Master Thesis

Ability of *Campylobacter* to persist in a viable but nonculturable state (VBNC)

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30 ECTS

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Preface and acknowledgement

This thesis is the product of 30 ECTS, as the final product of the masters in Biotechnology at the Technical University of Denmark (DTU), National Food Institute. The project was carried out at the research group Food Microbiology and Hygiene, from September 2023 to February 2024. Prior to this thesis, a special course presented as a literature study was conducted in collaboration with the research group. With a great focus on the viable but nonculturable state of *C. jejuni*. Parts of the experimental work for this thesis were conducted in collaboration with Sofie Pauly Kofoed Mose, for both our theses. First, I would like to thank my supervisors, Nao Takeuchi-Storm, Brian Lassen, and Annette Nygaard Jensen, for letting me do the project and for guidance through it through weekly meetings. Furthermore, I would like to thank the laboratory technicians Pia Engelsmann, Margrethe Carlsen, and Resadije Idrizi for helping me in the laboratory and for always answering all my stupid questions. I would like to thank Sofie for the collaboration of joyful and fascinating experimental work for both of our theses.

Abstract

Campylobacter species are one of the leading causes of food-borne gastrointestinal infections with poultry meat serving as the primary source of transmission. Detection of *Campylobacter* is currently dependent on culturing methods. Hence, when exposed to environmental stressors, *Campylobacter* can adopt a survival state known as viable but nonculturable (VBNC). The VBNC state challenges the detection of the bacteria, as it cannot be cultured using conventional media. Despite being unable to grow, the VBNC cells remain metabolically active and retain membrane integrity. VBNC bacteria have been found to resuscitate when exposed to favorable conditions, posing a risk to human health. Thus, alternative non-culturing dependent methods have been investigated, to determine VBNC bacteria.

This study assessed the combination of propidium monoazide (PMA) and quantitative polymerase chain reaction (qPCR) ability to differentiate viable and heat-inactivated *C. jejuni* cells. The differentiation was obtained using a PMA concentration of at 20 µM. A standard curve for *C. jejuni* cells with PMA-qPCR was established to quantify viable cells from unknown samples. Additionally, the LIVE/DEAD *Bac*Light membrane integrity method was tested, where a ratio of (1:2) of the two DNA binding dyes, SYTO 9 and propidium iodide (PI), was applied. The LIVE/DEAD *Bac*Light ineffectively distinguished the viable and heat-inactivated *C. jejuni* cells. This may be attributed to the dye ratio utilized. Therefore, further investigation is needed.

Various environmental stressors were investigated to assess the potential induction of the VBNC state. Desiccation for 48 h of *C. jejuni* in 0.9% NaCl resulted in VBNC cells. Osmotic stress by 7% NaCl and tap water, indicated to induce the VBNC state of *C. jejuni* after 7 days of incubation. *C. jejuni* was exposed to nutrient deprivation by incubation in 0.9% NaCl over a 14-day period, where VBNC cells were observed. The addition of chicken juice to the bacterial solutions to simulate a dirty environment containing nutrients, showed an increase in viable cells when exposed to the stressors compared to without chicken juice. The formation of VBNC *C. jejuni* cells indicate that the environment at the broiler farm can induce the VBNC state of *C. jejuni*. The formation of the VBNC cells can potentially lead to underestimation by traditional culturing-dependent methods.

Dansk resume

Campylobacter er en af de førende årsager til fødevarebårne mave-tarm infektioner, hvor kylling er den primære smittekilde. Hvis *Campylobacter* udsættes for miljømæssig stress, kan de gå ind i et overlevelsesstadie,, hvor de er levedygtige men ikke kan dyrkes. I dette stadie er *Campylobacter* stadig metabolisk aktiv og bevarer celle membranens integritet. Cellerne kan dog ikke dyrkes, og derved undgår de påvisning ved de traditionelle dyrkningsmetoder der anvendes i fødevareindustrien. Forskning har påvist at celler i dette overlevelsesstadie, kan genoplive og forårsage sygdom hvis de eksponeres for favorable forhold, hvilket derved udgør en risiko med menneskets sundhed. Derfor søges efter metoder til påvisning af *Campylobacter* der ikke er dyrkningsbaseret.

Dette studie undersøgte brugen af propidium monoazide (PMA) og kvantitativ polymerase kædereaktion (qPCR), til at påvise levedygtige men ikke dyrkningsbare *Campylobacter* celler, samt at adskille levedygtige og varmebehandlede (døde) celler, ved brug af PMA med en koncentration på 20 µM. Derudover blev en standardkurve etableret for metoden, til at kunne kvantificere levedygtige celler i en ukendt prøve. LIVE/DEAD *Bac*Light er en metode der er baseret på membran integritet, hvor to fluorescens farver SYTO 9 og propidium iodide (PI) farver henholdsvis levedygtige og døde celler. Denne metode blev undersøgt ved at adskille levedygtige og varmebehandlede *Campylobacter* celler, hvor farve forholdene var 1:2 (SYTO 9:PI). Det var dog ikke muligt at adskille cellerne, dette kan skyldes farve forholdene og skal derved undersøges nærmere.

Dette studie testede tilstedeværelsen af bakterier i overlevelsesstadiet (levedygtige men ikke dyrkningsbare), ved at udsætte *C. jejuni* celler for forskellige miljømæssige stress faktorer. Her blev det observeret at *C. jejuni* gik ind i overlevelsesstadiet ved udtørring i 0.9% NaCl over 48 timer. *C. jejuni* blev også udsat for osmotisk stress, hvor *C. jejuni* blev udsat for 7% NaCl og vand fra hanen. Resultaterne indikerer at overlevelsesstadiet blev observeret efter 7 dages inkubation. Derudover blev *C. jejuni* udsat for et forsøg hvor bakterien blev udsat for mangel på næringsstoffer over 14 dage. *C. jejuni* blev inkuberet i 0.9% NaCl, og efter 14 dage blev levedygtige men ikke dyrkningsbare bakterier observeret. Det blev også observeret at tilføjelsen af kyllingesaft i de forskellige forsøg, havde en positiv indvirkning på levedygtigheden af *C. jejuni* celler. Mængden af levedygtige celler faldt langsommere over tid, sammenlignet med opløsninger uden kyllingesaft. De observerede resultater indikerer at de miljømæssige forhold *Campylobacter* bliver udsat for på kyllingegården og gennem fødevare forarbejdning kan inducere overlevelsesstadiet.

Contents

1 Abbreviations

BA	:	Blood Agar
CFU	:	Colonony-forming unit
C_t	:	Threshold cycle
DC	:	Heat-inactivated control
DTU	:	Technical University of Denmark
EFSA	:	European Food Safety Authority
IAC	:	Internal Amplification Control
mCCDA	:	Modified charcoal cefoperazone deoxycholate agar
NaCl	:	Sodium chloride
NDC	:	Non dye control
NG	:	No growth
OD	:	Optical density
ON	:	Overnight
PI	:	Propidium iodide
PMA	:	Propidium monoazide
qPCR	:	Quantitative polymerase chain reaction
RH	:	Relative humidity
Saline	:	0.9% (w/v) NaCl
SD	:	Standard deviation
VBNC	:	Viable but nonculturable
VC	:	Viable control
WHO	:	World Health Organization

2 Introduction

Campylobacter is the most commonly reported cause of human foodborne gastroenteritis, called campylobacteriosis [1]. In 2021, there were 127,840 confirmed cases of campylobacteriosis in the European Union [2]. The symptoms of campylobacteriosis are characterized by diarrhea, vomiting, cramps, and fever, with the possibility of developing severe diseases such as Guillain-Barre syndrome [1, 3]. The main transmission route of *Campylobacter* is through poultry meat, as poultry is a major reservoir of *Campylobacter*. Poultry is estimated to be responsible for up to 30% of foodborne human infections, where *Campylobacter jejuni* is the primary species causing infections [1]. Effective methods to diminish *Campylobacter* in the broiler production are needed. Therefore, a partnership between DTU, Danpo, and Thor Ice was established on a joint project, SafeChicken, to investigate methods and technologies to potentially reduce *Campylobacter* in the broiler production [4]. *Campylobacter* has the ability to enter a viable but nonculturable state (VBNC), possibly as a survival strategy when exposed to stressors. Furthermore, *Campylobacter* in the VBNC state, may be undetected when using conventional culturing methods, as it will not multiply and create colonies in culture media [5, 6, 7]. VBNC *Campylobacter* can potentially resuscitate when exposed to favorable conditions and regain pathogenicity, thereby posing a risk to human health [8].
Potential methods for the detection of *Campylobacter* in its VBNC state have been suggested through research. PMA-qPCR combines propidium monoazide (PMA) and quantitative polymerase chain reaction (qPCR). It is a non-culturing dependent method where PMA, an intercalating dye, penetrates damaged cell membranes and binds to the DNA of dead cells. The binding inhibits the amplification of DNA from dead cells, but viable, and VBNC cells will be detected [5, 9]. LIVE/DEAD *Bac*Light is a non culturing-dependent staining method. It combines two fluorescence dyes, SYTO 9 and propidium iodide (PI). The combination enables direct enumeration and differentiation of viable and injured/dead cells with the use of fluorescence microscopy or flow cytometry [5].

