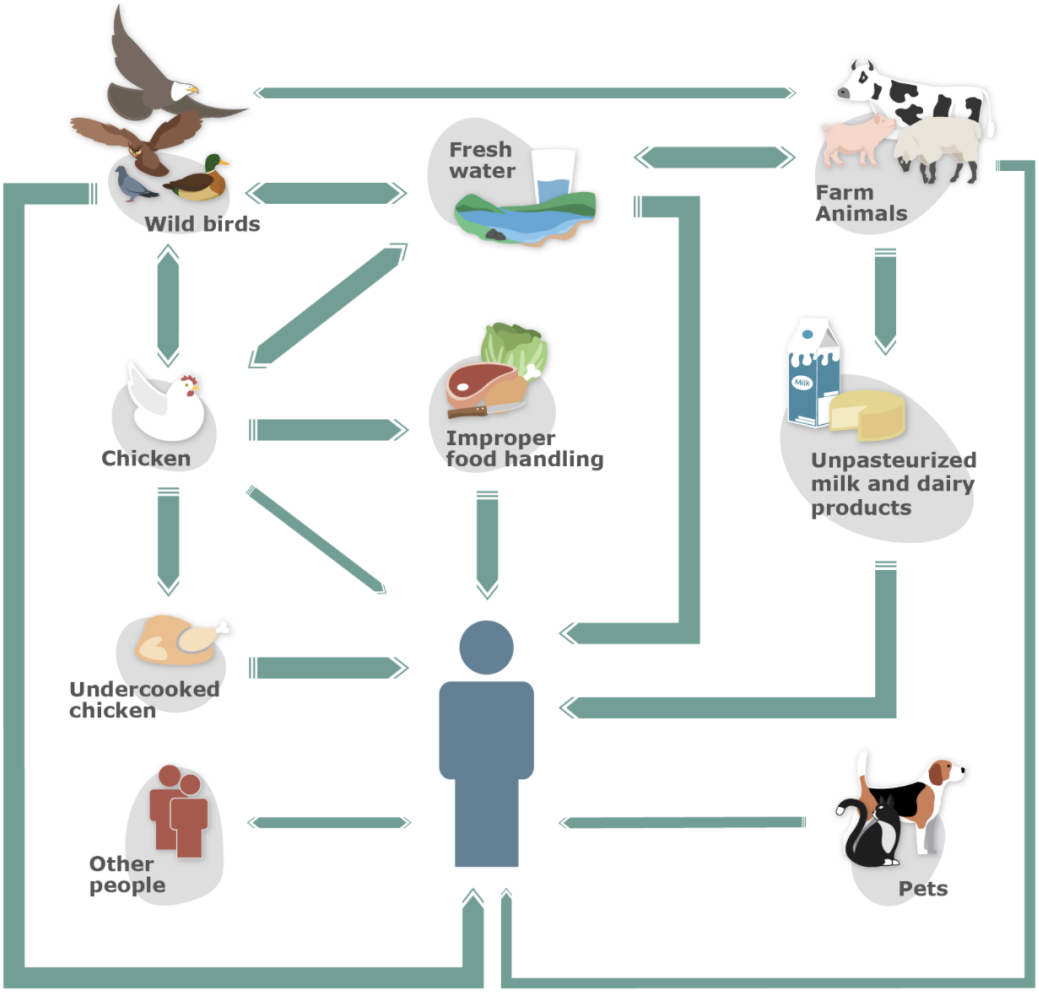


Figure 1: *Model over the different transmission route where Campylobacter can infect humans. The size of the arrow illustrates the relevance of the transmission route. The illustration is taken from Campylobacter Story map, ESFA [11]*

Campylobacteriosis is caused by *Campylobacter*, which is a gram-negative, non-spore-forming, zoonotic, and microaerophile pathogen. Two of the 27 species in the *Campylobacter* genus are considered the most relevant when referring to human health [1]. The two species are *Campylobacter jejuni* and *Campylobacter coli*, which are responsible for the majority of *Campylobacter* infections in humans, *C. jejuni* 80-90% and *C. coli* 5-10% respectively [1]. Under laboratory conditions *Campylobacter* spp. require strict conditions for optimal growth. They require a microaerobic atmosphere with an oxygen concentration of approximately 5%, temperature at 42℃, and a pH of 6.5-7.5 for optimal growth [12].
Its natural reservoir is warm-blooded animals (including domesticated and wild), where it rarely causes illness [11]. *Campylobacter* has been considered a commensal organism in different animals' gastrointestinal tracts, where poultry is the most common reservoir [13]. Many factors influence how poultry gets infected, but it is known to rapidly spread once *Campylobacter* is introduced to a broiler flock. Potential sources of infection can be

inadequate cleaning and disinfection, farmers wearing inappropriate clothing, environmental contamination, or poor house maintenance. Still, it is common for *Campylobacter* to be transmitted horizontally to the flock [7]. This may indicate that *Campylobacter* has a much higher survival rate in its natural habitat than what is detected in the laboratory [14]. The phenomenal indicates that *Campylobacter* may use a survival mechanism called viable but non-culturable (VBNC) when exposed to non-favorable conditions.

4.2 Viable but nonculturable state

Many different bacteria can enter this survival mechanism called the viable but non-culturable (VBNC) state, such as *Listeria, Escherichia* and *Campylobacter* [15]. Studies have shown that when the bacteria are in the VBNC stage, the bacteria are still viable even though they are non-culturable. In this stage, the bacteria retain their membrane integrity and metabolic activity, but it is unknown to what extent the VBNC cells are still virulent [12, 15]. Nevertheless, when resuscitated, the cells regain their full infective phenotypes. How long the bacteria can stay in the VBNC stage is still unknown, but researchers have shown that several months may pass before the bacteria are resuscitated under favorable conditions [15]. Different factors may lead the bacteria to enter the VBNC state, meaning some bacteria are more tolerant than others. Researchers have found that *Campylobacter* may be a less tolerant bacteria, which means it may enter the VBNC state earlier than others [15].

4.3 Detection of bacteria in VBNC

Despite the knowledge that fastidious bacteria may go into VBNC when exposed to different conditions, the current standard methods for detecting bacteria are still based on culturing methods [14]. The detection of *Campylobacter* is done by following the ISO 10272-1:2017 and ISO 10272-2:2017 [16, 3], where the colony forming units (CFU) is quantified by plating the bacteria on a selective agar plate under favorable conditions. The selective agar plate for *Campylobacter* is the Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) plate. The method is effective on fresh samples such as fecal samples, but if the sample is frozen or somehow affected, it may affect the detection of *Campylobacter* [14, **?**]. Studies have shown that growth on laboratory agar plates ceases when *Campylobacter* is exposed to different stress conditions [15]. This means that *Campylobacter* is either dead or has gone into the VBNC state. If *Campylobacter* has entered the VBNC state, it may not be detected using traditional culturing methods and can give an incomplete picture of how many viable *Campylobacter* cells there are at that given time [7]. This underestimation of viable cells can significantly affect epidemiological studies and public health efforts. It may lead to continued spread of the disease within the population without being able to detect it. A response to an outbreak may be delayed or inadequate. It can lead to misguided policies and recommendations and a lack of public awareness and education about the risks and preventive measures.

Overall, it is important to detect bacteria in a valid, qualitative, and quick way to minimize the risk to public health. In this study, two different detection methods (PMA-qPCR and LIVE/DEAD *Bac*Light) are tested to investigate if they can be used to detect *Campylobacter* even if they are in the VBNC state.

4.3.1 PMA-qPCR

PMA-qPCR is a method that combines Propidium monoazide (PMA) and qPCR to help distinguish between dead and viable cells in a sample. PMA is a photoreactive DNA binding dye, and by exposing it to light, PMA covalently modifies the DNA, making it unavailable for PCR amplification.[5]. PMA is membrane-impermeant, meaning it can only bind to DNA with compromised membranes while leaving cells with intact membranes unmodified [17]. A normal qPCR reaction cannot distinguish between live and dead cells but amplifies the total DNA in the sample [18]. Treating the sample with PMA before the qPCR reaction, only the viable cells will be amplified, whereas the number of dead cells will stay unchanged. As illustrated in figure 2, only the viable cells' DNA will be amplified using the PMA-qPCR methods. By combining PMA-qPCR with traditional plating, one should be able to distinguish between the three different stages of a bacteria: viable, dead, and VBNC cells. The viable cell can be found using plating. The VBNC cells are found by adding PMA to the sample and running qPCR, whereas the dead is found by subtracting the quantity of cells estimated by PMA-treatment from the total amount estimated wtihout PMA-treatment.

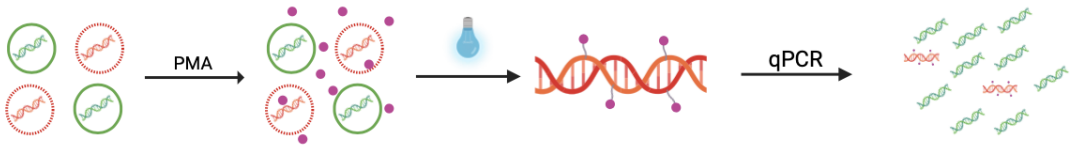


Figure 2: *The model illustrates how PMA only interacts with DNA from cells with compromised membranes. After PCR, only the cells with intact membranes are amplified. Inspiration for the illustration is from Biotum [19]*

This study uses a multiplex qPCR, using the two TaqMan probes simultaneously. The two fluorescent reporter dyes are 6-carboxyfluorescein (FAM) and hexacholoro-6-carboxyfluorescein (HEX). The advantage of using TaqMan is that different probes can easily be labeled with different reporter dyes. The TaqMan probes are also specific, consisting of a single-stranded oligonucleotide complementary to a sequence within the target template. The probe hybridizes the sequence on the target template and gets digested by the 5' exonuclease activity of the Taq DNA polymerase [20]. FAM is the Campy probe to target *Campylobacter* in the sample. If a negative response is indicated, it means no target sequence is present in the reaction. HEX is used to target an Internal Amplification Control (IAC). The IAC is a nontarget DNA sequence, and is amplified alongside the target DNA. Its primary purpose is to monitor the reliability and efficiency of the qPCR reaction. If IAC gives a negative response, there is a

failure in the qPCR reaction [21]. An completed qPCR reaction with the two signals are illustrated in figure

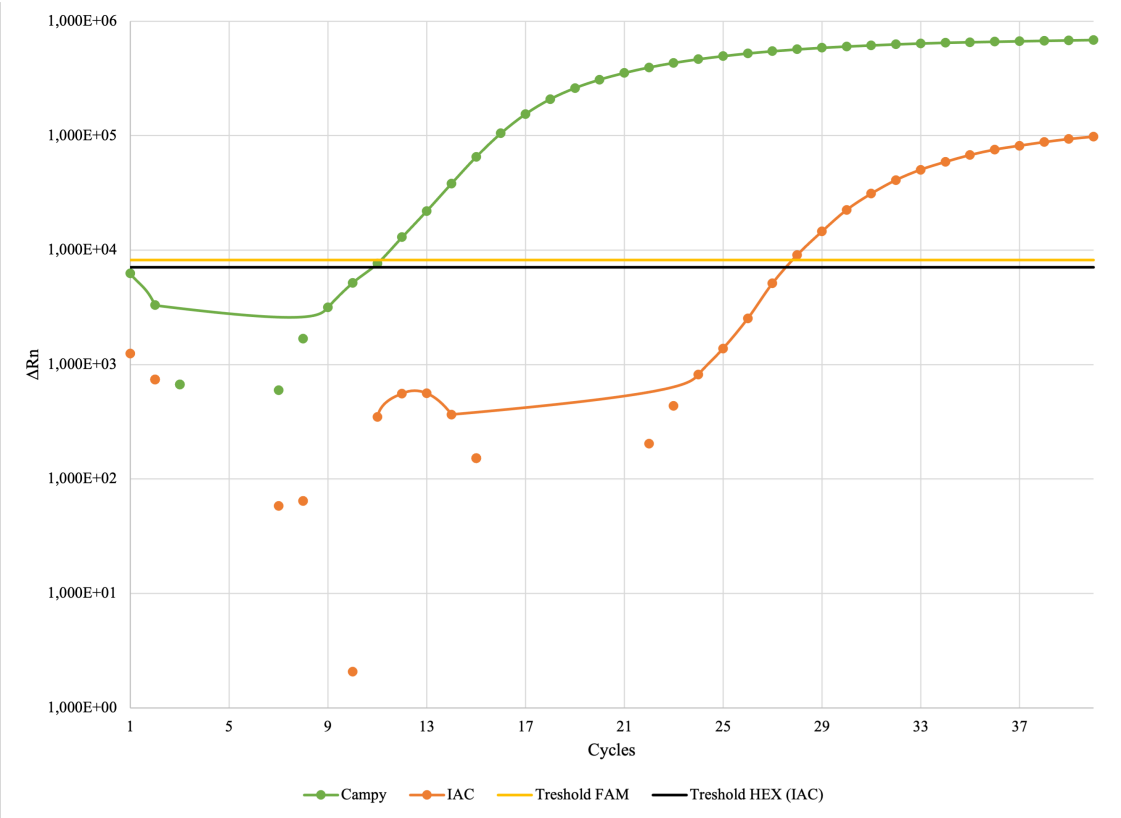


Figure 3: *An amplification plot over a FAM signal(Green) and a HEX signal(Orange). The corresponding thresholds is indicated as yellow for FAM and black for HEX.*

4.3.2 LIVE/DEAD Bacterial Viability Kit

Combined with the traditional plating technique, the LIVE/DEAD Bacterial Viability Kit (*Bac*Light) could also be a promising candidate to detect bacteria in the VBNC state. LIVE/DEAD *Bac*Light is a rapid and visual method to distinguish between live and dead cells based on membrane integrity [6]. The staining method utilizes two nucleic acid-binding stains, SYTO9 and propidium iodide (PI), together with a fluorescence microscope. SYTO9 stains the cells green and can penetrate all bacterial membranes, whereas PI can only penetrate cells with compromised membranes. The combination of the two stains causes a reduction of the SYTO9 stain, which lead the cell to stain fluorescent red [22]. The manufacturer instruction from the LIVE/DEAD *Bac*Light kit indicate that viable cells with intact membranes stain green, while dead cells with damaged membranes are stained red. Combining the plating methods with LIVE/DEAD *Bac*Light, the difference between culturable and green viable cells should indicate the cells in the VBNC states.

4.4 Cleaning product and Biocides

Through centuries, it has been known that proper hygiene can decrease the risk of becoming sick. High hygiene is important in avoiding contamination, especially when handling animals, food, and water. Hygiene often involves using different kinds of cleaning materials, which all affect the surroundings differently depending on the active substrate. Biocides and cleaning agents/detergents must be used for specific tasks to achieve the best result. The difference between a biocidal product and a cleaning agent is that a biocidal product is intended to prevent, destroy, repel, or mitigate bacteria, fungi, or pests. In contrast, a cleaning agent is used to remove unwanted substances [23]. The overall meaning is that biocidal affects the harmful microorganism intended, and cleaning agents "just" remove it from the surroundings. Whether or not a product is classified as biocidal, it is determined in the European Union (EU) by the Biocidal Products Regulation (BPR) [23]. Biocidal products are divided into 22 different product types, with four main groups: disinfectants, preservatives, pest control, and other biocidal products. All active substances must be evaluated by BPR and classified into a product type. After the substance is approved, it may be used in all products of that type [24].
Disinfectants typically have multiple target sites to attach/enter the bacterial cell, affecting many bacterial species. [25]. The bactericidal effect of the biocides depends on the number of targets affected and the severity of the damage done by them [8]. This means that to achieve a product's full potential, it is necessary to understand which kind of active substance is needed for different situations. Besides understanding the active substance in the product, proper handling of the product is necessary. Different factors like pH or concentration may affect the biocide's overall effect. A poor understanding of the product may lead to bacterial survival, adaptation to the active substance, and, ultimately, bacterial resistance to the effects [8].
A biocide's different mechanisms of action can be divided into two major types: highly reactive and less reactive. As indicated, does the Highly reactive biocide interact strongly through chemicals or ionic binding, whereas a less reactive biocide has a weak physical interaction with the organism [8]. Highly reactive biocides are, e.g., alkylating and oxidizing agents, whereas alcohols, metal ions, and phenolics are classified as Less reactive biocides [26].