2.1 Aim of the study and objectives

The overall aim of the study was to investigate and provide insight if *C. jejuni* has the potential to persist in the broiler farm environment by entering a VBNC state. *C. jejuni* were exposed to various simulations of environmental stressors to investigate which factors induce the VBNC state of *C. jejuni* using PMA-qPCR.
To explore the overall aim of the study, six study objectives were formulated:

- To assess PMA-qPCR's ability to differentiate viable and heat-inactivated *C. jejuni* cells.
- To produce and verify a standard curve for the PMA-qPCR method using live and heat-inactivated *C. jejuni* cells both treated and non-treated with PMA.

- To assess LIVE/DEAD *Bac*Light's ability to differentiate viable and heat-inactivated *C. jejuni* cells.
- To investigate the potential induction of the VBNC state in *C. jejuni* through desiccation.
- To test if osmotic stress can induce the VBNC state in *C. jejuni*.
- To investigate whether nutrient deprivation can induce the VBNC state in *C. jejuni*.

3 Background and theory

For the past five years, 3,740-5,389 cases of *Campylobacter* infections have been registered in Denmark every year [10, 11]. *Camppylobacter* is the highest registered gastrointestinal bacterium in Denmark, compared to other gastrointestinal pathogens such as *Salmonella*, *STEC*, *Yersinia enterocolitica*, *Shigella* and *Listeria* (figure 1). A substantial increase in *Salmonella* cases was registered in 2008, where the number of registered cases exceeded the registered cases of *Campylobacter*, due to a long-lasting outbreak. Nevertheless, *Campylobacter* have been the dominating cause of gastroenteritis for the last 10 years in Denmark [2, 11, 12]. Foodborne gastroenteritis caused by *Campylobacter* spp. is considered highly underreported, as it is usually self-limiting. The infectious dose is as low as 500 colony-forming units (CFU), to cause infection. There are 16 species of the *Campylobacter* genera, where *C. jejuni* and *C. coli* are the most common species causing infections in humans. *C. jejuni* is estimated to cause 95% of the campylobacteriosis cases [1, 13, 14, 15].

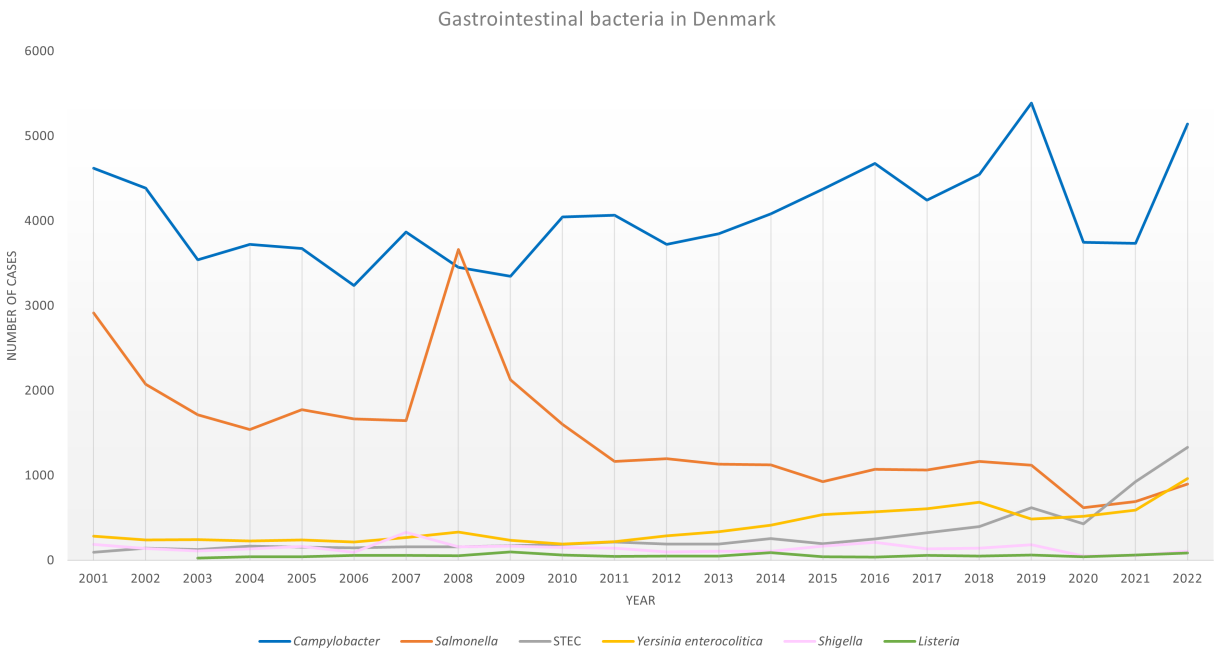

Figure 1: *Number of laboratory registered cases of gastrointestinal bacterial infections in Denmark in years 2001-2022. Campylobacter (blue), Salmonella (orange), STEC (grey), Yersinia enterocolitica (yellow), Shigella (pink) and Listeria (green).*
Numbers are from Statens Serum Institut [11].

3.1 *Campylobacter jejuni*

C. jejuni is a Gram-negative, spiral-shaped bacterium. It is non-sporeforming and can have a single flagellum or bipolar flagella [2, 16]. It is a microaerophilic bacterium, signifying it requires 5-10% oxygen to thrive. *C. jejuni* is classified as thermophilic and has optimal growth at temperatures between 37 ℃ and 42 ℃, but does not grow at temperatures below 30 ℃ [1, 2, 17]. *C. jejuni* is considered to be a bacterium that is sensitive to changes in oxygen, pH, desiccation, acids, salts, disinfectants, nutrient deprivation, osmotic stress, and the fluctuation of temperatures [14, 16]. Despite the high sensitivity to environmental conditions, *C. jejuni* is highly prevalent in poultry farms and food processing environments, which include aerobic environments, refrigeration, freezing, scalding, and use of antimicrobials. The ability of *C. jejuni* to survive makes the elimination of the bacteria in the food industry a challenge [1, 8, 16].

3.2 Reservoir and transmission routes

C. jejuni is often found in warm-blooded animals, such as cows, sheep, birds, and poultry. The animals are considered reservoirs for the pathogen, where *C. jejuni* rarely causes disease symptoms. Birds and poultry have a higher metabolic temperature, around 42 ℃, facilitating the growth of *C. jejuni*. It mainly colonizes the small intestine and ceca of the animals and makes the bacterium detectable in the feces [2, 14, 18, 19].

The typical transmission route of *C. jejuni* is foodborne transmission, which is considered the main route to human infections. It covers contaminated or undercooked food, unpasteurized milk, dairy products, and cross-contamination, for instance, through the slaughtering process. Suggested transmission routes can be seen illustrated in figure 2, where the thickness of the arrows illustrates the relevance of the transmission routes. The transmission routes also include waterborne transmission contaminated by animal feces, contact with infected pets, and livestock as *C. jejuni* resides in the intestinal tract of these animals, and person-to-person contact with an infected person with poor hygiene [2, 8, 18, 20].

The introduction of *C. jejuni* into poultry flocks is not fully understood. It is suggested that the most common cause of the introduction of *C. jejuni* to commercially reared flocks, is through horizontal transfer. *C. jejuni* is ubiquitous in the environment, therefore it can easily be introduced into the poultry house through various means, like through routine management and maintenance of the flock. The organic/free-range broiler flocks have a higher percentage of positive *Campylobacter* cases than conventional flocks in Denmark [10, 18, 21]. However, when a chicken within a broiler flock is first detected positive, the bacterium will transmit rapidly within days, and most of the birds will get infected with *Campylobacter*. This often spreads through feces. Furthermore, the season is considered a factor, as more cases of *Campylobacter* positive flocks are registered in the summertime [18].

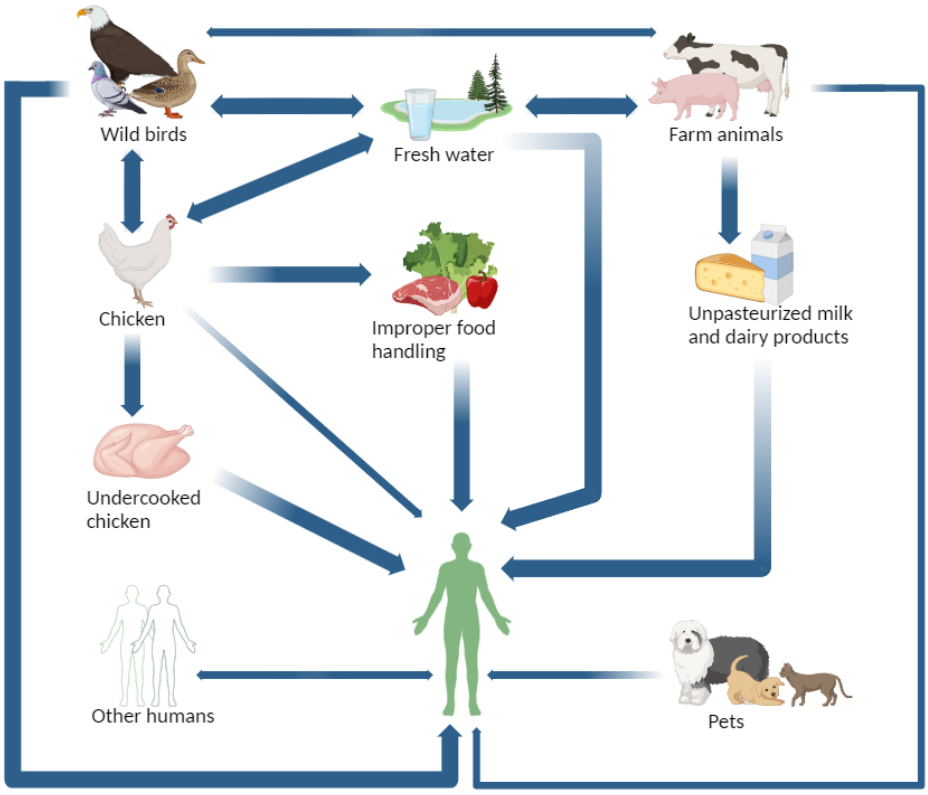

Figure 2: *The transmission routes of C. jejuni. The arrow size represents the relevance of the transmission route. Figure inspired from EFSA, Story map on Campylobacter [2], created in BioRender.*

The European Food Safety Authority (EFSA) [2] suggests prevention measures such as good hygiene at the manufacturing and food distribution levels. Furthermore, that the consumer handles the food properly to avoid cross-contamination. At farm level, EFSA suggests slaughtering the chickens at a younger age (35 days) and dedicated changing facilities for the farm workers together with specific indoor clothes and footwear. The Danish Veterinary and Food Administration has initiated an action plan from year 2022-2026 for *Campylobacter* in broilers, food products, and the environment to reduce the occurrence of *Campylobacter* in food and in the environment. This type of action plan has been implemented in the early years [22]. However, an increase in reported *Campylobacter* human cases is still seen yearly [1, 2].
A variety of prevention strategies and interventions are used against *C. jejuni* in food processing and industry. These include scalding, freezing, chilling, and the use of antimicrobials that are used as treatments in the processing plant [1]. Furthermore, food preservatives are also used, where Sodium Chloride (NaCl) is frequently utilized. The high salt concentrations expose food-borne pathogens such as *C. jejuni*, for osmotic stress, which studies of model bacteria have shown can lead to rapid efflux of cellular water, dehydration, and membrane distortion [23, 24]. Both through food processing and at a chicken stable, *C. jejuni* can get exposed to desiccation. Desiccation can occur when *C. jejuni* is exposed to air-drying, particularly if it remains in a water droplet in the chicken stable, that gradually evaporates over time. This drying process can have various effects on *C. jejuni*. Studies with model bacteria have shown that desiccation can lead to shrinkage of the capsular layer, shrinkage in the volume, and potentially alter cell morphology. The intracellular salt concentrations might increase, and the pili and membrane may be altered or damaged due to desiccation [25]. As a response to the environmental stressors, *C. jejuni* can enter the VBNC state [1].

3.3 The viable but nonculturable state of *C. jejuni*

Certain bacterial species, including species belonging to the *Campylobacter* genera, have been discovered to enter the VBNC state [8, 6]. Previously, the VBNC state was described as a dormant state, but the metabolic activity in the VBNC state is measurable compared to the dormant state [6, 26]. *C. jejuni* can enter the VBNC state, where it is not able to grow in the laboratories and is thus not detectable when using traditional culturing methods [8]. The VBNC state of *C. jejuni* was first described by Rollins and Colwell in 1986 [27]. Up until 2020, 85 bacterial species have been reported to be capable of entering the VBNC state when exposed to stressful conditions [8].

Stressful environmental conditions, such as temperature and pH fluctuations have been demonstrated to induce the VBNC state in *C. jeuni*. For instance, prolonged incubation at 4 ℃ resulted in the development of VBNC *C. jejuni* [3, 28]. Osmotic shock, desiccation, and nutrient deprivation have also been identified to induce the VBNC in *C. jejuni*. *C. jejuni* is sensitive to high concentrations of NaCl but can tolerate it to some extent. The ability to

tolerate and grow in NaCl has shown to be temperature-dependent. Research has reported that *C. jejuni* can survive longer at 4.5% (w/v) NaCl at 4 ℃ compared to a temperature at 42 ℃ [1, 29].

Numerous genes contribute to the induction of the VBNC state in *C. jejuni*. A down-regulation in the transcription of virulence-associated genes such as *cdtA, cdtB, cdtC, flaA, flaB, cadF* and *ciaB* have been observed for *C. jejuni* in the VBNC state. The genes are associated with adhesion and invasion in the intestinal tract of the host. Despite these genes being downregulated, they are still maintained in the VBNC bacteria [3, 30]. Research suggests that *C. jejuni* down-regulates possible genes, such as virulence genes, to conserve energy for metabolism [1, 30].
Environmental stressors have previously been reported to promote upregulation of virulence gene expression in various pathogens. This adaptive response has also been observed for *C. jejuni*, as a response to nutrient deprivation among other stressors. If the stress persists *C. jejuni* may eventually entering the VBNC state [28, 31, 32]. Studies have demonstrated that *C. jejuni* switch morphological appearance from spiral shape to coccoid form when entering the VBNC state [3, 28].

The VBNC cells are suggested to be avirulent when being the VBNC, as a result of the reduced rate of gene expression and protein translation. Furthermore, VBNC cells can be dormant for several months and then resuscitated when exposed to favorable conditions, regaining pathogenicity [6, 8]. VBNC cells have been demonstrated to resuscitate only within a limited period of a certain time, the resuscitation window. The resuscitation window has been shown to be related to the age of the VBNC cells. If the cells do not enter favorable conditions within the resuscitation window, they lose the ability and will at some point die [5, 33]. It has been reported that VBNC *C. jejuni* have been resuscitated in mouse intestines and embryonated eggs [33].

C. jejuni have adopted other survival mechanisms to overcome environmental stressors than the VBNC state. There have been suggested mechanisms, such as the development of aerotolerance and biofilm formation [1]. As *C. jejuni* is a microaerophilic bacterium that thrives best in low oxygen levels, aerotolerance enables it to survive in oxygen-rich environments. Furthermore, *C. jejuni* can develop biofilm formation. A biofilm is a bacterial community that is encapsulated by a matrix produced by the bacteria that adhere to surfaces and the bacteria. The biofilm protects the bacteria from environmental stressors such as antimicrobials, disinfectants, and dehydration. Research suggests biofilm formation is a survival strategy contributing to its ability to persist in the environment. Research indicates that the ability of *C. jejuni* to form biofilms is strengthened when food or organic components in food are present, such as chicken meat exudate (chicken juice) [1, 34].