The two cleaning agents tested in this project are those used on the broiler Farm where field experiment in the project Safe Chicken is carried out. The products (Kombinon Special and Glutack) are used for cleaning between rotations of broilers flocks. It is necessary to validate the efficacy of these cleaning agents to remove *Campylobacter* from the broiler environment to avoid *Campylobacter* infection of subsequent flocks.

4.4.1 Kombinon Special

Kombinon Special(NCÅ-Verodan A/S, Hadsund, Denmark) is an alkaline foam cleaning agent with a pH of around 13.2 in its concentrated form. It consists of Sodium Hydroxide and β-Alanine, N-(2-carboxyethyl-)-,N-coco alkyl derivs., disodium salts. According to the manufacturer instruction, the concentration must be at 2-5%, the activation time 5-20min, and the temperature must be between 0-60℃[27].
By ECHA (European Chemicals Agency) under REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals Regulation), it has been given the product category PC35, Washing and Cleaning Products [28]. This means the product does not contain a biocidal active substance, which means the product does not intend to harm the organism. Nevertheless, sodium hydroxide is known for removing protein and nucleic acids and can inactivate different kinds of bacteria, viruses, yeast, fungi, and endotoxins. [29].

4.4.2 Glutack (NCÅ-Verodan A/S, Hadsund, Denmark)

Glutack is a broad-spectrum disinfectant with a pH of around 5 in its concentrated form. It consists of Glutaral (Glutaraldehyde), Alkyl(C12-16) dimethylbenzyl ammonium chloride, Ethanol, Didecyldimethylammoniumchlorid and Poly(oxy-1,2-ethanediyl),alpha-(2-propylheptyl)-omega-hydroxy-. The contact time is at least 1 hour. The concentration depends on the temperature and the instrument used with spraying or fog equipment [30].
By EACH under REACH, it has classified as the product category PC8, Biocidal products [28]. As mentioned above, BPR divides biocidal products into 22 different product categories. Glutack is Product type 3 according to BRP, which is for use in veterinary hygiene, under the main group 1 [**?**].

4 out of 5 of the compounds Glutack components are active biocide chemicals: Glutaral, Alkyl(C12-16) dimethylbenzyl ammonium chloride, Ethanol, and Didecyldimethylammonium chlorid.
Glutaral is a highly reactive biocide and is classified as an alkylating agent. It is known to strongly interact with the outer layer of cells because of its ability to cross-link, which can inhibit the transport of processes into the cell [31].
Alkyl(C12-16) dimethylbenzyl ammonium chloride and Didecyldimethylammonium chloride are both classified as a quaternary ammonium substance (QAS), which are less-reactive biocides [8]. QAS is known to target the cytoplasmic membrane in bacteria, leading to a disruption of the inner membrane, which causes a leakage of the intracellular substance, resulting in cell death. [31, 32].
Ethanol is also described as a less-reactive biocide. It disrupts the cytoplasmic membrane, which leads to disorganized membranes and dehydration of the bacteria[26].

5 Material and Methods

The following experiment used the *Campylobacter jejuni* ATCC 33560 stain, originally isolated from bovine feces [33]. The experiment has three parts: DNA extraction methods, testing the detection methods (PMA-qPCR and LIVE/DEAD *Bac*Light), and investigating cleaning products' effect on *C. jejuni* different states (live, dead, and VBNC). The first two parts of the experiment were done in cooperation with Agnes Sigrid Bjørnstad (s183469), which means the standard curve was the same in both of our Master Thesis.
Due to time limited was the field experiment not done.

5.1 Bacterial stock solution

5.1.1 *Campylobacter jejuni*

C. jejuni (ATCC 33560) were stored at −80℃ in Lysogeny broth medium containing 15% glycerol as a cryoprotectant. They were thawed by plating them onto a selective solid medium, Modified Charcoal Cefoperazone Deoxycholate agar (mCCDA) plate(Art. no. 18209, SSI Diagnostica, Hillerød, Denmark) and incubated at 41.5℃ under microaerobic conditions for 48 hours. After incubation, they were isolated on a Blood agar (BA) plate (TSA Agarplade m. 5% calf's blood, Art. nr.98802, SSI Diagnostica, Hillerød, Denmark) and incubated at 41.5℃ under microaerobic conditions for 24 hours.

A stock solution was prepared for *C. jejuni* by pouring 1 ml of saline (0.9% w/v NaCl) on the BA containing the bacteria. The solution was mixed using a Drigalski spatula, and the bacterial solution was collected into a 10 ml tube. The optical density (OD) was measured on the solution and adjusted to OD_{600}=0.6 ($\sim 1.0 \times 10^9$ CFU/ml).

5.1.2 *E. coli* (Negative Control)

For negative control *E. coli*(ATCC 11229) was used. It was stored at −80℃ in Lysogeny broth medium containing 15% glycerol as a cryoprotectant. *E. coli* was thawed by plating it onto a BA and incubated at room temperature for 24 hours. After incubation, it was isolated on a BA plate and incubated at 37℃ for 24 hours.

A stock solution was prepared for *E. coli* by pouring one ml of saline onto the BA plate containing the bacteria. The solution was mixed using a Drigalski spatula, and the bacterial solution was collected into a 10 ml tube. The optical density (OD) was measured on the solution and adjusted to OD_{600}=0.6 ($\sim 1.0 \times 10^9$ CFU/ml).

A 10-fold dilution series ranging from 10^2 to 10^8 CFU/ml was created by mixing 100 µl of bacteria culture and 900 µl of saline. For enumeration of the stock solution, 100 µl of dilution 10^2 to 10^4 CFU/ml was spread onto a BA plate and incubated at 37℃ for 24 hours.

One ml of *E. coli* in 10^6 CFU/ml was added to an Eppendorf tube and centrifuged at 7,500g for 7 min. The supernatant was removed, and the pellet was stored at −20℃ until DNA extraction.

5.2 DNA extraction methods

Three DNA extraction methods were tested to investigate whether they could extract DNA from PMA-treated samples. All three methods were tested on *C. jejuni* in two different concentration (10^7 and 10^6 CFU/ml). The DNA extraction was performed on samples with and without PMA sample treatment (section 5.3.1). After DNA extraction, NanoPhotometer® NP80 (Implen, Munich, Germany) with a range of 600 nm was used to measure the concentration and quality of the extracted DNA.

5.2.1 Fast DNA Stool Mini kit

DNA extraction of the samples with and without PMA sample treatment was performed using the QIAamp® Fast DNA Stool Mini kit (Qiagen, Hilden, Germany). The DNA was extracted using the following methods: One ml inhibits buffer was added to the bacteria pellet and vortexed until homogenized. The suspension was heated to 70℃ for 10 min. The sample was then centrifuged for one minute at 13,000 rpm. 25 µl Proteinase K was added to a tube. 600 µl of the supernatant from the sample was transferred to the tube with Proteinase K. Followed by 600 µl Buffer AL and vortex for 15 sec. The sample was then incubated at 70℃ for 10 minutes. After incubation, 600 µl ethanol ($96 - 100\%$) was added to the lysate and mixed with vortexing. 600 µl of the lysate was added to the QIAmp spin column and centrifuged for one minute at 13,000 rpm. After centrifugation, the supernatant was discarded. This step was repeated until all the lysate had been infiltrated. After the lysate had run through the QIAmp spin column, it was placed in a new collection tube. After filtration, 500 µl of Buffer AW1 was added to the QIAamp spin column. Then, it was centrifuged for one minute at 13,000 rpm, and the collection tube with the filtrate was discarded. The QIAmp spin column was placed in a new collection tube, and 500 µl of Buffer AW2 was added. The column was then centrifuged for three minutes at 13,000 rpm. The collection with the filtrate was discarded, and the QIAmp spin column was placed in a new collection tube. To avoid carryover of the Buffer AW2, the tube was centrifuged for three min at 13,000 rpm one more time. After centrifugation, the QIAmp spin column was transferred over in a 2 ml microcentrifuge tube, and 100µl Buffer ATE was added to the column. It was then incubated at room temperature for one minute and centrifuged for two minutes at 14,000 rpm. The eluted DNA was stored at −20℃ until qPCR was run. For each DNA extraction, a Process control(a tube with no sample) was made to control the extraction.

5.2.2 DNeasy Blood & Tissue Kit

DNA extraction using QIAamp®, DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) was performed using the method for Gram-negative bacteria. 180 µl ALT Buffer was added to the bacteria pellet and vortexed. 20 µl proteinase K was added and vortexed. The substrate was incubated at 56℃for 60 minutes while shaking. Afterward, the substrate was vortexed for 15 seconds, and 200 µl Buffer AL was added and mixed by vortexing. 200 µl of Ethanol (96-98%) was added, followed by vortexing. The substrate was transferred to the DNeasy-column in a 2 ml collection tube and then centrifuged for 1 min at 10.000 rpm. The DNeasy-column was placed in a new 2ml collection tube, whereas the old one was discarded. 500 µl of Buffer AW1 was added to the DNeasy-column and centrifuged for one minute at 10.000 rpm. The DNeasy-column was placed in a new 2 ml collection tube, whereas the old one was discarded. 500 µl of Buffer AW2 was added to the DNeasy-column and centrifuged for three minutes at 14.000 rpm. The DNeasy-column was placed in a 1,5 ml Eppendorf tube and 100 µl of Buffer AE was added. Then, it was centrifuged for two minutes at 10.000 rpm. The eluted DNA was stored at −20℃ until qPCR was run. For each DNA extraction, a Process control was made to control the extraction.

5.2.3 Cell lysis

The DNA was extracted using cell lysis by adding 200 µl of 10X TE buffer to the bacteria pellet and then mixed thoroughly by vortexing. The mixture was incubated at 100℃for 10 minutes. A rack was placed on top of the tubes to prevent the lid from opening. Afterward, the mixture was centrifuged at 10.000 rpm for five minutes. The supernatant was transferred to the new Eppendorf tube and stored at −20℃ until qPCR was run. A Process Control was made to control the cell lysis.

5.3 PMA-qPCR

5.3.1 PMA treatment

20 mM PMA was dissolved in sterile distilled water to reach a concentration of 200 µM PMA. Of this was 100 µl added to 900 µl from a sample for PMA treatment. The final concentration of PMA was 20 µM. The samples were then incubated on a shaker for 5 min in the dark. After incubation, the samples were placed in an LED-active blue machine (IB, Applied Science) for 15 minutes and shaken by hand every 5 minutes. The samples were then centrifuged at 7,500xg for 7 min. The supernatant was removed, and the pellet was kept on −20℃ until DNA extraction.
The samples without PMA treatment got the same amount of saline and were given the same treatment as the PMA-treated samples.

5.3.2 qPCR setup

A multiplex TaqMan qPCR with two probes was used: a Campy IAC probe and a Campy-locked nucleic acid (Campy LNA) probe. Table 1 illustrates the specific primers and probes used for the qPCR reaction in this study. The primers(TAG Copenhage A/S, Denmark) were diluted with water to achieve the right concentration, whereas the Campy LNA and the Campy IAC probes were diluted with TE buffer. The primers and Campy IAC probe were chosen based on Josefsen *et al.* (2004, [34]). The Campy LNA probes were chosen based on Josefsen *et al.* (2010, [17]).

Table 1: *The different primers and probes used in this study.*

Prime/probe	**Name (ID)**	**Sequence**
Forward primer	OT-1559	5'-CTG CTT AAC ACA AGT TGA GTA GG-3'
Reverse Primer	18-1	5'-TTC CTT AGG TAC CGT CAG AA-3'
Probe	Campy LNA	5'-[6FAM]CA[+T]CC[+T]CCACGC-CGC[+T] TGC[BHQ1]
Probe	Campy IAC	5'-[HEX]TTCATGAGGACACCTGACTTG [BHQ1]
Primer	Campy IAC	5'-CTG CTT AAC ACA AGT TGA GTA GGC AAC TCA GGT GCT CTC AGT AAT T..G AA-3'

(a) *The three primers were produced and purchased from TAG Copenhagen A/S, Denmark. The two probes were produced and purchased from Merck Life Science A/S, Denmark. The forward, reverse primer, and Campy IAC probe were selected based on the study Josefsen et al. (2004, [34]. The Campy LNA probe was selected based on the study Josefsen et al. (2010,[17]*

The qPCR was performed with a reaction volume of 25 µl. Table 2 indicates the different reactions used per. sample for in the qPCR. The following controls were used for the standard curve and the DNA extraction experiment: Three positive controls (PC) (*C. jejuni* in dilution (1:10, 1:100, and 1:1000), one negative control (NC) (*E. coli* in dilution 1:100) and two NTC (Non-template control). In the experiment with cleaning products, the following controls were used: One PC (A sample from the standard curve), one NC (*E. coli* in dilution 1:100), two NTC, one heat-treated *C. jejuni* sample with PMA and one heat-treated *C. jejuni* without PMA both in a 10^7 CFU/ml concentration.

Table 2: *The volume of the different reactions used in the qPCR*

Reaction	**Final concentration in qPCR solution**	**Volume (µl) per sample**
H_2O		2.22
PCR buffer for Tth DNA polymerase	10x conc.	2.50
$MgCl_2$	25 mM	2.50
Glycerol		2.00
dNTP Mix with dUTP	12.5mM	1.20
Forward primer OT-1559	10 pmol/µl	1.25
Reverse primer 18-1	10 pmol/µl	1.25
Campy LNA probe	5 pmol/µl	0.38
Campy IAC probe	6 pmol/µl	0.25
Boivine serum albumin (BSA)		0.25
Tht enzyme (polymerase)	12.2 U/mol	0.20
Sum (Without CAmpy IAC)		14.00
Campy IAC	10^{-9} pmol/µl	1.00
Sum (With Campy IAC)		15.00
DNA template		10.00
Total		25.00

The mastermix was prepared by adding the different reactions to an Eppendorf tube and mixed by vortexing. 15 µl of the Mastermix was added to a 96 PCR well plate, followed by 10 µl of the DNA template. The PCR well was closed using an Optical Adhesive Covers (Applied Biosystems, Life technologies, Singapore) and then centrifuged for two minutes at 14.000g. Afterward, the qPCR reaction was run as follows: An initiation of 3 minutes at 95℃ followed by 40 cycles with 15 seconds at 95℃ then 60 seconds at 60℃ and the last 30 seconds at 72℃. The Ct were collected at 60℃. The machine used for qPCR was QuantStudio 5 from Applied Biosystems (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

5.4 Discrimination of viable and heat-inactivated cells using the PMA-qPCR methods

The PMA-qPCR ability to discriminate between viable and heat-inactivated cells was tested on *C. jejuni* cells. The inspiration for the experiment was found in Josefsen *et al.* (2010,[17])).

An illustration of the procedure was found in figure 4, followed by a description below.
A standardized cell culture of *C. jejuni* at a 1.0×10^9 CFU/ml was created, as demonstrated in section 5.1.1. A 10-fold dilution series ranging from 10^1 to 10^7 CFU/ml was created by mixing one ml of bacteria culture and 9 ml of saline. For enumeration of the stock solution, 100 µl of dilution 10^2 to 10^4 CFU/ml was spread onto a BA plate and incubated at 41.5℃

under microaerobic conditions for 24 hours.