3.4 Detection of *C. jejuni* by non culturing-dependent methods

3.4.1 PMA-qPCR

The detection method for *Campylobacter* in food processing heavily relies on traditional culturing methods, where CFU are quantified by plating on selective media and incubated at microaerobic atmosphere with elevated temperatures. Although the culturing-dependent methods are cost-effective and simple, they require detectable growth to confirm the presence of the pathogen, a requirement that is not met by VBNC *C. jejuni* [35, 36]. A suggested method for detection of VBNC *C. jejuni*, is the use of PMA in conjunction with qPCR. Unlike the traditional culturing methods that rely on culturability, this approach evaluates the viability based on membrane integrity [8].
PMA, a derivative of propidium iodide, can pass through the cell membrane of dead cells. It binds strongly and irreversibly with a covalent bond to the double-stranded DNA when exposed to light (465-475 nm) [37]. The structural characteristics and positive charge of PMA, prevent it from passing through the intact membranes of viable cells. When PMA binds to DNA, it prevents amplification from the DNA polymerase through qPCR analysis. Which leads to the assumption that the binding of PMA ensures only PCR products from viable cells [8, 38, 39]. An illustration of PMA-qPCR is shown in figure 3.

qPCR provides real-time quantification of amplified DNA. A fluorescence signal is measured through each cycle to monitor real-time amplification of the DNA. Upon reaching a predetermined threshold (C_t value), the initial amount of DNA in the sample can be quantified. qPCR can not differentiate viable cells from non-viable cells alone, and therefore, PMA is used as a supplement to enable differentiation and reduce overestimation [39, 40].

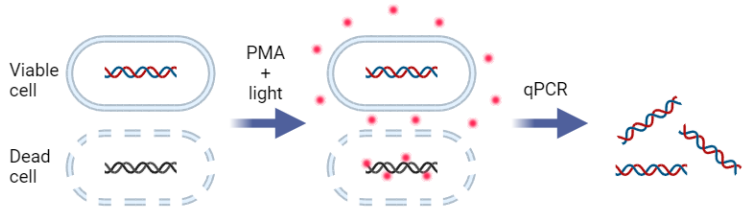

Figure 3: *The PMA enters the dead cells through the compromised cell membrane and binds to DNA upon photoactivation. The binding inhibits the DNA polymerase from amplifying the DNA of the dead cells, resulting in the polymerase only amplifies DNA from the viable cells [5, 8].*
Figure from special course: Viable but not culturable stage of Campylobacter bacteria, created by Agnes Sigrid Bjørnstad, inspired by figure 2 in Dong et al. 2020 [5]. Created in BioRender.

3.4.2 LIVE/DEAD *Bac*Light

LIVE/DEAD *Bac*Light is a staining method that, in combination with traditional culturing-dependent methods, can detect VBNC cells. LIVE/DEAD *Bac*Light can be used to distinguish viable and dead cells based on the membrane integrity using two nucleic acid fluorescent stains. SYTO 9, a green dye that permeates the cell membrane of both intact and injured

cells, upon binding of DNA will fluorescence green. PI is a red dye, that penetrates the membrane of only the injured cells and binds DNA, producing red fluorescence [5, 41, 42]. When both dyes are present in the injured cell, PI establishes a stronger affinity to the DNA and outcompetes SYTO 9. The fluorescence can then be examined using a fluorescence microscope or flow cytometry [5, 43]. The method is illustrated in figure 4.

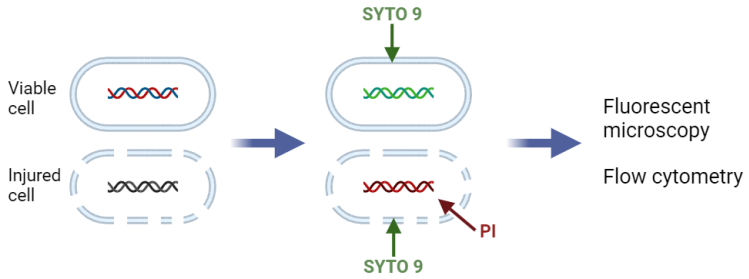

Figure 4: *SYTO 9 penetrates the membrane of both live and injured cells, emitting green fluorescence. PI penetrates the compromised membrane of injured/dead cells, emitting red fluorescence. The fluorescence can be examined using fluorescent microscopy or flow cytometry. Figure from special course: Viable but not culturable stage of Campylobacter bacteria, created by Agnes Sigrid Bjørnstad, inspired by figure 2 in Dong et al. 2020 [5]. Created in BioRender.*

4 Materials and methods

The experimental work of this study consisted of assessing three DNA extraction methods, along with PMA-qPCR and LIVE/DEAD *Bac*Light for detection of *C. jejuni*. Additionally, investigation of the influence of environmental stressors on the viability of *C. jejuni* such as desiccation, osmotic stress, and nutrient deprivation. Creation of the standard curve from PMA-qPCR, DNA extraction trials, and LIVE/DEAD *Bac*Light assay were conducted in collaboration with Sofie Pauly Kofoed Mose, with the purpose of producing a standard curve for the PMA-qPCR method and to test the LIVE/DEAD *Bac*Light method for both our theses.

4.1 Bacterial strains and cultivation conditions

The bacterial strains used in the project were *Campylobacter jejuni* subsp. *jejuni* ATCC 33560 and *Escherichia coli* ATCC 11229.
The bacterial strains were thawed from -80 ℃ cryo-stocks stored in lysogeny broth with 15% glycerol. The *C. jejuni* strain was streaked on selective media, modified charcoal cefoperazone deoxycholate agar (mCCDA, CCDA agar plate, SSI Diagnostica, Denmark), and incubated for 72 hours at 41.5 ℃ at microaerobic conditions. One *C. jejuni* colony was transferred to blood agar (BA, TSA agar plate with 5% bovine blood, SSI Diagnostica, Denmark) and incubated overnight (ON) at 41.5 ℃ at microaerobic conditions. *E. coli* was thawed from -80 ℃ and streaked on BA and incubated at 37 ℃ ON.

4.2 Bacterial stock solution

The bacterial stock solutions were prepared by 1 mL saline (0.9% (w/v) NaCl) was poured on the BA plate containing the bacterial colonies (section 4.1). The colonies were mixed with the liquid using a Drigalski spatula. The bacterial solution was collected and diluted with saline to obtain an OD_{600}=0.6 $\sim$ 1.0x10^9 CFU/mL.
The bacterial concentration of the working stock was confirmed by making a 10-fold dilution series in saline. The dilutions 10^{-5}-10^{-7} were plated on BA and incubated for 48 hours at 41.5 ℃ at microaerobic conditions to count CFU/mL.

4.3 Preparation of dilutions to the standard curve

To create a standard curve and investigate PMA-qPCR's ability to differentiate viable and heat-inactivated cells a 10-fold dilution series of *C. jejuni* was made (section 4.2), the procedure can be seen in figure 5. From the dilutions 10^{-2}-10^{-9}, 4x900 µL of each dilution were transferred to Eppendorf tubes, resulting in four rows with tubes. Half of them were heated in a heat block at 90℃ for 15 minutes. Resulting in two rows of Eppendorf tubes (10^{-2}-10^{-9}) as dead cell control (heat-inactivated) and two rows of tubes as live (viable) cell control. The

PMA treatment can be found in section 4.4. From the heat-inactivated cells, 100 µL from 10^{-1} and 10^{-3} were plated on BA and incubated at 41.5 ℃ at microaerobic conditions ON, to ensure heat-inactivation was achieved.

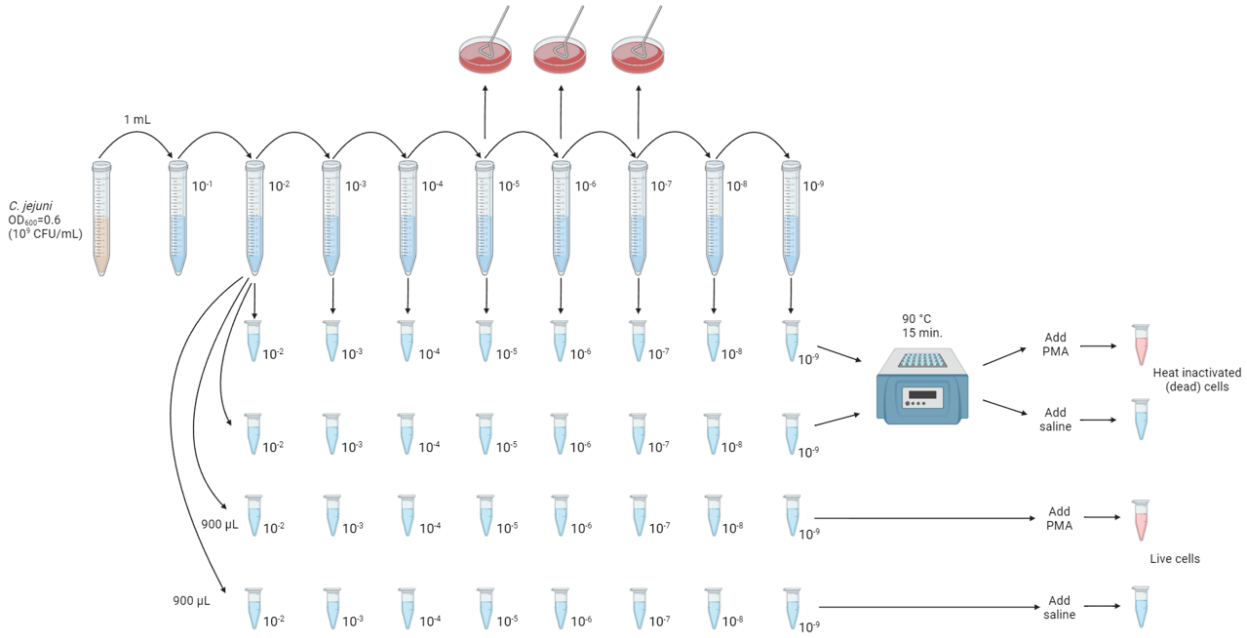

Figure 5: *Workflow of the 10-fold dilution series for the standard curve. Created in BioRender.*

Likewise, a control sample of *E. coli* was prepared by a 10-fold dilution series. Dilutions 10^{-5}-10^{-9} were plated on BA and incubated ON at 37 ℃ to count CFU/mL. Sample 10^{-3} was centrifuged at 7,500 x g for 7 min, the supernatant was removed, and the sample was stored at -20 ℃ until DNA extraction.

4.4 PMA treatment

From the solution to be tested, 900 µL was transferred to an Eppendorf tube, where 100 µL PMA (VWR International, US, Avantor) was added to obtain a final PMA concentration at 20 µM [8, 44]. The tube was shaken at room temperature at 150 rpm for five minutes in the dark and afterward exposed to light in a LED Active Blue instrument (IB Applied Science) for photo-crosslinking for 15 min. The tube was centrifuged at 7,500 x g for 7 min, and supernatant was discarded to remove the excess PMA. The PMA-treated sample was stored at -20 ℃ until DNA extraction. The procedure of the PMA-qPCR procedure can be seen in figure 6.
Likewise, the PMA-treated sample described above, a non-treated sample was collected, as a control. Where 100 µL saline was added instead of PMA and followed the same procedure as the PMA-treated sample.

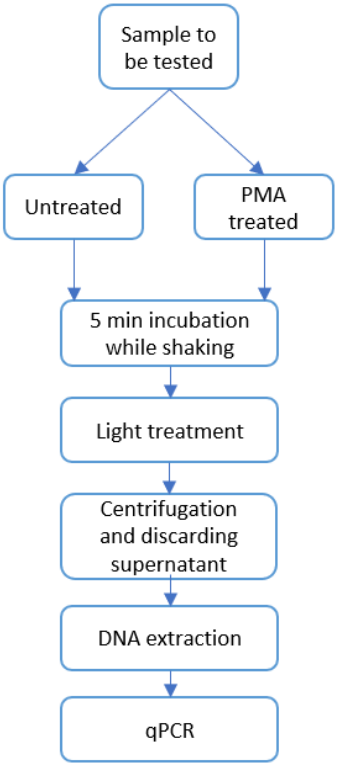

Figure 6: *Workflow for the PMA-qPCR procedure. The figure is inspired by the product information of PMA from the Biotium instruction manual [37].*

4.5 DNA extraction

Three DNA extraction methods were tested to determine which was most suitable for further experiments with PMA-qPCR. Boiling lysis and two extraction kits from Qiagen: DNeasy Blood & Tissue Kit and QIAmp Fast DNA Stool Mini Kit. The extraction methods were tested with *C. jejuni* samples at concentrations of 10^7 and 10^6 CFU/mL. These samples consisted of both a PMA-treated and non-treated representative (section 4.4). Prior DNA extraction, the frozen samples, including both PMA-treated and non-treated, were thawed at room temperature.

4.5.1 Boiling lysis

For the samples to be extracted, 200 µL of 10X TE buffer was added to the bacterial pellets and vortexed. The tubes were heated at 100 ℃ for 10 min. Lids were kept shut with a constant pressure to secure the lids did not open under the heating. The tubes were centrifuged at 10.000 x g for 5 min. The supernatant was transferred to another Eppendorf tube. The DNA was stored at -20 ℃ until qPCR analysis (section 4.6).

4.5.2 DNA extraction with DNeasy Blood & Tissue Kit

For the samples to be extracted, 180 µL Buffer ATL was added to each sample. 25 µL Proteinase K was added to the samples and vortexed. The samples were heated to 56 ℃ for 60 min and vortexed for 15 seconds. 200 µL Buffer AL was added and vortexed. 200 µL ethanol (96-98%) was added and vortexed. The lysate from the tubes was added to the spin columns, placed in a 2 mL collection tube, and centrifuged for 1 min at 10,000 rpm. The filtrate liquid in the spin columns after the centrifugation was discarded. This process was repeated until all the lysate had been through the spin columns. 500 µL AW1 Buffer was added to the spin columns and centrifuged for 1 min at 10,000 rpm. The spin columns were placed in a new 2 mL collection tube, and 500 µL AW2 Buffer was added, and the samples were centrifuged at 10,000 rpm for 3 min. The spin columns were transferred to an Eppendorf tube, and 100 µL of AE Buffer was added. The tubes were incubated for 1 min at room temperature and were then centrifuged at 14,000 rpm for 2 min. The DNA was stored at -20 ℃ until qPCR analysis (section 4.6).

4.5.3 DNA extraction with QIAmp Fast DNA Stool Mini Kit

For the samples to be extracted, 1 mL InhibitEX Buffer was added to each sample and vortexed. The samples were heated to 70 ℃ for 10 min and then vortexed. The samples were centrifuged 1 min at 10,000 rpm. 25 µL Proteinase K was added to new Eppendorf tubes. 600 µL supernatant from the sample tubes was transferred to the Eppendorf tubes containing Proteinase K. 600 µL buffer AL was added to the tubes and vortexed for 15 seconds. The tubes were incubated at 70 ℃ for 10 min. 600 µL 90% ethanol was added to each tube and vortexed. The lysate from the tubes was added to the QIAmp spin columns, placed in a 2 mL collection tube, and centrifuged for 1 min at 10,000 rpm. The filtrate liquid in the spin columns after the centrifugation was discarded. This process was repeated until all the lysate had been through the spin columns. 500 µL AW1 Buffer was added to the spin columns and centrifuged for 1 min at 10,000 rpm. The spin columns were placed in a new 2 mL collection tube and 500 µL AW2 Buffer was added. The samples were centrifuged at 10,000 rpm for 3 min. The spin columns were transferred to an Eppendorf tube, and 100 µL of ATE Buffer was added. The tubes were incubated for 1 min at room temperature and were then centrifuged at 14,000 rpm for 2 min. The DNA was stored at -20 ℃ until qPCR analysis (section 4.6).