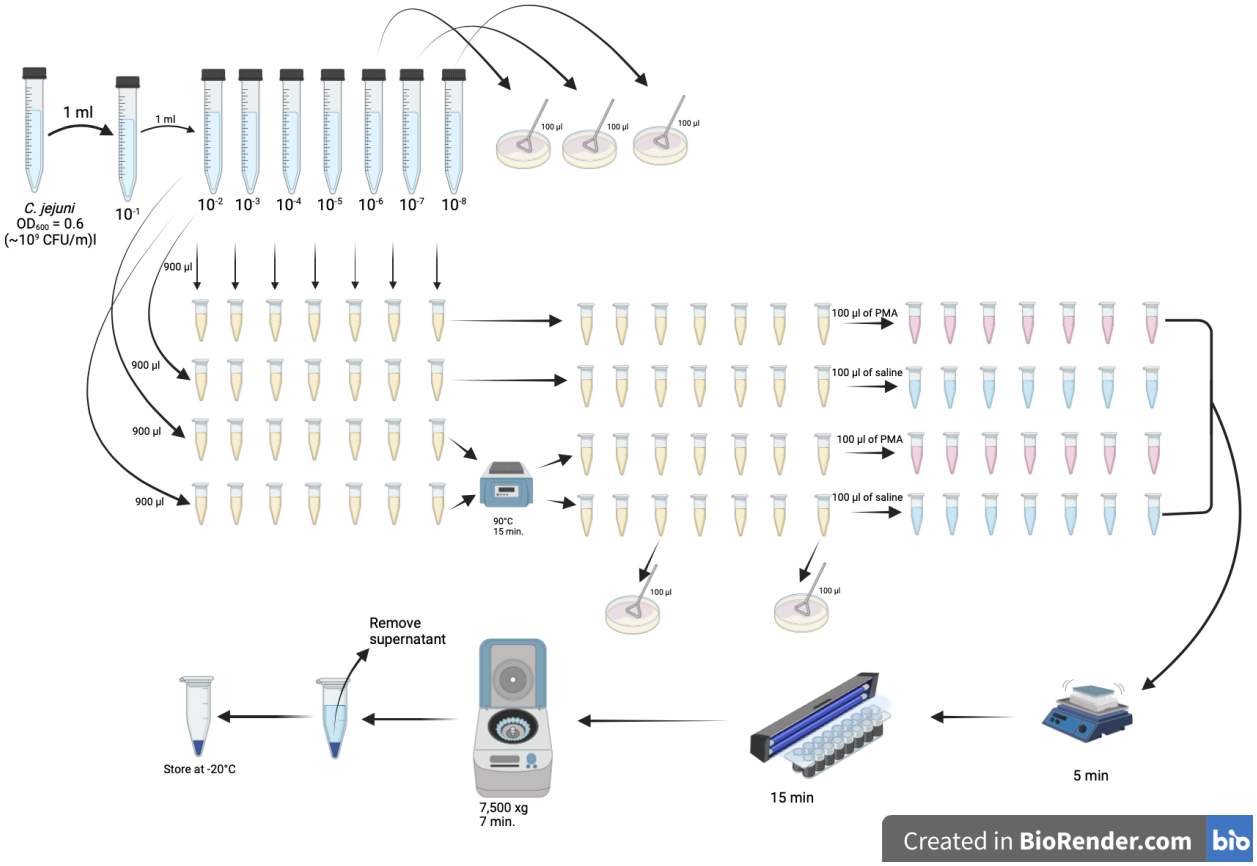


Figure 4: *Illustration of the procedure for discriminating viable and heat-inactivated cells using the PMA-qPCR method.*

5.4.1 Heat-treated cell control

1800 µl of each dilution (10^1 to 10^7 CFU/ml) of *C. jejuni* was heat-inactivated at 90℃ for 15 minutes. Afterward, 900 µl of each sample was added to two Eppendorf tubes. One tube was treated with PMA, whereas the other was treated with saline. The treatment is described in section 5.3.1. The DNA from both samples with and without PMA sample treatment was extracted using the Fast DNA Stool Mini Kit (described in section 5.2.1). The extracted DNA was subsequently analyzed by qPCR as described in section 5.3.2. The obtained Ct values from the qPCR reaction were plotted in an amplification plot to evaluate PMA influence on the heat-treated *C. jejuni* cells.

To control that the heat-inactivation affected the bacteria, 100 µl of the samples from the dilutions 10^2 and 10^4 CFU/ml were spread onto a mCCDA plate and incubated at 41.5℃ under microaerobic condition for 24 hours.

5.4.2 Standard curve over viable cells

From each stock dilution (10^1 to 10^7 CFU/ml), 900 µl was transferred into two Eppendorf tubes. One of the tubes was treated with PMA, whereas the other was treated with saline. The treatment is described in section 5.3.1. The DNA from both samples with and without PMA sample treatment was extracted using the Fast DNA Stool Mini Kit (described in section 5.2.1). The extracted DNA was subsequently analyzed by qPCR as described in section 5.3.2. The obtained Ct values from the qPCR reaction were plotted in an amplification plot to evaluate PMA influence on the viable *C. jejuni* cells.

For enumeration of the stock solution, 100 µl of dilution 10^4 to 10^2 CFU/ml was spread onto a mCCDA plate and incubated at 41.5℃ under microaerobic condition for 48 hours.

The Ct values of the different dilutions with PMA treatment were plotted against their respective Log(CFU/ml), calculated from the plates. A linear regression was performed to convert Ct values from future samples into corresponding concentrations. The limit of quantification (LOQ) was estimated from the standard curve.
Using the slope of the equation, the qPCR reaction efficiency was calculated using the following equation:

$$E = 10^{(-1/slope)} - 1 \tag{1}$$

An efficiency between 90-100% was accepted. The coefficient of determination, R^2, was accepted if R^2 was ≥ 0.98. A lower R^2 value may risk that the results are not reliable.

5.5 Discrimination of viable and heat-inactivated cells using LIVE/DEAD *Bac*Light

The experiment with LIVE/DEAD *Bac*Light was generated to investigate if the methods could discriminate between viable and heat-inactivated *C. jejuni* cells.
A stock solution of *C. jejuni* was created by following the instructions in section 5.1.1. For the experiment *C. jejuni* was in a 10^9 CFU/ml concentration.

One ml of *C. jejuni* in a 10^9 CFU/ml concentration was added to two tubes. One of the tubes was heat-inactivated at 90℃for 15 minutes. 300 µl of the heat-inactivated cell and 300 µl of the viable cells were mixed by vortexing(Called mix). A dilution series range from 10^5 to 10^8 CFU/ml was created from viable, heat-inactivated, and mix cells by adding 900 µl of saline and 100 µl of the bacteria suspension. 100 µl from each bacteria suspension mixture was added to separated wells in a 96-well flat-bottom microplate. This was done in triplicates. How they were placed was illustrated in figure 5

A solution of SYTO9 and PI was created in a ratio of 1:2 (10µl of SYTO9 : 20µl of PI) in 5 ml of filter-sterilized dH_2O and vortexed. 100 µl of the stain solution was added to

each well and thoroughly mixed by pipetting up and down several times.

A PC was created by adding 100 µl of *C. jejuni* in a 10 ^{9} CFU/ml concentration in a well. Three µl of SYTO9 dye was mixed with 500 µl of filter-sterilized dH_2O. 100 µl of the dye was added to the well and thoroughly mixed by pipetting up and down several times.

A NC was created by adding 100 µl of *C. jejuni* in a 10 ^{9} CFU/ml concentration in a well. Three µl of PI dye was mixed with 500 µl of filter-sterilized dH_2O. 100 µl of the dye was added to the well and thoroughly mixed by pipetting up and down several times.

Five None-dye-control was created by adding 200 µl of *C. jejuni* in different dilutions in each well. The dilution ranged was from ^{5} to ^{9} CFU/ml.
The 96-well was incubated at room temperature in the dark for 15 min and placed in ImageXpress Micro Confocal (Molecular Devices, California, United States) to measure the fluorescence intensity.

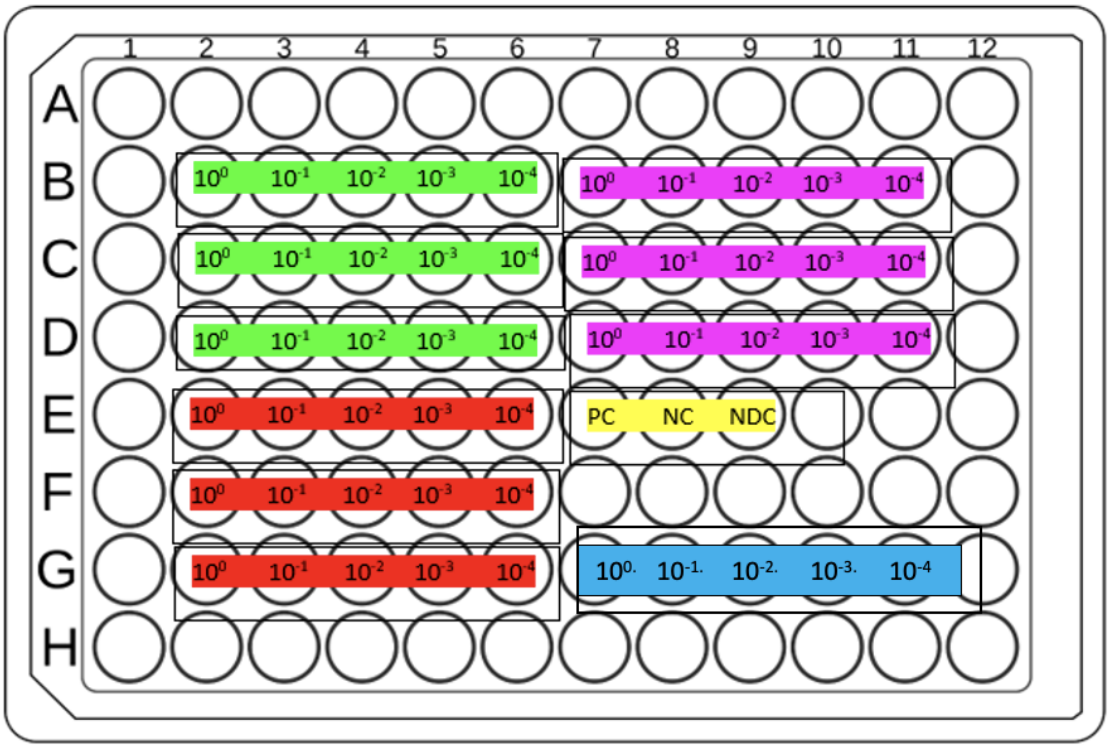


Figure 5: *The setup of each sample in the 96-well. Each color represents a sample of C. jejuni. Viable in green, Heat-inactivated in red, Mix in pink, control in yellow and None-dye-control in blue. NDC = None-dye-control. All of the test was done in triplicates.*

5.6 Cleaning agents effect on *C. jejuni*

The experiment with cleaning agents had three phases.
First, a pilot experiment to investigate the cleaning agent effect on *C. jejuni* using the

PMA-qPCR methods as a detecting method. Secondly, a washing experiment to investigate if the neutralization buffer and wash with saline affected the test material. Third, *C. jejuni* was exposed to different concentrations of the cleaning agent under different conditions over time. Due to the limited time, the experiment was only done on the cleaning product Kombinon Special.

5.6.1 Neutralization buffer

The neutralization buffer (NB) was prepared according to the Dansk standard DS/EN 14349:2012, [35]. NB was used to stop the reaction between *Campylobacter* and the cleaning product. It was assumed that the reaction was stopped when the NB was added. The NB was prepared by dissolving 3g/l of Lecithin, 30g/l of Tween80, 5g/l of sodium thiosulphate, 1 g/l of L-histidine and 30 g/l of Saponine in 0.9% w/v NaCl. The solution was mixed thoroughly and then autoclaved at 121℃for 15 minutes. Afterward, it was stored at 4℃until used.

5.6.2 Chicken Juice

The chicken juice was produced and used to simulate a "dirty" environment on the farm. A "dirty" environment with remains of organic material may affect the survival mechanism of the bacteria. Chicken juice was prepared according to the method used in Birk *et al.* (2004, [36].)

The chicken juice was prepared overnight by thawing commercial frozen chicken legs at room temperature. The thawing chicken juice (almost 50 ml) was collected and mixed before storage at −80℃ overnight. The frozen chicken juice was then thawed at 4℃overnight. The chicken juice was centrifuged at 10.000 rpm for 10 minutes to remove large particles. The supernatant was transferred to a new tube and then sterilized using a 0.45-µm filter. The sterilized chicken juice was stored at −20℃ until used. When used, the chicken juice was thawed at room temperature, and the OD was measured.

5.6.3 Phase 1: Pilot experiment with Kombinon Special

The pilot experiment with Kombinon Special and *C. jejuni* was performed as a single biological trial, but duplicates were made for the qPCR reaction. Two concentrations of Kombinon Special were tested: 0.5% and 2%. Samples were collected after 0, 5, 20, and 60 minutes of reaction time. A control with the same amount of saline was generated for both concentrations.

The overall experiment ran as illustrated in figure 6, while a more elaborate description was made below.
A stock solution of *C. jejuni* was created by following the instructions in section 5.1.1. The start concentration of *C. jejuni* was in a 10^7 CFU/ml. For enumeration of the stock solution, 100µl of dilution 10^4 to 10^2 CFU/ml was spread onto a BA plate and incubated at 41.5℃

under microaerobic condition for 24 hours.

The experiment started by adding 200 µl or 50 µl of Kombinon Special to 9.8 ml or 9.95 ml of *C. jejuni*, to achieve a concentration of either 2% or 0.5% of kombinon Special. The mixture was then vortex. At a specific time, two ml of the sample was collected and added to 2 ml of the NB. The mixture was then centrifuged at 7.500xg for seven min, and the supernatant was removed. One ml of saline was then added to the tube and centrifuged at 7.500xg for seven min. Afterward, the supernatant was removed, and 2.5 ml of saline was added to the tube. The 2,5 ml was referred to as the final sample. 900 µl of the final sample was added to two Eppendorf tubes: one for PMA treatment and one without PMA treatment. The PMA treatment was made as described in section 5.3.1. The DNA from both samples with and without PMA sample treatment was extracted using the Fast DNA Stool Mini Kit, as demonstrated in section 5.2.1, followed by qPCR analysis, as described in section 5.3.2. To emulate the culturable cells, 100µl of the mixture was plated onto a BA plate and incubated at 41℃ under microaerobic conditions for 24 hours.

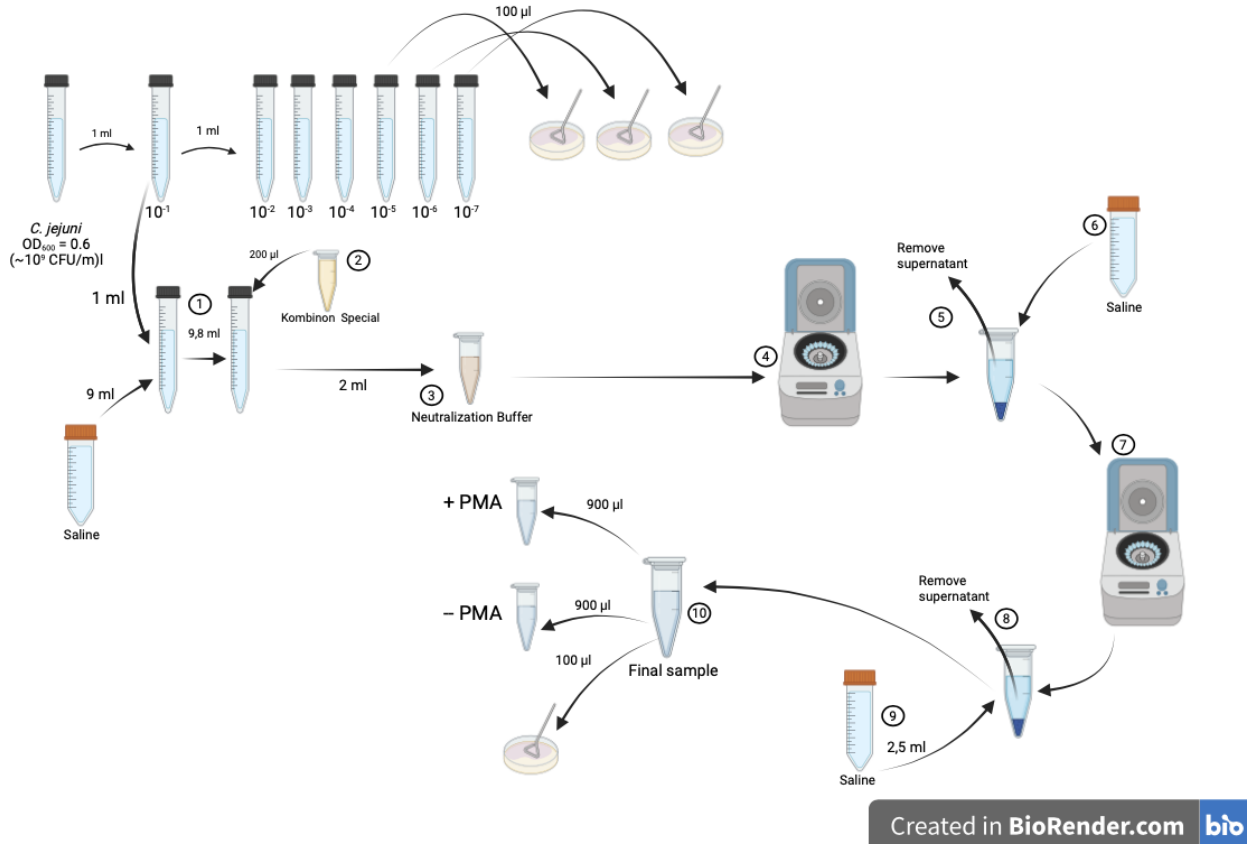


Figure 6: *Illustration over the washing test. The number indicates the different steps in the experiment. 1) Add the right amount of the bacteria mixture in a tube. 2) Add the cleaning productto reach the right concentration. 3) Take two ml of the mixture out and add it to the NB. 4) Centrifuge 5) Remove supernatant 6) Add saline 7) Centrifuge 8) Remove supernatant 9) Add 2.5 ml saline 10) Transfer 900 µl to two tubes, and 100 µl for plating. How the different settings for the centrifuge and amount of saline are described in sections 5.6.3 and 5.6.4.*

5.6.4 Phase 2: Instigation of the Washing step

Five washing experiments were created to determine the effect of the NB and washing step with saline on *C. jejuni*.
A stock solution of *C. jejuni* was created by following the instructions in section 5.1.1. For the experiment *C. jejuni* was in a 10^7 CFU/ml concentration. For enumeration of the stock solution, 100µl of dilution 10^4 to 10^2 CFU/ml was spread onto a mCCDA plate and incubated at 41.5℃ under microaerobic condition for 48 hours.