4.6 Real-time quantitative PCR

The primers and probes used for the qPCR in this study are shown in table 1. The primers for *C. jejuni* targets the 16s rRNA [45]. Two TagMan probes are used, where the Campy LNA (locked nucleic acid) probe for *C. jeuni* creates a FAM (6-Carboxyfluorescein) signal, and the IAC probe creates a HEX (Hexachlorofluorescein) signal in the qPCR analysis. To ensure and

monitor the efficiency of the qPCR reaction, an Internal Amplification Control (IAC) was included with a corresponding probe. The IAC serves as an internal amplification reference of the qPCR and is proposed as a requirement when PCR testing foodborne pathogens [46]. The composition of the master mix is shown in table 2. Fifteen µL master mix was added to the wells of a MicroAmp Fast optical 96-well Reaction Plate (Applied Biosystems, Life Technologies, Singapore). Ten µL of the DNA sample (template) was added to the wells in duplicates and mixed by pipetting. Positive controls of *C. jejuni*, one negative control of *E. coli*, and two non-template controls (PCR water) were also included. The MicroAmp plate was covered with an optical adhesive cover (Applied Biosystems, Life Technologies, Singapore) and centrifuged at 2000 rpm for 2 min. The PCR cycles were run in a QuantStudio 5 (Applied Biosystems, Thermo Fisher Scientific, US). The cycle profile was initial denaturation at 95 ℃ for 3 min, followed by a total of 40 cycles of 95 ℃ for 15 sec, then 60 ℃ for 60 sec, and afterward 72 ℃ for 30 sec. The data collection was performed in the exponential phase of the process. The fluorescence measurements were obtained and analyzed using QuantStudio Design & Analysis Software 2.7.0.

Table 1: *Primers and probes used in the study. IAC (Internal Amplification Control) was included to secure successful amplification of the PCR process.*

Primers and probes	**Sequence**	**Ref.**
Forward primer OT-1559	5'-CTG CTT AAC ACA AGT TGA GTA GG-3'	[47]
Reverse primer 18-1	5'-TTC CTT AGG TAC CGT CAG AA-3'	[47]
Campy LNA probe	5'-[6FAM]CA[+T]CC[+T]CCACGCGGCG[+T] TGC[BHQ1]	[47]
IAC	5'-CTG CTT AAC ACA AGT TGA GTA GGC AAC TCA GGT GTC CTC ATG AAT T...G AA-3'	
IAC probe	5'-[HEX]TTCATGAGGACACCTGAGTTG[BHQ1]	[47]

Table 2: *qPCR master mix reagents and the volumes pr sample. The primers were diluted with PCR water and probes with TE buffer. The qPCR was run with 10 µL template.*

Reagent	µL per sample	
Water	2.22	Merck, Germany
Buffer for Tth DNA polymerase (10x)	2.50	Roche Diagnostics Germany
$MgCl_2$ (25mM)	2.50	Roche Diagnostics Germany
Glycerol	2.00	Merck, Germany
dNTP (12.5 mM)	1.20	Thermo Fisher Scientific, US
Forward primer OT-1559 (10 pmol/µL)	1.25	TAG Copenhagen A/S Denmark
Reverse primer18-1 (10 pmol/µL)	1.25	TAG Copenhagen A/S Denmark
Campy LNA probe (5 pmol/µL)	0.38	Merck, Germany
IAC probe (6 pmol/µL)	0.25	Merck, Germany
BSA	0.25	Roche Diagnostics Germany
Tth enzyme (polymerase) (12.2 U/mol)	0.20	Roche Diagnostics Germany
IAC (100 pmol/µL)	1.00 (10^{-9} dilution used)	TAG Copenhagen A/S Denmark

4.7 LIVE/DEAD *Bac*Light

A bacterial stock solution of *C. jejuni* was prepared as described in sections: 4.1 and 4.2. From the stock solution, 2x1 mL was added to two Eppendorf tubes. One of the tubes was heat treated at 90 ℃ for 15 min to ensure heat-inactivated cells. 300 µL from the viable cells and 300 µL from the heat-inactivated cells were added to a new Eppendorf tube. The tube was vortexed thoroughly. A 10-fold dilution series (10^0-10^{-4}) was made for both viable, heat-inactivated and mixed cells. 100 µL of the bacterial cell suspensions were added to a sterile flat bottom 96-well polystyrene microplate (In Vitro, Denmark) in triplicates.
The stains used were from the LIVE/DEAD *Bac*Light Bacterial Viability Kit L7012 (Molecular Probes, Invitrogen, Thermo Fisher Scientific, Eugene, Oreg.). The SYTO 9 (3.34mM) and PI (20mM) were mixed in 1:2 ratio [42], 10 µL SYTO 9 were mixed with 20 µL PI. The dye mixture was added to a 5 mL filter sterilized dH_2O and mixed by vortexing. 100 µL of the dye mixture was added to each well and mixed by pipetting. Viable (live) and heat-inactivated (dead) cell controls were added by adding 100 µL of viable and heat-inactivated cells to

separate wells in the microplate. For the viable control (VC), 3 µL of SYTO 9 was mixed with 500 µL filter sterilized dH_2O. 100 µL of the dye was added to the viable control and mixed. The same was conducted with PI and added to the heat-inactivated control (DC). A non-dyed control (NDC) was added, where the 10-fold dilution series of viable cells (10^0-10^{-4}) was added to the microplate without adding the dyes. The setup in the microplate can be seen illustrated in figure 7.
The microplate containing the samples was incubated at room temperature in the dark for 15 min. The plate was inspected with no lid, by confocal microscopy at 60x magnification using an ImageXpress Micro Confocal & MetaXpress 6 (Molecular Devices) microscope. The inspection was done using FITC or Cy5 filters, or no filter, for the visualization of viable and heat-inactivated cells, respectively. Images were taken at room temperature with the MetaXpress software (Molecular Devices LCC) with laser-based focusing to define offset and utilize the auto-exposure settings. The images were taken with only one filter at a time, and then the MetaXpress software was used to overlay the images, to enable showing both stains. The images were taken with help from the laboratory.

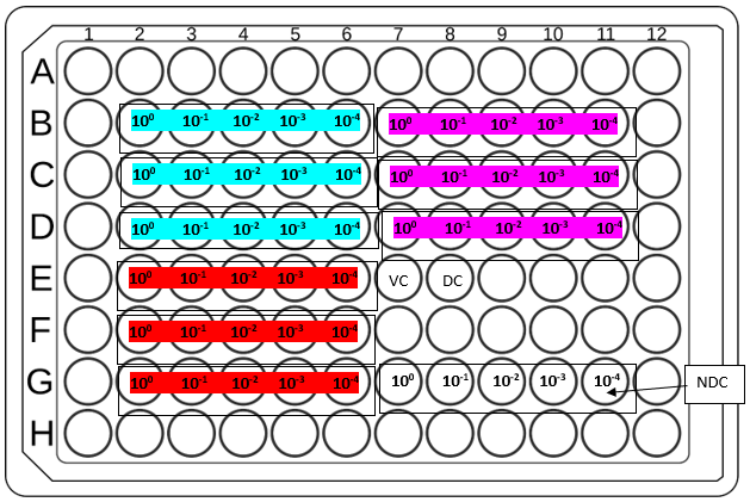

Figure 7: *The positions of the LIVE/DEAD BacLight samples in the 96-well microplate. The numbers correspond to 10-fold dilution series of C. jejuni. Viable cells (blue), heat-inactivated cells (red) and mix (50:50) of viable and heat-inactivated cells (purple). Viable control (VC), heat-inactivated control (DC) and non-dye control (NDC).*

4.8 Environmental stressors inducing the VBNC state in *C. jejuni*

4.8.1 Preparation of chicken juice

Chicken juice was prepared for the assays to mimic the nutrient source environment at the chicken stable with organic material from chickens. Commercially frozen chicken thighs were thawed at room temperature ON in a plastic bag. The chicken juice was collected and stored at -80 ℃ for 48 h. The chicken juice was thawed at -4 ℃ and centrifuged at 10,000 x g for 10 min to eliminate large particles. To sterilize the chicken juice, it was filtrated using a 0.45

µm filter. The optical density (OD) of the chicken juice was measured and the chicken juice was stored at -20 ℃ until use [48].