The experiment was run at timepoint zero, i.e., the sample was collected right after Kombinon Special was added. The cleaning agent Kombinon Special with a 2% concentration was used. A control with the same amount of saline was also run for all 5 experiments. The overall approach is the same as in phase 1 (section 5.6.3) and was illustrated in figure 6. The different settings for the centrifuge and amount of saline for the five variations were shown in table 3.

Table 3: *The amount of NB and saline together with the time and centrifugation used in the five different experiments*

	NB (mL)	**Time and centrifugation with NB**	**Number of washes with saline**	**saline (mL)**	**Time and centrifugation with saline**
W1	2	7 min. at 7500g	1	1	7 min. at 7500g
W2	4	7 min. at 7500g	1	1	7 min. at 7500g
W3	2	7 min. at 7500g	2	1	3 min. at 7500g
W4	2	7 min. at 7500g	3	1	3 min. at 7500g
W5	2	7 min. at 7500g	1	2	7 min. at 7500g

5.6.5 Phase 3: Testing Kombinon Special effect on *C. jejuni* under different conditions

C. jejuni were exposed to two different growth conditions. One "clean" (saline and *C. jejuni*), and one "dirty" (saline, *C. jejuni* and 5% chicken juice). Afterwards was *C. jejuni*(in the two conditions) exposed to three concentration of Kombinon Special (0.1%, 2% and 10%). This was done to investigate the chicken juice effect on *C. jejuni* growth when exposed to a cleaning agent. For each condition, a control was created, meaning four samples from each concentration were created: The four samples were: Clean control, Dirty control, Clean and Dirty. The sample conditions are described in table 4. Samples were collected after 0 min, 5 min, 20 min, 1 hour, and 24 hours of reaction time. The cleaning product was prepared by mixing it with saline to achieve the right concentration. An illustration of the experiment was found in figure 7, while a description in more detail can be found below.

Table 4: *The four conditions used in the experiment with cleaning products*

Clean control	*C. jejuni* in saline
Dirty Control	*C. jejuni* in saline + 5% Chicken juice
Clean	*C. jejuni* in cleaning product
Dirty	*C. jejuni* in cleaning product + 5% Chicken juice

A stock solution of *C. jejuni* was created by following the instructions in section 5.1.1. The start concentration of *C. jejuni* was at 10^8 CFU/ml.

9,5 ml of *C. jejuni* in a 10^8 CFU/ml concentration was added to four tubes. For the Dirty control and Dirty, 500 µl of chicken juice was added to the tubes to reach a 5% chicken juice concentration, whereas for the Clean control and Clean, 500 µl saline was added. Afterward, five ml from each tube was added to four new tubes. Five ml of Cleaning product was added to reach a 1:1 ratio between the bacteria substrate and the test subject in the Clean and Dirty tubes. In the two controls, 5 ml of Saline was added. The reaction started when the bacteria and test subjects were mixed and vortexed for 15 seconds. At different time points, a sample of two ml was taken from the mixture. The sample was added to two ml of NB and vortex for 15 seconds. The mixture was then centrifuged at 7500g for seven minutes, and the supernatant was removed. One ml of saline was added to the tube. The sample was vortexed for 15 seconds and centrifuged at 7500g for seven minutes.

Afterward, the supernatant was removed, and 2.5 ml of saline was added to the tube and then vortexed for 15 sec. 2.5 ml of bacteria substrate was referred to as the final sample. 900 µl of the final sample was added to two Eppendorf tubes: one for PMA treatment and one without PMA treatment(saline). The treatment was made as described in section 5.3.1. The DNA from both with and without PMA sample treatment was extracted using the Fast DNA Stool Mini Kit, as demonstrated in section 5.2.1, followed by qPCR analysis demonstrated in section 5.3.2.
For cell count, 100 µl of the sample was plated onto a BA plate and incubated at 41℃ under microaerobic conditions for 24 hours.

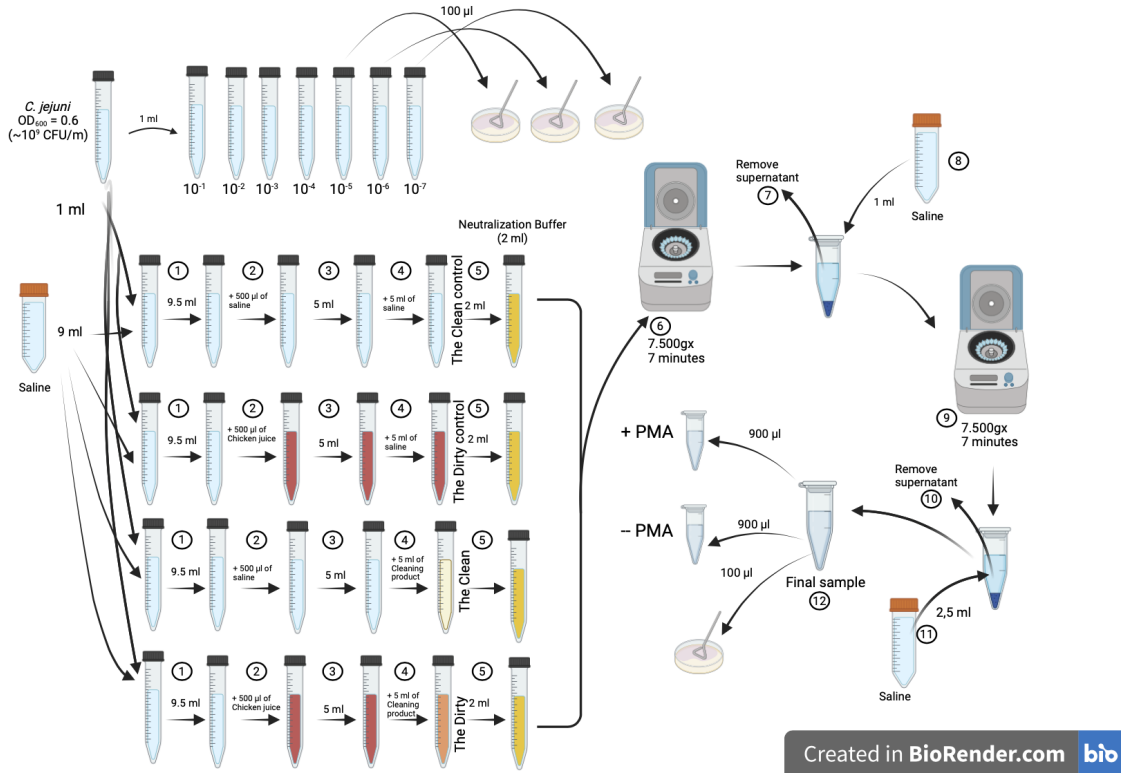


Figure 7: *Illustrating over the experiment in phase 3. The number indicates the different steps in the experiment. Four tubes with C. jejuni in a 10^8 CFU/ml concentration where created. 1) Transfer 9.5 ml of the bacteria substrate to a tube. 2) 500 µl of saline was added to two tubes, while the other two got 500 µl of chicken juice. 3) 5 ml from each tube was transferred to a tube. 4) The two controls got saline, while the test got a specific amount of cleaning product to reach the right concentration. 5) Transfer two ml of the sample to two ml of NB. 6) Centrifuge 7) Remove supernatant 8) add one ml saline. 9)Centrifuge 10) Remove supernatant 11) add 2.5 ml saline. 10) Transfer 900 µl to two tubes and 100 µl for plating.*

5.7 Data analyses

All plating was done in triplicates. The colonies were counted and converted to CFU/ml. Colonies on plates with less than 300 colonies were used to calculate CFU/ml. The qPCR analysis of each sample was performed in duplicate, except for the experiment where the DNA methods were tested. They were done as a single trial.

The Ct values from the qPCR reaction were analyzed using Design & Analysis 2.7.0 Real-Time PCR system from applied biosystems (Thermo Fisher Scientific). All quantitative data were generated in a Microsoft Excel spreadsheet.
dCt was calculated by subtracting the samples with PMA from those without PMA. This was done on both viable and heat-treated cells from the standard curve. dCt was used to determine PMA's ability to inhibit the dead cells' DNA amplification. According to the product information about PMA ([19]), should the dCt for the viable cells be close to 0 $(+/-1)$, whereas for the heat-inactivated, was dCt > 4.
The percentage of viable cells on an unknown sample were calculated using the following

equation:

$$dCt = Ct_8 = Ct_{sample,with\ PMA} - Ct_{sample,without\ PMA}$$

$$Fold\ reduction = 2^{dCt}$$

$$\%\,Viability = \frac{100}{Fold\ reduction}$$

The threshold in the experiment with the cleaning product was set according to the Ct value from the standard curve, which was used as a PC. This was done to ensure the Ct value from the standard curve always had the same value in all experiments. The Ct from each sample was converted to CFU/ml using the linear regression from the standard curve. To compare the enumerated CFU/ml with the CFU/ml from the qPCR results, they were both converted to Log(CFU/ml).

If no signal was given from a sample above the threshold, the Ct was not detectable and was marked as Undetermined or UND. UND indicates that *C. jejuni* was not present in a high enough amount or not present at all. The Ct of the IAC signal from each sample was checked every time to ensure the qPCR reaction was run properly. The qPCR was run again if the Ct value was not detectable for the IAC signal. The VBNC cells were calculated by subtracting the enumerated Log(CFU/ml) from the PMA-treated Log(CFU/ml). The percentage different was calculated to compare two samples using the following equation:

$$Percentage\ different = ABS\left(\frac{reference\text{-}sample}{reference \times 100}\right)$$

6 Results

6.1 Comparison of DNA extraction methods

The three DNA extraction methods were tested using two different concentrations of *C. jejuni*, 10^7 and 10^6 CFU/ml. The test was performed on viable and heat-inactivated (dead) cells with and without PMA treatment. The Ct values from the qPCR reaction was shown in table 5. If the Ct value was undetermined, it was indicated with UND. All samples were tested in the same qPCR run.
The FAST DNA Stool Mini Kit was the only one made in biological duplicates, meaning the Ct values in table 5 are the average of the two replications. The two individual results are in the appendix 1.
The IAC signal was apparent in all samples, which indicates that the qPCR reaction ran as expected. The results are not shown.

Table 5: *The Ct values obtained by the qPCR reaction from the three DNA extraction methods.*

DNA extraction method	Initial CFU/ml	Viable cells		Dead cells	
		- PMA	+ PMA	- PMA	+ PMA
FAST DNA Stool Mini Kit	10^7	16.3	17.9	21.3	UND
	10^6	20.1	21.3	24.3	UND
DNeasy Blood & Tissues Kit	10^7	UND	15.9	13.7	26.4
	10^6	17.1	18.3	17.0	UND
Cell lysis	10^7	UND	UND	UND	38,64
	10^6	UND	UND	UND	UND

(a) *UND means the Ct was Undetermined. "- PMA" indicates samples without PMA treatment, and "+ PMA" indicates samples with PMA treatment.*

6.2 Evaluation of PMA-qPCR discrimination of viable and heat-inactivated *C. jejuni* cells

The influence of PMA on cells with compromised membranes was investigated on a 10-fold dilution series of *C. jejuni* ((10^1 to 10^7 CFU/ml). The cells were divided into four groups: [Viable +PMA], [Viable -PMA], [Dead +PMA] and [Dead -PMA]. The cells were heat-inactivated by incubation for 15 minutes at 90℃. Two samples, 10^2 and 10^4 CFU/ml, were plated to check if the heat-inactivating worked. None of *C. jejuni* grew on any of the plates, which indicated that the heat-inactivation worked in preventing colony growth. The plates are not shown in this study. Figure 8 and 9 illustrated the amplification plot over the obtained FAM signal by the qPCR reaction from the chosen standard curve. The standard curve is chosen based on the description below (section 6.3). The samples with(red) and without(blue) PMA sample treatment were shown in the duplicates from the qPCR reaction. Only the dilution in 10^5, 10^6 and 10^7 CFU/ml gave a detectable Ct value for the dead cells, whereas the viable cells gave detectable Ct value from 10^4 to 10^7 CFU/ml. No signal was obtained from the qPCR reaction for the dead PMA-treated samples.
All samples obtained a detectable signal from the IAC signal, indicating that the qPCR reaction proceeded flawlessly. A graph of all IAC signals from each sample is shown in appendix 2.

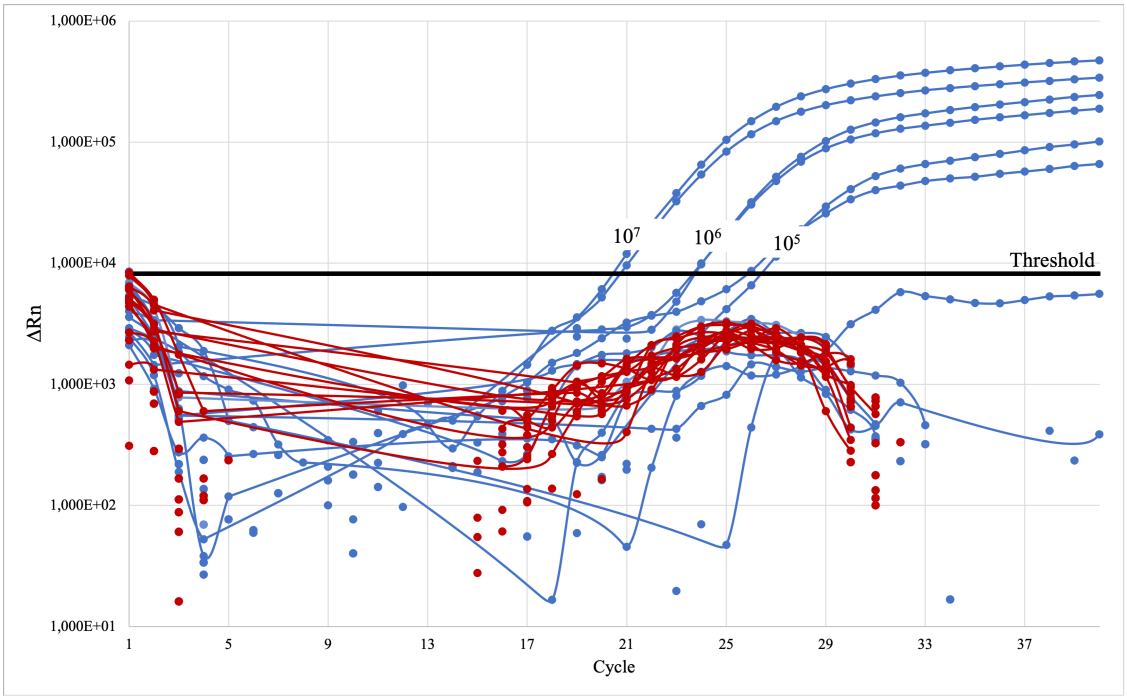


Figure 8: *Amplification plot over FAM signal obtained by the qPCR reaction from heat-treated C. jejuni cells in a 10-fold dilution (10^1 to 10^7 CFU/ml) with(red) and without(blue) PMA sample treatment.*

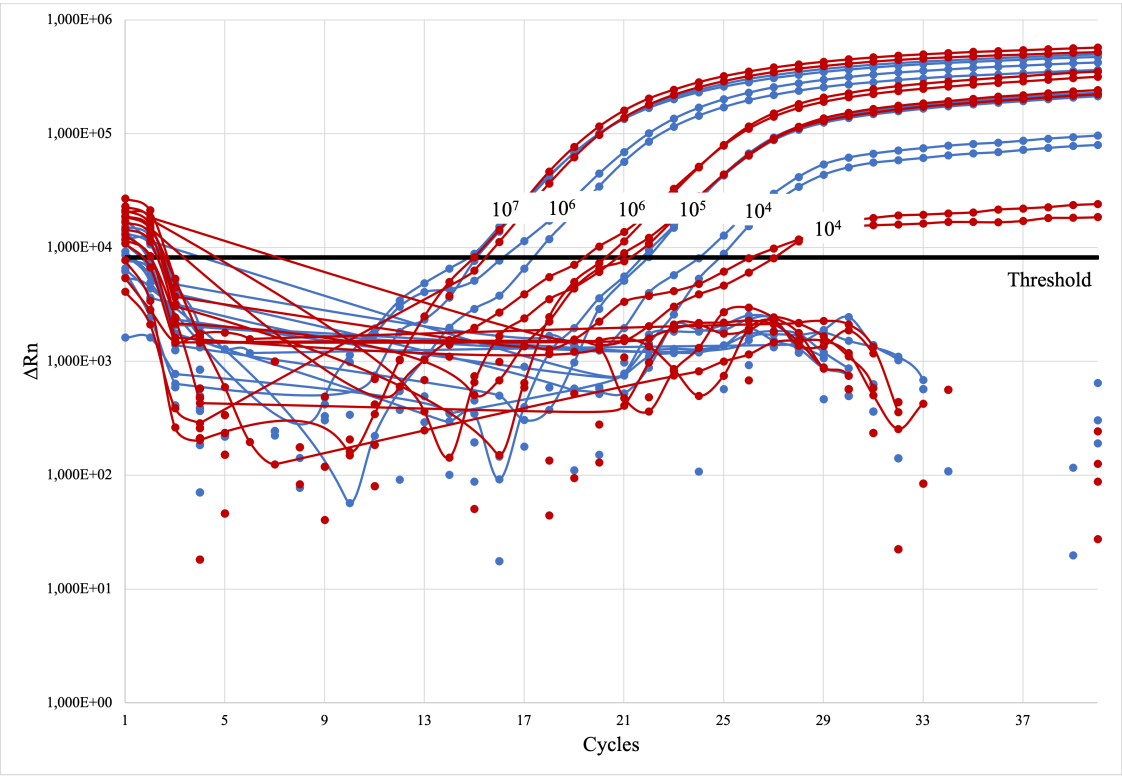


Figure 9: *Amplication plot over FAM signal obtained by the qPCR reaction from the viable C. jejuni cells in a 10-fold dilution (10^1 to 10^7 CFU/ml) with(red) and without(blue) PMA sample treatment.*

The dCt was used to determine whether PMA adequately inhibited the amplification of the

Table 6: *The obtained Ct values from both samples with and without PMA, together with the calculated dCt*

CFU/ml	Ct (-PMA)	Ct (+ PMA)	dCt
$\mathbf{10^7}$	14.9	15.3	0.3
$\mathbf{10^6}$	16.8	19.9	3.0
$\mathbf{10^5}$	21.8	21.0	-0.8
$\mathbf{10^4}$	24.4	26.5	2.2

dead cells. A dCT close to 0 $(+/-1)$ for the live cells indicates that the PMA treatment was not affecting the viable cells. Two of the dCT values were close to 0, where two of them are above (tabel 6).

6.3 Standard curve over the viable PMA treated cell

The standard curve was produced over the obtained Ct value from the viable PMA-treated *C. jejuni* cells in a 10-fold dilution series (10^1 to 10^7CFU/ml). They were plotted against the calculated Log(CFU/ml) and were illustrated in figure 10. Log(CFU/ml) was calculated using the plated *C. jejuni* in a 10-fold dilution series from 10^2 to 10^4 CFU/ml. The counted colonies from the plating were converted to Log(CFU/ml).

The efficiency of the qPCR reaction was found to be 93.2 %. The LOQ for the standard curve was estimated to 10^4 CFU/ml. The samples were run through qPCR reaction three times to evaluate the efficiency and R^2 value. The two other standard curves were illustrated in appendix 3, and their efficiency was calculated to $A = 88.36\%$ and $B = 84.18\%$, respectively. None of the standard curves R^2 value was above the acceptable value. Therefore, the standard curve was based on the calculated efficiency. From the chosen standard curve, the concentration of *C. jejuni* was expressed by the equation:

$$Log(CFU/ml) = (Ct - 34,278)/(-3,491)$$

.

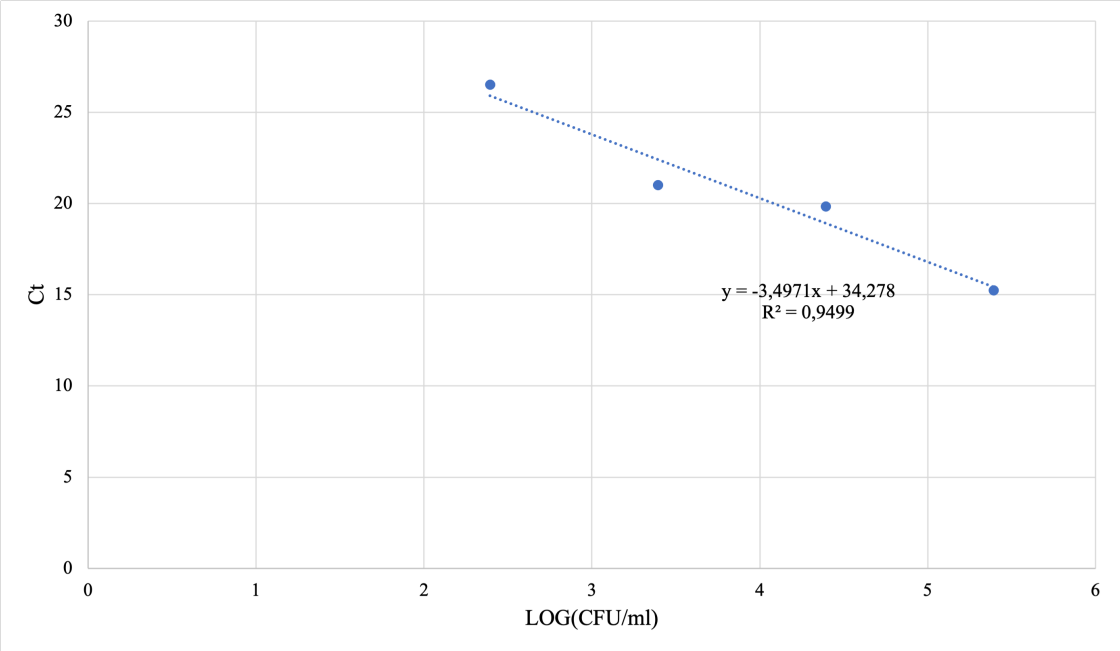
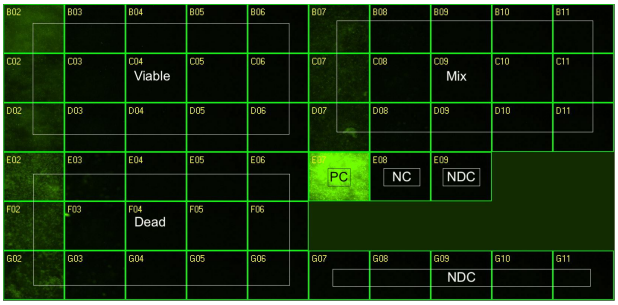


Figure 10: *Standardcurve over the viable PMA treated C. jejuni cells. The ct value obtained from the qPCR reaction was plotted against the initial Log(CFU/ml).*

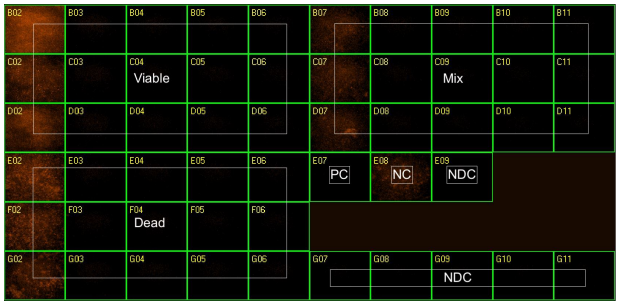
6.4 Evaluation of LIVE/DEAD *Bac*Light Discrimination of viable and heat-inactivated *C. jejuni* cells

The LIVE/DEAD *Bac*Light methods tested on *C. jejuni* in a 10-fold dilution serie ranged from 10^5 to 10^9 CFU/ml. The cells were divided into three groups: [viable], [Dead], and [Mix(50:50 of viable:dead)]. Three controls were constructed (PC, NC, and none-dye-control) in a 10^9 CFU/ml concentration. Five none-dye-controls were also constructed in a 10-fold dilution series (10^5 to 10^9 CFU/ml). The none-dye-controls were created to investigate if *C. jejuni* were detectable without the stains. The position of the samples was illustrated in figure 5. The fluorescence intensity of the green dye was high in the PC when the green dye was detected only (figure 11a). For the NC the fluorescence intensity was slightly lower for the red dye, as shown in figure 11b. No signal for either the green or red light was obtained when the concentration of *C. jejuni* was lower than 10^9 CFU/ml. In the combination of the two dyes, no clear signal was obtained for any of the samples (figure 11c).

(a) ***Emission of SYTO9 (green dye)***



(b) ***Emission of PI(red dye)***



(c) *Emission from both SYTO9 and PI*

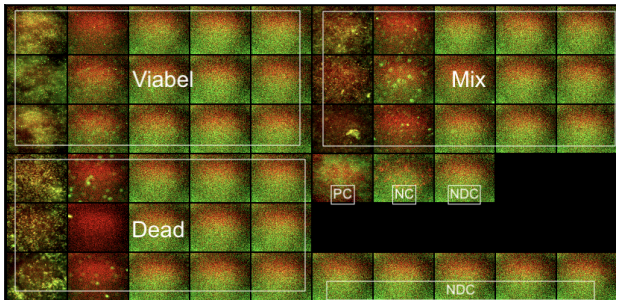


Figure 11: *Figures a and b illustrate the emission of the two stain individuals, while figure c illustrates the emission of both stains. All samples were done in triplicate.*

6.5 Effect of cleaning agents on *C. jejuni*

The experiment was constructed to investigate what effect the cleaning product has on the status of *C. jejuni*(alive, dead, or VBNC). The experiment was divided into three phases to easier follow the process of the experiment with the cleaning agents. All experiments were run as a single biological trial but with duplicates in the qPCR reaction. Two things were assumed through the experiment. First, the experiment started when the cleaning product and *C. jejuni* got mixed. Second, the reaction stopped when the test substance came in contact with the NB.

6.5.1 Phase 1: Pilot experiment with Kombinon Special

The experiment was generated to investigate the cleaning agent, Kombinon Special, effect on *C. jejuni* different states (live, dead and VBNC) Figure 12 illustrates the results obtained from the qPCR reaction over the pilot experiment. The Log(CFU/ml) was plotted against the time in minutes. The culturable cells (grey) represent the alive and culturable cells. Without PMA (blue) represents everything in the samples, while with PMA (red) represents only the viable cells. The Log(CFU/ml) for the culturable cells was calculated based on the detected colonies on the plate, while the Log(CFU/ml) from the samples with and without the PMA sample treatment was calculated from the Ct value obtained in the qPCR reaction.

All of the plated controls gave detectable colonies. No colonies were formed, when *C. jejuni* were exposed to 2% Kombinon Special, which indicated that all of the cells were in the VBNC state. There were no detectable colonies at time zero when *C. jejuni* were exposed to 0.5% Kombinon Special, but colonies were detected the rest of the time.

Figure 12a and 12b over the two controls illustrated that the Log(CFU/ml) for the culturable cells was around 40% higher than the Log(CFU/ml) obtained from the samples without PMA. Besides that does the results indicate for all three groups (culturable, with PMA and without PMA) no major variation over time, meaning the graph shows almost a straight line. When *c. jejuni* was exposed to Kombinon Special an instant decrease occurred in the number of viable *C. jejuni* cells for both concentration (figure 12c and 12d). At time zero, the 0.5% decreases around 25 %, whereas the 2% decreases around 36%. After five minutes, a further reduction in the viable cells happened, but after this time-point, it looked like it flattened out, possibly with a small increase in the number of viable cells over time.

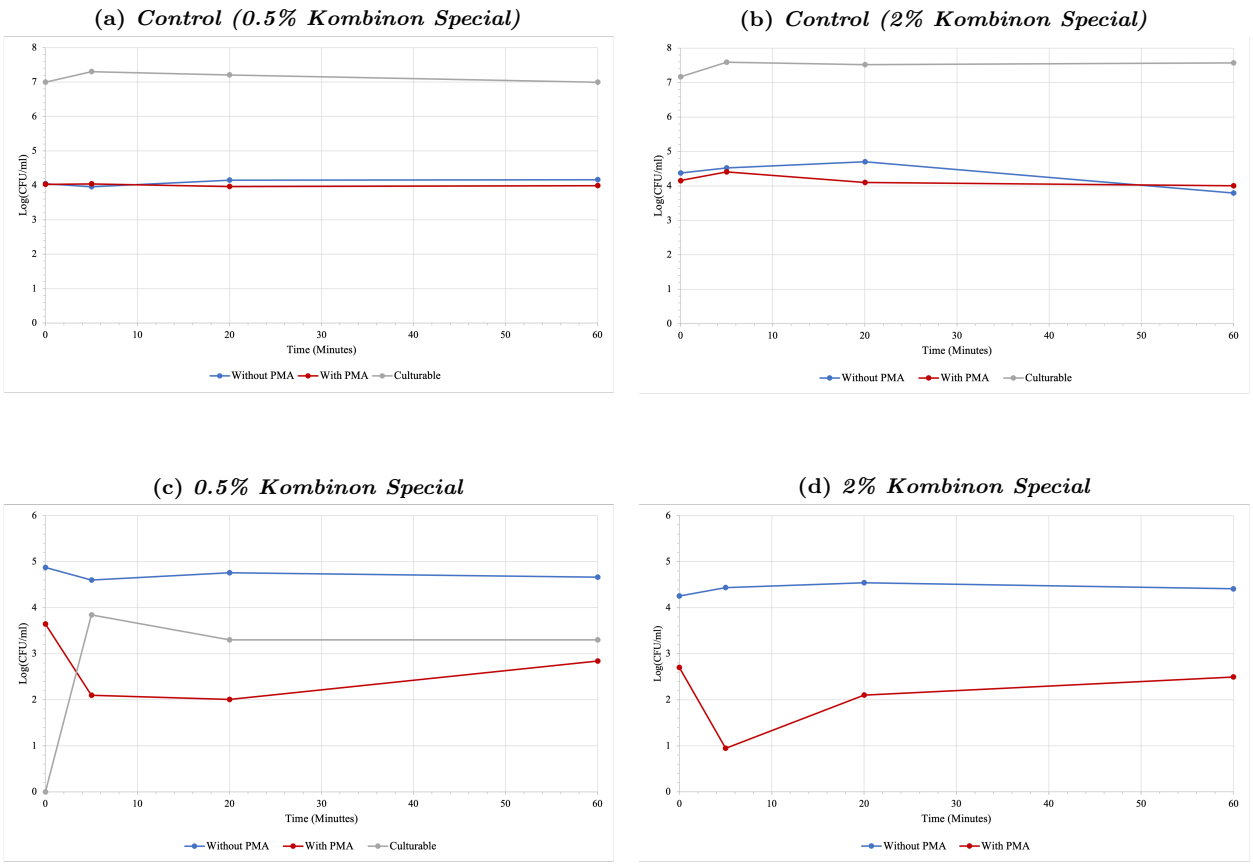


Figure 12: *The four graphs (Controls: a+b and test: c+d) illustrates the calculated Log(CFU/ml) from the pilot experiment over time in minutes. The culturable cell (grey) are determined using the plated assays. The Log(CFU/ml) over each sample with (red) and without(Blue) PMA sample treatment were obtained from the Ct value found using the qPCR reaction.*

6.5.2 Phase 2: Effect of washing step on the test procedure

W1 (table 3) was used as the reference washing test since that procedure was used in phase 1. The tests were compared individually between the samples with PMA and without PMA and compared to the reference. Both control and test for each experiment were used to investigate the influence of the washing step. The mixture of *C. jejuni* and Kombinon Special is referred to as "test."