4.8.2 Desiccation of *C. jejuni*

4.8.2.1 Desiccation trial of *C. jejuni*

Prior to the arrival of a new flock of chickens to the stable, it gets washed and disinfected followed by heating to a minimum of 25 ℃ [49]. It was assumed that *C. jejuni* could be left in a droplet at the stable after washing to desiccate. Therefore, the effect of desiccation of *C. jejuni* was investigated. As a preliminary experiment, 1 mL of saline was added to an empty Petri dish at room temperature to observe the desiccation of the liquid.
To investigate if *C. jejuni* would survive to fully dry out and to test if it was possible to get qPCR results from the desiccation, a bacterial stock solution of *C. jejuni* was prepared as described in sections 4.1 and 4.2.
From the *C. jejuni* stock solution (10^9 CFU/mL), 500 µL was transferred to two empty Petri dishes (plates) (⌀ 55 mm) and 1 mL onto two other empty Petri dishes. The dishes were then swirled to distribute the contents on the bottom.
The setup and workflow of the assay are illustrated in figure 8. One of the plates containing 500 µL bacterial solution and one of the plates containing 1 mL, were placed together in an anaerobic jar. The lids for the Petri dishes were set at an angle on the lower plate to allow a gradual evaporation. To ensure optimal microaerobic conditions for *Campylobacter*, CampyGen Compact sheets (Fisher Scientific, Thermo Scientific, Denmark) were placed in an anaerobic jar together with the Petri dishes. The same setup was repeated with the other 500 µL and 1 mL plates, where a glass beaker (⌀ 106 mm) containing 1 L distilled water was placed inside the jar to increase humidity. The plates were incubated inside the jars at 41.5 ℃ for 48 h. The stock solution of *C. jejuni* was kept at 4 ℃ (±3 ℃) for 48 h as a control.

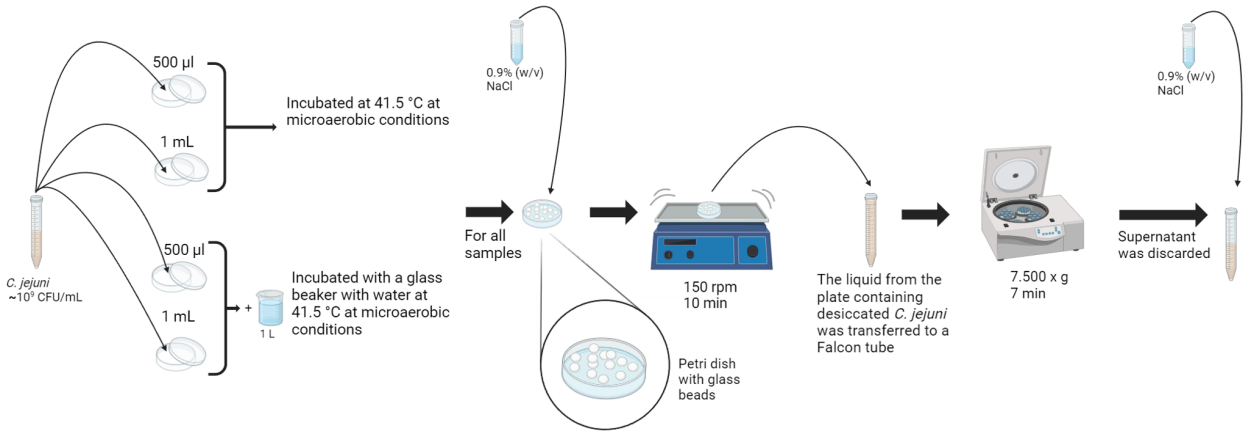

Figure 8: *Desiccation assay, incubated at 41.5 ℃ for 48 h until the solutions were completely desiccated. A glass beaker with water was placed together with two of the plates through incubation. Figure created in BioRender.*

After 48 hours, 10 mL of saline was added to each plate together with 5 g sterilized glass beads. The plates were shaken at 150 rpm for 10 min with saline and glass beads. The bacterial solutions were transferred from the plates to 15 mL tubes. 1 mL was transferred from the control tube to a new 15 mL Falcon tube, and saline was added to obtain the same volume as the tubes from the plates. The tubes were centrifuged at 7,500 x g for 7 min. The supernatant was discarded and 2.5 mL saline was added to the pellet and vortexed for 30 sec. A 10-fold dilution series was made for each solution and was plated on BA and incubated at microaerobic conditions at 41.5 ℃ ON. A 10-fold dilution series and plating were also performed for the control (10^9 CFU/mL).
Each solution, including the control, was treated with PMA and non-treated following the procedure in section 4.4. The samples were stored at -20 ℃ until DNA extraction, using the QIAmp Fast DNA Stool Mini Kit, following the procedure in section 4.5.3. Followed by qPCR analysis described in section 4.6.

4.8.2.2 Desiccation of *C. jejuni* with chicken juice

A similar desiccation assay was performed with the addition of chicken juice to simulate the farm environment. Therefore, the following procedure is the same as in section 4.8.2.1, where the differences are described below. The bacterial stock solution of *C. jejuni* was divided into two tubes, and chicken juice was added to obtain a bacterial solution containing 5% chicken juice. Saline was added to the other tube to obtain equal volumes.

The assay was performed with duplicates. 1 mL from each tube (with and without chicken juice) was added to two empty Petri dishes (plates). The plates were placed in an anaerobic jar, with the lid of the jar half open to ensure atmospheric conditions. An illustration of the experimental setup can be seen in figure 9. Together with the plates, the tubes containing the bacterial stock solutions (with and without chicken juice) were placed within the jar with no lid on as controls, to not desiccate (the controls are not demonstrated in the illustration, but appear in the incubation setup picture in figure 9). A glass beaker (⌀ 69 mm) with 250 mL water, was placed inside the jar to increase humidity. The humidity was monitored using a data logger (Tinyview Plus TV-1500, Gemini Data Loggers) during the assay. The measurements were initiated with reference to a broiler farm's chicken stable, wherein a chart illustrating % relative humidity (%RH) measurements within the stable is provided. The chart can be seen in appendix 10.3. At the chicken farm, the humidity in the chicken stable is monitored and maintained at 49-70% from the introduction of a flock at week 1 until week 5. The jar with the samples was incubated for 48 h at 25 ℃ until the plates were completely desiccated. The retrieval of the desiccated *C. jejuni* cells follows as described in section 4.8.2.1. After retrieval of the cells, 100µL from each solution and the controls, was plated on BA and incubated at 41.5 ℃ at microaerobic conditions ON, to register growth.

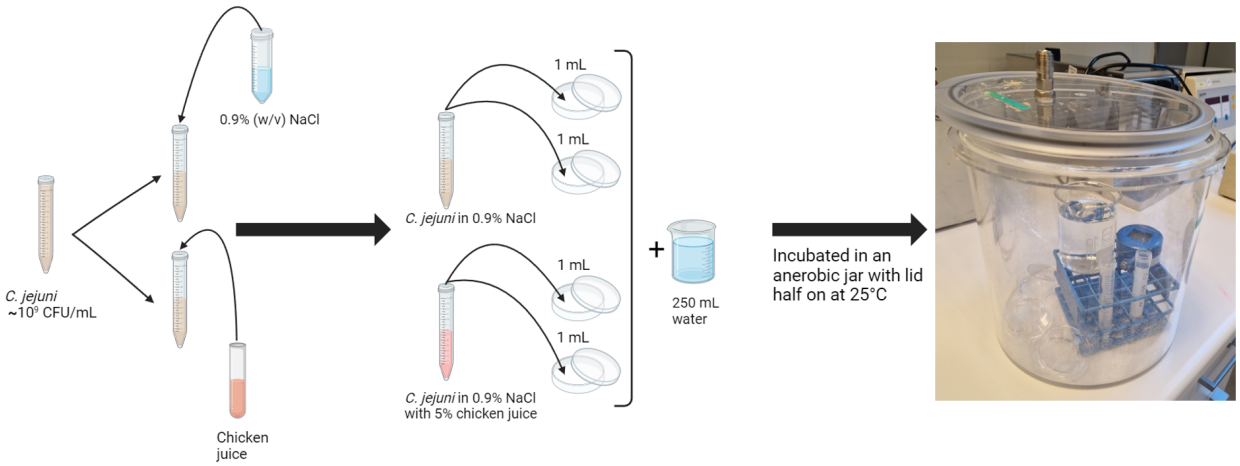

Figure 9: *Desiccation assay, left for incubation at 25℃ for 48 h until the solutions were completely desiccated. The controls incubated together with the plates in the jar. A glass beaker with water and a data logger was placed together with the plates and control tubes through incubation. The experimental procedure follows as in figure 8, at the arrow stating "For all samples". Figure created with BioRender and Powerpoint.*