Colonies were formed on all the controls, but none was formed from the test. The results is not shown. Figure 13b illustrate the samples with and without PMA for the five controls. Control from W4 gave the highest percentage different at around 14% between the samples with PMA and without PMA (table 8a). The percentage of the viability of *C. jejuni* cells of the other four controls was around 79-88% (table 7).

Table 7: *The percentage of viability of C. jejuni over the test and control from the five washing test*

	Test	**Control**
W1	30.2%	79.5%
W2	7.1%	85.6%
W3	17.2%	92.4%
W4	20.1%	66.9%
W5	20.3%	88.2%

(a) ***2% Kombinon Special.*** **(b)** ***Control***

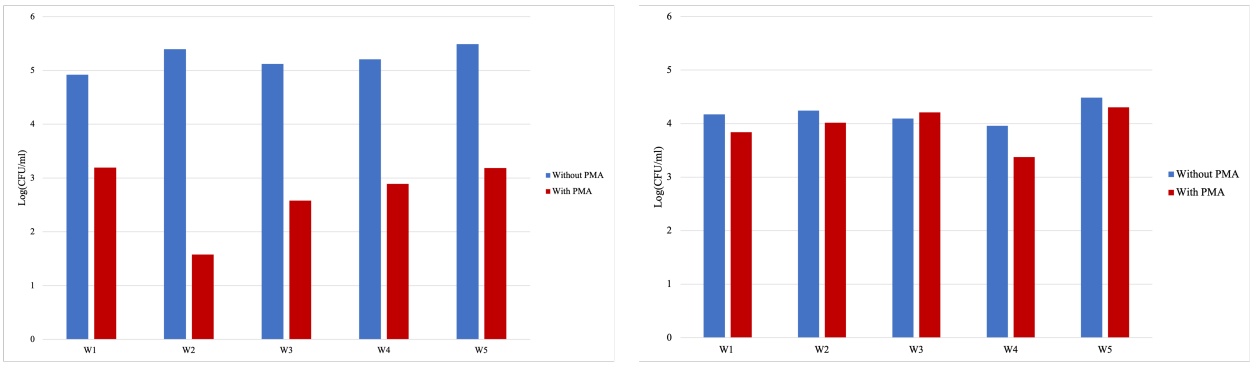


Figure 13: *Estimated Log(CFU/ml) after treatment of C. jejuni with 0.5% Kombinon Special. Both the five controls (a) and the five test (b) were presented as the PMA treated (red) and without PMA treatment (Blue) from each sample.*

The estimated Log(CFU/ml) of the test were illustrated in figure 13a. W5 gave the highest amount of Log(CFU/ml) for both the sample with and without PMA. The biggest reduction between a test own with and without PMA sample was found in W2 with a percentage difference at 70.8%. The highest percentage of the viability was found in W1 (table 7). The smallest difference was found between W5 and W1, with a percentage different at only 0.3 %.

Table 8: *The absolute percentage difference over the five washing tests, both control and test.*

Percentage difference				
	Control		**Test**	
	Between each test's own +PMA and -PMA	**Between W1 and each test (+PMA)**	**Between each test's own +PMA and -PMA**	**Between W1 and each test (+PMA)**
W1	7.9 %	–	35.1 %	–
W2	5.3 %	4.6 %	70.8 %	50.6 %
W3	2.8 %	9.6 %	49.6 %	19.2 %
W4	14.7 %	12.08 %	44.5 %	9.5 %
W5	4.0 %	12.07 %	42 %	0.3 %

(a) *The test is indicated as the mixture of C. jejuni and 2% Kombinon Special. Columns 2 and 4 [Between W1 and each test (+PMA)] indicate the absolute percentage difference between each test's PMA-treated and untreated samples. Columns 3 and 5 [Between each test's own +PMA and-PMA] indicate the absolute percentage difference between the reference (W1) and the rest of the test for the PMA-treated samples*

6.5.3 Phase 3: Experiment with cleaning product under different conditions and concentrations over time

Two different condition were tested: One without chicken juice and one with chicken juice. Each test had one control and one test. Table 4 describes the two condition with its individual control and test. The name of each condition will be used as reference to that specific condition, such as "Clean control" referring to the substrate with "*C. jejuni* and saline". The pH were taking for each concentration of Kombinon Special and gave the following results: 10 (0.1%), 12 (2%) and 13 (10%)

For each experiment, all conditions were plated. Colonies were formed on both controls, which indicated that all viable cells were culturable. The results were not shown because they were plated in a to high solution to count. For Clean and Dirty was colonies form at a concentration of 0.1 %, Kombinon Special. Figure 15a and 15b illustrates that when exposing *C. jejuni* to 0.1 % Kombinon Special more culturable cells were observed in Dirty then in Clean. The Log(CFU/ml) at time-point zero was around 7 for Dirty, whereas for Clean it were calculated to 4.3 Log(CFU/ml). After 24 hours, none of the *C. jejuni* cells were culturable in Clean, whereas Dirty still got 2.5 Log(CFU/ml). No colonies were formed in Clean or Dirty after increasing the concentration of Kombinon Special to 2% and 10%.

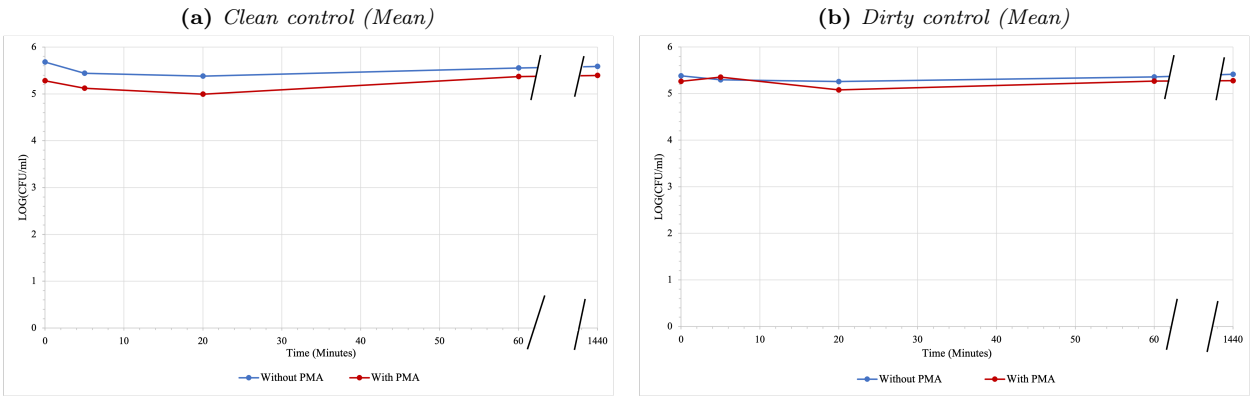


Figure 14: *The mean of the two different control conditions, Clean control, and dirty control. Their respective Log(CFU/ml) was plotted against the time in minutes. The individual results were shown in appendix 4. Both graphs present the results with PMA(red) and without PMA(Blue).*

Three replicates were constructed for Clean control and Dirty control, respectively. The mean from each was illustrated in figure 14. The individual results were shown in appendix 4. The difference between the samples with PMA(red) and without PMA(blue) was larger for the Clean control than the Dirty control, as shown in 14a and 14b. The percentage of viable cells was calculated from each time point of both controls, shown in 9. The overall percentage of viable cells was higher for Dirty control than Clean, where Dirty control varied from 88-96%, while Clean varied from 75-88%.

Table 9: *The calculated percentage of viable cells for the Clean control and Dirty control.*

Time (Minutes)	**Clean Control**	**Dirty Control**
0	75.9%	91.9%
5	79.9%	96.3%
20	76.4%	88.2%
60	88,1%	94.0%
1440	87,3%	90.9%

While being in the two conditions was *C. jejuni* exposed to three different concentrations of Kombinon Special (0.1%, 2%, and 10%). All of the samples without PMA obtained a Ct value from the qPCR reaction (figure 15), whereas only Dirty at 0.1% and both Clean and Dirty at 10% obtained a valuable Ct value from the qPCR after PMA-treatment (figure 15b, 15e and 15f).

Table 10: *The percentage of the viability of C. jejuni in Clean and Dirt for the three concentration of Kombinon Special.*

		Clean			Dirty		
Time (Minutes)	**Concentration of Kombinon Special**	**0.1%**	**2%**	**10%**	**0.1%**	**2%**	**10%**
0		67.5%	-	9.6%	88.6%	18.1%	34.3%
5		32.1%	9.0%	37.1%	96.1%	18.2%	46.8%
20		-	9.5%	20.5%	89.8%	47.1%	45.0%
60		-	-	32.3%	95.9%	-	37.6%
1440		-	11.7%	57.3%	28.8%	-	47.8%

(a) *"-" indicate that it was not possible to calculate the percentage of the viability.*

To quantify the viability of *C. jejuni* in each sample the percentage of the viable cell, were calculated for both Clean and Dirty in all three concentration (Table 10). The viability for *C. jejuni* was general higher in Dirty then in Clean for all three concentration.
In a 0.1% concentration of Kombinon Special more viable *C. jejuni* cells more viable were obtained in Dirty compared to Clean. At time-point zero a 4.2 Log(CFU/ml) were calculated from the sample with PMA in the Clean, whereas the Dirty were calculated to 5.3 Log(CFU/ml). After five minutes there was a reduction of the viable cells by half in the Clean, while the Dirty still showed a viability at 96.1%. Between 1 hour and 24 hours, there was a reduction in the viability of the cell from 95.9% to 28.8% in the Dirty.

As mentioned above was no colonies formed when increasing the concentration of Kombinon Special to 2% and 10%. It was therefor assumed that all of the viable cells obtained from the samples with PMA were in a VBNC state. After exposing *C. jejuni* for a 10% concentration of Kombinon Special there were still 3.3 Log(CFU/ml) viable cells obtained in the Dirty after 24 hours. The clean indicated that between 1 hour and 24 hours of exposing *C. jejuni* to 10% Kombinon Special the viable cells increased from 3.1 Log(CFU/ml) to 4.3 Log(CFU/ml). Beside the value obtained after 24 hours, was the the viability higher for the Dirty than the Clean for *C. jejuni* in a 10% concentration of Kombinon Special (figure 15e and 15f.

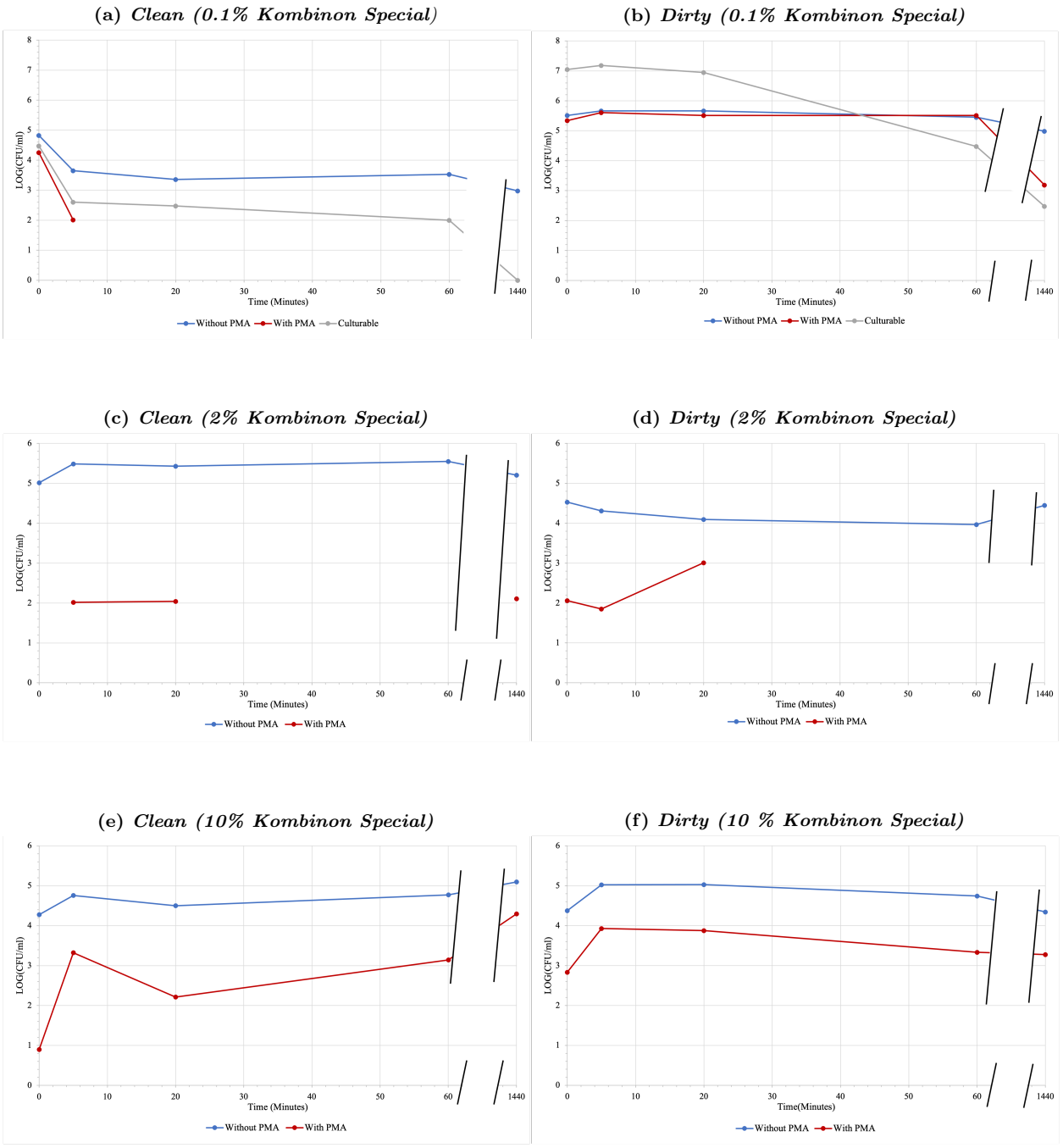


Figure 15: *The six plots illustrate the estimated Log(CFU/ml) over time in minutes. For each concentration (0.1%, 2% and 10%) was, two different conditions tested: Clean (C. jejuni and Kombinon Special) (Graph: a, c, and e) and Dirty (C. jejuni, 5% chicken juice and Kombinon Special) (Graph: b, c, and f). The Log(CFU/ml) over the samples with(red) and without(blue) PMA treatment was calculated from the obtained Ct values in the qPCR reaction. The Log(CFU/ml) over the culturable cells (grey) was calculated using the plating assay.*

7 Discussion

7.1 Comparison of DNA extraction methods

The three DNA extraction methods were compared to investigate if the right DNA extraction methods were used. The original protocol used the FAST DNA Stool Mini Kit, but other methods were tested on the samples due to undetectable DNA concentration when using the Nanophotometer instrument. The results from the Nanophotometer instrument are not shown. The experiment was short but could have been optimized by testing all three methods at least twice as biological tests and in the qPCR reaction.
Nevertheless, the results in table 5 indicated that the Cell lysis did not gave any detectable signal in the qPCR reaction. The different between the C_t results from FAST DNA Stool Mini Kit and DNeasy Blood & Tissue Kit did not gave the biggest difference, if the result from DNeasy Blood & Tissue Kit viable cell in 10^{7} CFU/ml was excluded.
In the end, the FAST DNA Stool Mini Kit was chosen based on the fact that the method was faster than the DNeasy Blood & Tissue Kit. The FAST DNA Stool Mini Kit were incubated for 10 minutes, whereas DNeasy Blood & Tissue Kit were incubated for 60 minutes.

7.2 Evaluation of LIVE/DEAD *Bac*Light detection of *C. jejuni*

In combination with the traditional plating methods other studies has successfully been capable to detect bacteria in the VBNC state using the LIVE/DEAD *Bac*Light methods. The methods distinguish the viable (green) and dead (red) cells using two different stains. By measuring the fluorescence intensity of the two dye, the total amount of cell and the viable cell can be found. No measurement was done doing the experiment, only the overall picture of the fluorescence intensity were investigated (figure 11).

The PC gave a strong fluorescence intensity, where as the NC was slightly decreased (figure 11a and 11b). As excepted was no signal obtain in the none-dye-controls for either of the dyes. The overall results from the experiment is not very clear since both stain obtained signal in the viable and dead cell samples. The combination of the two dye should have decreased the SYTO9 signal leaven all the dead cell stained red. By looking closer at the samples with only the dead cells in figure 11c both green and red emission has occurred. A 1:2 (SYTO9:PI) raio between the two stain was used, but the result may indicate that a higher ratio was needed to obtain the wished result. On the other hand Magajna and Schraft (2015, [4]) were using LIVE/DEAD *Bac*Light in conjunction with confocal scanning laser microscopy and got successfully results when using a ratio 1:2 (SYTO9:PI). In the study they investigate LIVE/DEAD *Bac*Light capability to detect *C. jejuni* cells in the VBNC state, and compared it up against their results using the PMA-qPCR methods. The initiated concentration of *C. jejuni* was at around 7 CFU/ml in Magajna and Schraft (2015, [4]), whereas no clear signal was obtained for *C. jejuni* with a concentration of 10^{9} CFU/ml in this study. Overall does

the results from this experiment indicate that furthered reaches were needed, but based on the result from other studies should it be possible to use LIVE/DEAD *Bac*Light methods to detect *C. jejuni* in its different states.

7.3 Evaluation of PMA-qPCR detection of *C. jejuni*

PMA was known for binding to DNA from cells with compromised membranes. This means when using the PMA-qPCR methods to distinguish between live and dead cells, it must be assumed that cells with intact membranes were viable. The reason was that PMA only provide knowledge about the integrity of the cell membrane and not anything about the bacteria's pathogenicity, infectivity, or metabolic activity [18]. A study from Krüger *et al.* (2014,[37]) has suggested that cells harboring intact membrane could be called "intact and putatively infectious units" (IPIU), which comprising CFU and VBNC. This definition could help enhance the detection capability of *Campylobacter* through the whole food chain and maybe uncover some of the unknown transmission routes of the pathogen.

This study defines the VBNC cells as the PMA-treated cells subtracted by the culturable cells. If no colonies were on the plate, all of the PMA-treated cells were indicated as VBNC.

The protocol for the PMA-qPCR methods was created by taking inspiration from the study Josefsen *et al.* (2010, [17]). Their study successfully obtained Ct values from all the samples in the generated 10-folded dilution series of *C. jejuni* CCUG 11284 in chicken rinse (10^2 to 10^6 CFU/ml). Even though a different *C. jejuni* strain was used and the fact that the bacteria were not rinsed in chicken in this study; there was a major difference in how many of the samples which gave a valuable Ct value. As mentioned, all of the tested dilutions gave a Ct value in the Josefsen *et al.* (2010, [17]) study, whereas only 10^5 to 10^7 CFU/ml gave detectable Ct values from the heat-treated and 10^4 to 10^7 CFU/ml from the viable cells in the present study. Different things may have influenced these results, such as DNA loss, too few tests, or the concentration of PMA.

Loss of DNA may occur throughout the whole process, such as through DNA extraction or when pipetting for the qPCR reaction. To monitor the loss of DNA, Pacholewicz *et al.* (2019, [38], has developed an internal sample process control (ISPC). The ISPC was added to each sample and contained a specific number of peroxide-killed *C. sputorum* cell. ISPC was added right before the PMA treatment of the samples. By adding ISPC to the sample, it would be possible to calculate the accurate number of DNA loss throughout the whole process.

In this study, the experiment to test how the PMA influences *C. jejuni* was only done as a single biological trial, but three qPCR reactions were generated from it. To get a more accurate results more then one biological trial were needed. Doing more than one biological trial gives a more are reliable results

Using the optimal PMA concentration may also influence the outcome of the results. Treating the sample with a too high PMA concentration may lead to the PMA starting to inhibit the DNA amplification of viable cells, resulting in an underestimation of the viable cells. A concentration of PMA that is too low could lead to an overestimation because the PMA may not be able to inhibit the DNA amplification of the dead cells fully. The concentration of PMA vary from study to study. The product information about PMA recommend a concentration of PMA at 50 µM [19]. The used PMA concentration in this study was based on the Josefsen *et al.* (2010, [17]) study, together with the study from Lv *et al.* (2020 [15]). In Josefsen *et al.* (2010, [17]), they used a concentration of PMA at around 23.71 µM, whereas at Lv *et al.* (2020 [15]) they ended up with using a PMA concentration at 20 µM. Lv *et al.* (2020 [15]) tested different concentrations of PMA on viable and heat-inactivated cells at a 6 Log(CFU/ml). Their results showed that by using a concentration of PMA at 20µM, PMA could effectively inhibit the DNA amplification of the dead cells without influencing the viable cells significantly. Besides using different PMA concentrations, the two studies used different strains of *C. jejuni*. Josefsen *et al.* (2010, [17]) used *C. jejuni* (CCUG 11284), where as Lv *et al.* (2020 [15]) where using different *C. jejuni* strains here among *C. jejuni* (ATCC 33560). Bacterial strains within the same species may exhibit genetic variation, meaning to find the optimal PMA concentration for the specific strain used in a study, different concentrations must be tested to find the most promising for that specific one.

As figure 8 indicated, no signals were obtained from the qPCR reaction of the heat-inactivated *C. jejuni* cells after they were treated with PMA. Based on that it may look like the PMA successfully inhibited the DNA amplification from cells with a compromised membrane. To determined if the PMA concentration was appropriate the dCt were calculated from the viable cells. As figure 9 illustrate gave two of the viable samples not acceptable dCT value. In table 6 was shown that the sample for 10^6 CFU/ml and 10^4 CFU/ml gave a dCT at 3 and 2.2, receptively. This indicate that the PMA may penetrate the living cells, hence lowing the live cells estimation. If that was the case a lower PMA concentration may be the solution. Either way further testing is needed to find the right DNA concentration for the *C. jejuni*

7.4 Standardcurve over PMA treated cells

Three standard curves were produced based on three replications of qPCR reactions on the same set of samples. The samples were viable PMA treated *C. jejuni* cells from a dilution series range from 10^1 to 10^7 CFU/ml. Figure 10 illustrates the chosen standard curve, whereas the two others are in appendix 3. The viable *C. jejuni* cells with PMA were chosen for the standard curve, because the PMA-qPCR method should be used to detect the VBNC cells of the bacteria. It was assumed that the sample with PMA only detected the viable cells, and the sample without PMA indicated everything in the sample. Therefore were, the sample with PMA the most obvious choice.

The efficiency and R^2 of the three curves were compared to determine which of the standard curves was most efficient to use when calculating Log(CFU/ml) for unknown samples. The reason for looking into these parameters was that both were important when quantifying if a qPCR reaction was acceptable. The efficiency of the qPCR determined how well the target DNA was amplified during each cycle. In contrast, the R^2 (coefficient of determination) describes how well the Ct value aligns with the expected concentrations [39]. As mentioned earlier, efficiency is accepted at 90-100% whereas R^2 needs to be ≥ 0.98 [40].

It was determined that the efficiency of the qPCR had the highest influence on which standard curve was chosen. The reason for that was the important impact on the quantification of the target DNA in an unknown sample [39]. Because the PMA-qPCR method should be used to detect *C. jejuni* cell in the VBNC state, the qPCR needs to be effective and precise when targeting the DNA. Therefore, the efficiency of the qPCR reaction needed to be as high as possible. By looking closer at the three generated standard curves, only one gave an efficiency above 90%, which determined the outcome of the chosen standard curve. Even though the R^2 was not above the acceptable value, the standard curve in figure 10 was chosen and used for conversion of Ct to cell count estimation for future experiments.

7.5 Evaluation of the cleaning agents effect on *C. jejuni*

Due to time-limited only one cleaning product was tested throughout the whole experiment. If there had been more time, more cleaning products would have been tested using the last experiment described in phase three. Through the experiment was it assumed that the sample with PMA was all viable cells, whereas the sample without PMA contained both dead and viable cells.

7.5.1 Phase 1: Evaluation of pilot experiment

The first experiment with *C. jejuni* and the cleaning product Kombinon Special was a pilot experiment to test was effect Kombinon Special had on *C. jejuni*.

When exposing *C. jejuni* to Kombinon Special in the concentration 0.5% and 2% most of the bacteria does not get killed (Figure 12c and 12c). Most of the *C. jejuni* cells were still culturable at a 0.5% concentration, whereas all of them had entered the VBNC state when the concentration was reached to 2%. Figure 12c illustrate that over time was there in decrease in the culturable cells, indicating that over time more and more of the cells had enter the VBNC cells. This is based on the fact that the sample with PMA were increasing whereas the culturable cell count were decreasing. Since the cell was exposed to a constant stress from Kombinon Special it was excepted that more and more cells would enter the VBNC state.

The controls for both experiments indicate no significant difference between the samples with and without PMA sample treatment (figure 12a and 12b). Nevertheless, it looks like there was a loss of DNA in the process. For both controls was it observed that the calculated Log(CFU/ml) over the culturable cells where slightly higher than the Log(CFU/ml) obtained from the qPCR reaction. Beside DNA loss may the results also indicate an underestimation of the PMA-qPCR methods, based on the fact that the PMA concentration may was to high.

7.5.2 Phase 2: Evaluation of the washing step effect on the procedure

The experiment was constructed to investigate if the amount of NB and wash with saline affected the outcome of the combination of cleaning product and *C. jejuni*. Three aspects of the washing step were tested: The amount of NB, the times of washing with saline, and the amount of saline. The setting of the five test were found in table 3.

All five controls gave culturable cells, whereas no colonies were formed from the five tests. It was therefor assumed that all of the viable *C. jejuni* cells obtained from the five test were in their VBNC state.

The results from the controls indicated that the NB alone does not affect the *C. jejuni*. Even by double the amount of NB, no significant decreasing occurred in the viable *C. jejuni* cells. The largest difference between the samples with PMA and without PMA was found in W4.

The amount of NB seems to influence the outcome of viable cells in the combination of 2% Kombinon Special and *C. jejuni*. By double the amount of NB, the viable cells go from 3.19 Log(CFU/ml) to 1.58 Log(CFU/ml), a reduction of 50.6% 13a. As mentioned, did the NB not affect the bacteria. Still, in combination with cleaning products, it may not just stop the reaction of the cleaning product but also start affecting the bacteria if the ratio is too large. When testing the effect of disinfectant using the EU standards, both DS/EN 1656:2019, [41], and DS/EN 14349:2012,[35] use a much higher amount of NB compared to the test substrate. DS/EN 1656:2019 used a ratio of 8:1, (NB:test substance), whereas DS/EN 14349:2012 used a ratio of 10:1 (NB:test substance). The results from W2 in figure **??** indicate an increase in dead cells compared to W1. It's unclear what specific had caused the increase in dead cells, but the ratio between NB and test substrate seems to play an unspecific role.

Experiments W3 and W4 were produced to investigate if the number of washes with saline affected the outcome. The results gave an ambiguous result. For W4 gave the controls a decrease in the viable cells, whereas the test have a increase. On the other hand was the largest decrease found in W3. It was expected that fewer cells may was obtained in W4 then W3, since some of the bacteria may have been washed away. The control gave therefore a more realistic picture of the process, then the test. Nevertheless, it is unclear what effect the amount of washes with saline had on the process.

Increasing the amount of saline from one ml to two ml in the washing step seems to increase the total amount of cells. Both the control and test from W5 had obtained a higher Log(CFU/ml) for both viabel cells and the total cell count compared to W1. This may indicate that more cells were gained in the final sample using a higher volume of saline.

In retrospect, it would have been more favorable to use 2 ml of saline in the washing step, based on the fact that both the control and test gained a higher amount of total cells count. Nevertheless, the washing step used for future experiments was determined to be W1. This was done, because the percentage of viability of *C. jejuni* was highest for W1.

7.5.3 Phase 3: Evaluation of *C. jejuni* influence of Kombinon Special in different conditions and concentrations over time

After the washing experiment was done, the ratio between the cleaning product and *C. jejuni* was changed. This was done for two reasons. First, when the farms use the cleaning product, it's diluted in tap water, meaning it's diluted before coming into contact with the bacteria. The cleaning product was diluted in saline instead of Tap water to avoid stressing the bacteria even more. Secondly, in DS/EN 1656:2019, [41], when testing a disinfectant, they use a ratio of 1:1 between the bacteria substance and the test substance. To follow the guideline used in DS/EN 1656:2019, the ratio between the cleaning product and *C. jejuni* were therefore changed to 1:1.

C. jejuni were exposed to two different conditions: a clean attempt and a dirty attempt. The dirt attempt was created for trying to stimulate the conditions on a chicken farm. To create the dirty condition *C. jejuni* was exposed to 5% chicken juice. This gave four different test samples, two controls(Clean control and Dirty control), and two tests (Clean and Dirty) (Table 4. As mentioned in section 6.5.3 will, the names of the four tests be used to compare the different results.

The two controls (Clean control and Dirty control) were used to investigate if the influence of chicken juice would have an effect on *C. jejuni* viability over 24 hours at room temperature. The PMA-treated cells from the Dirty control in figure 14b are clearly more aligned with the samples without PMA, compared to the Clean control in figure 14a. Based on the percentage viability of the two controls, there was an overall pattern that more of the *C. jejuni* cells were viable after 24 hours in the Dirty control compared to the Clean control. Based on that assumption it looks like the *C. jejuni* grows better in combination with Chicken juice than without. In T. Birk *et al.* (2003, [36]) they studied the *C. jejuni* strain (NCTC11168) capability of surviving on different agar plates, whereas one of them contained chicken juice. The experiment was constructed by incubating *C. jejuni* at two different temperature (5℃and 10℃), and was examined over 30 days. Here they discover that chicken juice prolonged the viability of *C. jejuni*, which correspond with the result obtained in figure 14b. Moreover,

it does not look like the chicken juice affect the PMA treatment and the following qPCR reaction in a negative way. Since all of the samples gave detectable Ct values from the qPCR reaction.

When exposing *C. jejuni* to a 0.1% concentration of Kombinon Special, was it still possible to detect 3.2 Log(CFU/ml) of culturable cells after 24 hours, when *C. jejuni* was in combination with 5% chicken juice (Figure 15b). Without 5% chicken juice the culturable level of cells where down to 2 Log(CFU/ml) after 1 hour, while none culturable cell where detected after 24 hours (Figure 15a). The calculated culturable cell were around 1.5 Log(CFU/ml) higher than the obtained Log(CFU/ml) from the samples with and without PMA in the Dirty condition at time point zero. This difference between the calculate Log(CFU/ml) could be due to various factors. The Log(CFU/ml) of the culturable cells are calculated based on the plating assay, whereas the sample with and without PMA were calculated based on the obtained Ct value from the qPCR reaction. Since this were two difference ways of calculating the Log(CFU/ml) the results may not add up. Another thing could be, as mentioned in section 7.3, that DNA loss may occur though the process of PMA-treatment, DNA extraction and the qPCR reaction. Nevertheless, the result from the Dirty condition with a 0.1% concentration of Kombinon Special indicate that all of the *C. jejuni* cell were in their culturable state after been exposed for 20 min. After 1 hour has 18.8% of the viable cell enter the VBNC state, while after 24 hours 22.2% were in the VBNC state. This indicate that more and more is entering the VBNC state over time.

The results from both the Clean and Dirty with 2% Kombinon Special indicate clearly a human error, since most of the results from the PMA-treated samples was not able to obtain any valuable Ct values from the qPCR reaction. Together with that was it already investigated how *C. jejuni* react to the clean condition in phase 1(12d and there all of the sample with PMA gave valuable Ct values. The error could have happened in difference steps of the process, but when doing the DNA extraction it was noted that the homogenization and afterwards the first heating process may had needed more time. The combination of cleaning product and chicken juice may influence the homogenization of the inhibitEX buffer with the bacteria substrate. Nevertheless, figure 15c indicate that there were still viable *C. jejuni* cells after 24 hours of been exposing to 2% Kombinon Special in the Clean condition. Since figure 15b indicate the chicken juice may prolonged the viability of the *C. jejuni* it would therefor have been excepted to see the same results when increasing the concentration to 2%. It may therefor be assumed that there still would be viable cells after 24 hours of exposing of 2% Kombinon Special in the Dirty condition.

By increasing the concentration of Kombinon Special to 10% VBNC cell were obtained from both the Clean and Dirty condition. Figure 15e over the Clean results obtained from the qPCR reaction, indicate that after 24 hours of been exposed to 10% Kombinon Special the viability of *C. jejuni* was almost as high as the initiated total amount of cell in the sample

(Without PMA-treatment). On the other hand the results from the Dirty increase from zero to five, where it afterwards diminishes quietly over time (figure 15f). After 24 hours the viable cells were calculated to 3.3 Log(CFU/ml).

Kombinon Special was known to be an alkaline foam cleaning agent, which does not contain any biocidal active substance[27]. This explain why most of the *C. jejuni* cell does not get killed, but go into the VBNC state. The high pH developed a stress environment for the *C. jejuni* when gets it to activate the survival mechanism. According to a study from Kelana *et al.* (2002, [42]), were they investigated *C. jejuni* (strain ATCC 35921) in different concentration of pH, where *C. jejuni* still capable of being culturable after being exposed to a pH at 8.5 at a temperature of 22℃after one day. In this study were *C. jejuni* exposed to a pH 10 and above.(0.1% = 10pH, 2% = 12 pH and 10% = 13pH) It was shown that *C. jejuni* still were culturable at a pH around 10 after exposing it for 1 hour without 5% chicken juice, whereas in the combination with 5% chicken juice they were still culturable after 24 hours. When increasing the pH to 12 or above, none of the cell were culturable but there were still viable cell detected using the PMA-qPCR methods. This indicate that if the cleaning product where tested on *C. jejuni* with the tradition culturable method, the test would had shown that *C. jejuni* was dead when increasing the pH to 12. This indicate that PMA-qPCR can be used to detect *C. jejuni* in its VBNC state, when it is assumed that all of the sample with PMA are viable cells.

This means if the farm only uses Kombinon Special, when cleaning between the broiler rotations *C. jejuni* will not be killed. It may go into VBNC state, but if favorable condition is reach it may become virulence and start infection the next broiler flock.

8 Conclusion

In this master thesis the protocols for of PMA-qPCR and *Bac*Light was examied for their ability to discriminate between viable and heat-inactivated *C. jejuni* cells based on a 10-fold dilution series (10^1 to 10^7 CFU/ml). This study found that the DNA of the heat-inactivated cells were modified making them unable to be amplified using qPCR reaction. On the other hand was it unclear if the LIVE/DEAd *Bac*Light methods cuold differentiate live, dead and VBNC *C. jejuni* cells sufficiently. An underestimation of viable cells was seen with PMA treatment, when comparing with the culturable cells. This may indicate that the concentration of PMA using in this study was too high, but future studies needed to verify that. Even though the PMA concentration was not optimal, a standard curve over the samples with PMA was produced. The standard curve was used to calculate the viable cells in an unknown sample

The NB did not affect the bacteria but in combination with the Kombinon Special it may affect the viability of the cells. The amount of NB may therefore have an influence on the washing step when testing the effect of cleaning agents on *C. jejuni*. The amount of saline may have a positive effect on the total outcome of the cell count, but future testing are needed to give a clear picture of that.

The tested cleaning product, Kombinon Special, was not capable of killing *C. jejuni* according to the PMA-qPCR methods. Instead, exposure of Kombinon Special seemed to stimulate *C. jejuni* into entering the VBNC state. By exposing the *C. jejuni* to 5% chicken juice the viability was prolonged even further. This may indicate that *C. jejuni* in the combination with chickens juice, as a proxy for a dirty environment, may improve survival. The PMA-qPCR method can be developed to assess the effectiveness of cleaning agents to inactivate *C. jejuni*, because it has the ability to detect the bacteria in its VBNC state. This can be a major advantages in the action towards fighting *C. jejuni*.

9 Future perspectives

The thesis has proved that PMA-qPCR is capable of detecting *C. jejuni* in its different states(live, dead and VBNC), but improvements of the method is needed. The concentration of PMA needs to be optimized to avoid underestimating the viable cells. This can be done using the PMA-qPCR method on *C. jejuni* at different concentrations and calculated the dCt to find the optimal concentration.

Based on the results from this study, it is unclear if the LIVE/DEAD *Bac*Light methods are capable of detecting *C. jejuni* in its different states (live, dead and VBNC). Future studies with different concentrations of the two stains must be done to find the right ratio to detect *C. jejuni* using the methods.

The PMA-qPCR methods have great potential for determining a cleaning agent's effect on *C. jejuni* viability. The experiment in phase 3 showed promising results, although repeated experiments are needed to confirm the results. However the developed protocol can be used on other cleaning agents. By using the experiment it is possibleto assess the ability of cleaning agents to effectively inactive *C. jejuni*. In the future, this experiment could be compared with a field experiment, where the different cleaning agents used on a broiler farm could be tested to see their effect on *C. jejuni*.

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11 Appendix

Appendix 1

Table 11: *The C_t value from the two biological tests of FAST DNA Stool Mini Kit.*

DNA extraction method	Biological test	Initial CFU/ml	Viable cells		Heat-treated cells	
			- PMA	+ PMA	- PMA	+ PMA
FAST DNA Stool Mini Kit	1	10^7	16,98	17,67	21,04	UND
	2	10^7	15,77	18,15	21,61	UND
	1	10^6	19,74	20,72	23,1	UND
	2	10^6	20,53	21,98	25,6	UND

Appendix 2

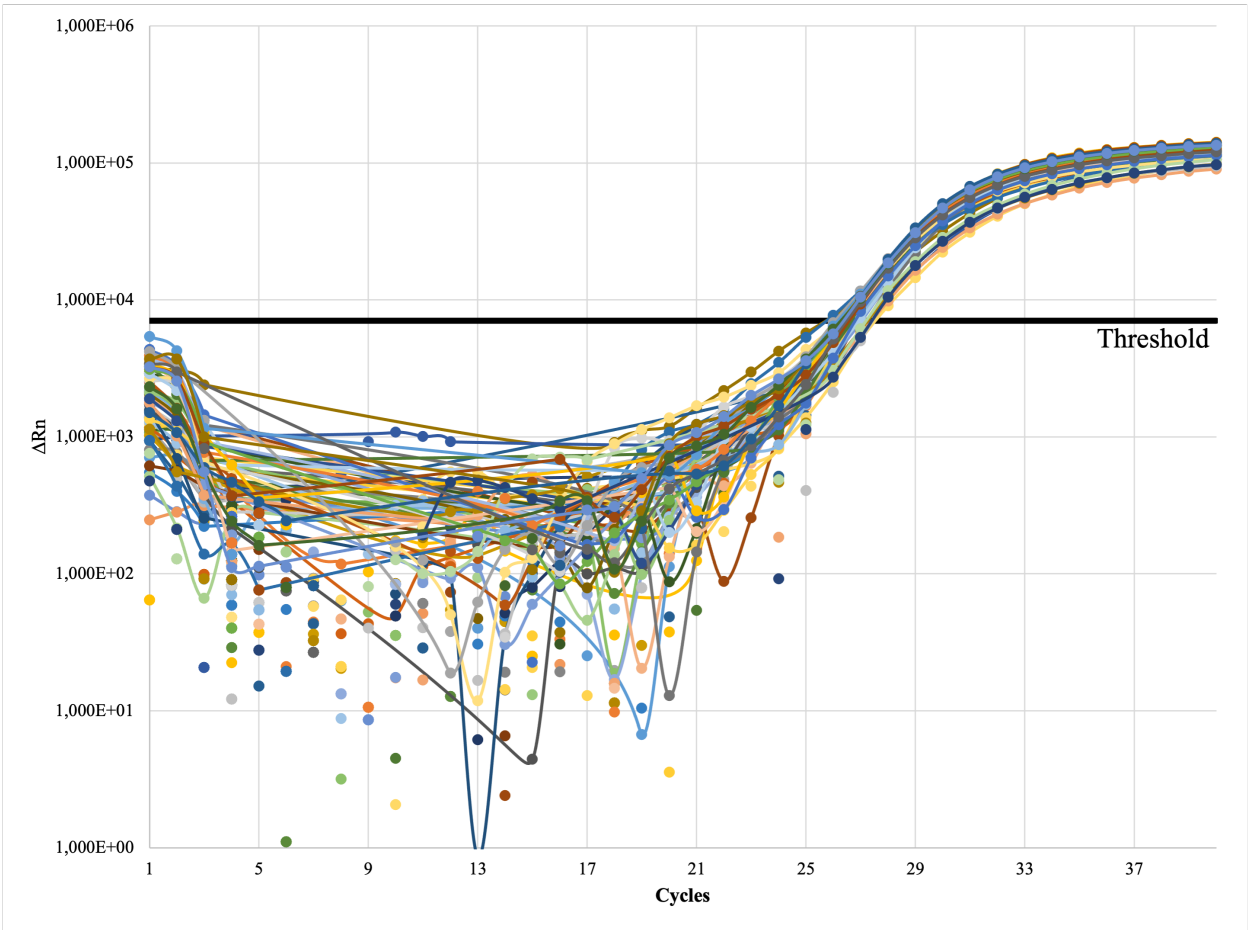
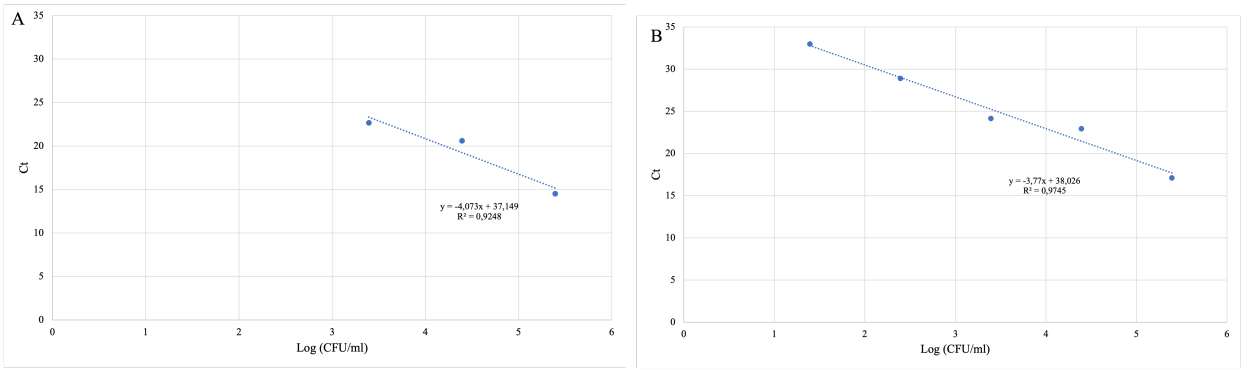


Figure 16: *Amplification plot over the IAC signal from each sample, both PMA and without PMA. The thick black line indicates the threshold.*

Appendix 3

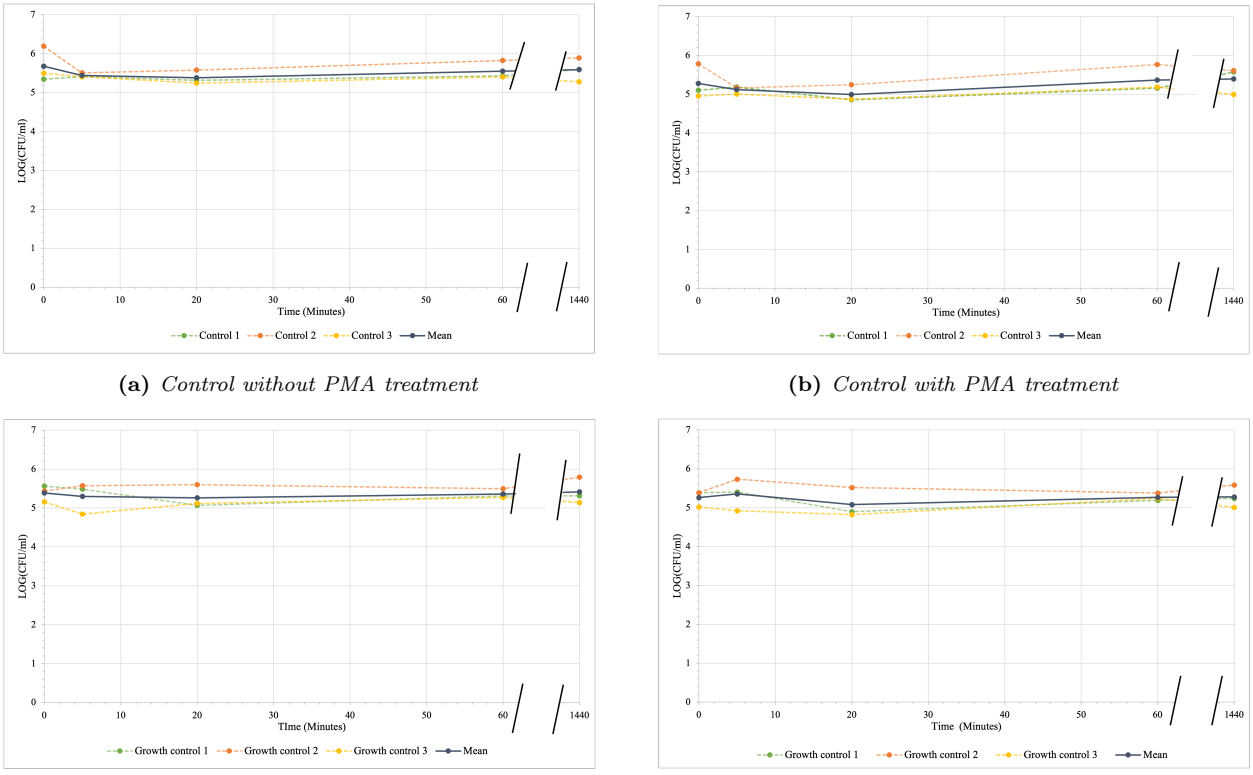


(a) *Ct values from the obtained qPCR reaction on viable PMA treated C. jejuni. The Ct value are plotted against the LOG(CFU/ml)*

(b) *Ct values from the obtained qPCR reaction on viable PMA treated C. jejuni. The Ct value are plotted against the LOG(CFU/ml)*

Figure 17: *The other two standard curve produces over other qPCR reactions but the same samples as used in figure 10*

Appendix 4



(a) *Control without PMA treatment*

(b) *Control with PMA treatment*

(c) *Growth control without PMA treatment*

(d) *Growth control with PMA treatment*

Figure 18: *The three individual and their means plotted from each sample with(B and D) and without(A and C) PMA treatment. All four graphs present the three controls in green, orange, and yellow, whereas the mean is present in grey. They are plotted with the LOG(CFU/ml) against time in minutes.*