4.8.3 Osmotic stress of *C. jejuni*

The potential induction of the VBNC state of *C. jejuni* through osmotic stress was investigated. The assay was performed with biological duplicates; therefore, two bacterial stock solutions of *C. jejuni* were prepared as described in sections 4.1 and 4.2 and the duplicate assay was performed simultaneously. An illustration of the assay is shown in figure 10.
The bacterial stock solution was divided into 3 tubes. The tubes were centrifuged at 7,500 x g for 7 min and supernatant was discarded. In one of the tubes, 10 mL 7% (w/v) NaCl was added. For the second tube, 10 mL tap water (from DTU, Lyngby) was added, and for the third tube, 10 mL of saline solution was added. The tube containing the saline solution was divided into two tubes. In one of the tubes, 500 µL chicken juice (section 4.8.1) was added, so the solution contained 9.09% chicken juice. In the other tube 500 µL saline was added to obtain equal volumes. All the tubes were incubated at room temperature (∼ 20 ℃) with the lid off to ensure atmospheric conditions. A box with holes at the side was placed over the tubes to prevent contamination. Samples for PMA treatment (following the procedure described in section 4.4) were collected after 0 h, 5 h, 24 h, 48 h, and 7 days. 100 µL from each tube was plated at every sample time on BA and incubated ON at 41.5 ℃ microaerobic conditions to register growth. The PMA-treated and non-treated samples were stored at -20 ℃ until DNA extraction, using the QIAmp Fast DNA Stool Mini Kit, following the procedure in section 4.5.3, followed by qPCR analysis described in section 4.6. When the samples were collected after 5 h, 5 mL saline was added to the tubes containing only the saline solution and saline with chicken juice to ensure enough bacterial solution for the rest of the sample times, this dilution was corrected in the data processing.

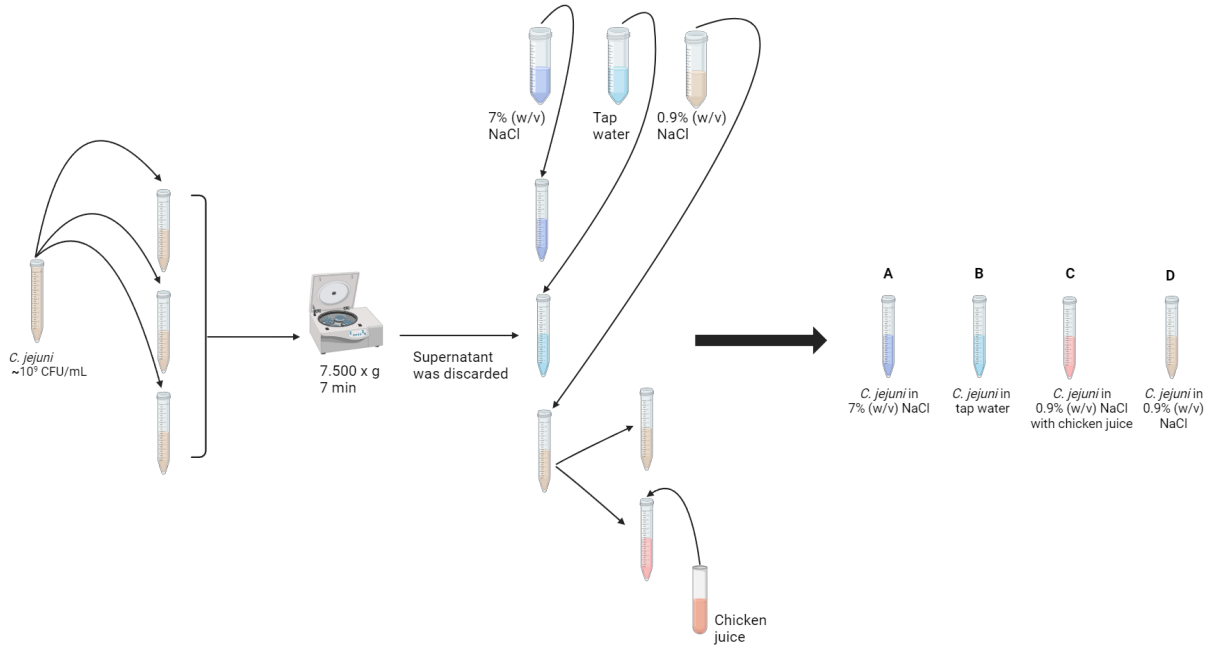

Figure 10: *Osmotic stress assay, the experimental workflow. C. jejuni was incubated in four different solutions. A: 7% (w/v) NaCl, B: tap water, C: saline and D: saline with chicken juice. The tubes were left for incubation at room temperature for 7 days. Figure created in BioRender.*

4.8.4 Nutrient deprivation

The influence of nutrient deprivation of *C. jejuni* was investigated by incubation in 0.9% NaCl, with and without chicken juice. *C. jejuni* was cultivated and a bacterial stock solution was prepared, following the procedure in sections: 4.1 and 4.2. The bacterial stock solution was divided into two tubes. Chicken juice was added to one of the tubes to obtain a solution with 5% chicken juice. Saline was added to the other tube to obtain the same volume in both tubes. The tubes were incubated at 25 ℃ with no lid on to ensure atmospheric conditions. Samples were collected after 2, 7, and 14 days. The samples were treated with PMA following the procedure described in section 4.4. At every sampling time, 100 µL from both tubes were plated on BA and incubated ON at 41.5 ℃ at microaerobic conditions to register growth The PMA-treated and non-treated samples were stored at -20 ℃ until DNA extraction, using the QIAmp Fast DNA Stool Mini Kit, following the procedure in section 4.5.3, followed by qPCR analysis described in section 4.6.

4.9 Data processing

The bacterial stock solutions were plated, to confirm the bacterial concentrations. CFU/mL was calculated from the plate counts using plates with 1-300 colonies; the results from plate counts are not included in this study.

Cells were assumed viable based on retained membrane integrity. Therefore, for a sample that was treated with PMA (+PMA) and returned a signal in the qPCR analysis, were assumed as viable cells. The corresponding sample, that was not treated with PMA (-PMA) and returned

a signal in the qPCR analysis, was considered a mixture of viable and dead/non-viable cells (all cells). If a sample was treated with PMA and did not return a signal from the qPCR analysis, but the corresponding non-PMA treated sample returned a signal, the cells were considered dead/non-viable. A PMA-treated sample that returned a signal from the qPCR analysis and did not show growth at BA incubated ON at 41.5 ℃ microaerobic conditions were assumed as VBNC cells.
The C_t values used in the data through this study are all based on an average of duplicate determination, except for the DNA extraction experiment where the DNeasy Blood & Tissue Kit and Boiling lysis were analyzed by qPCR as a single trial. The QIAmp Fast DNA Stool Mini Kit was tested with duplicates. All plots and calculations were made using Microsoft Excel.

4.9.1 PMA efficiency and standard curve

To determine if PMA inhibited the amplification of the DNA from the viable cells, the delta Ct (dCT) was calculated with the equation [37]:

$$dCt = Ct_{PMA-treated} - Ct_{untreated} \tag{1}$$

According to the Biotium PMA product information [37], the live (viable) cell control should have a dCt close to 0 (± 1), indicating the PMA treatment did not affect the amplification of DNA from the viable cells.
The standard curve was created by the C_t values for the 10-fold dilution series of the PMA-treated *C. jejuni* cells were plotted against calculated Log CFU/mL. A linear regression was obtained from the standard curve. The Efficiency of the qPCR reaction for the standard curve was calculated from the slope using the following equation [50]:

$$Efficiency = 10^{\frac{-1}{slope}} - 1 \tag{2}$$

An efficiency was accepted between 90-110%, and R^2 coefficient (coefficient of determination) was considered acceptable >0.98 to ensure reliable results [50, 51, 52].
The limit of detection (LOD) was estimated as the lowest concentration of *C. jejuni* that could be detected using qPCR from the serial dilutions. The limit of quantification (LOQ), was estimated as the lowest concentration of *C. jejuni* that could be quantified and included in the linear regression for the standard curve. Both values were estimated from the standard curve [50].

4.9.2 Converting C_t values to Log CFU/mL and standard deviations

For the environmental stressors assays performed in this study, a sample from the standard curve was included in the qPCR runs, and the threshold of the qPCR analysis was adjusted to the same as in the standard curve: 8176.147 ΔRn. The C_t values were converted to Log CFU/mL by inserting the C_t value from the unknown sample into the linear regression

obtained from the standard curve, then isolating for x (Log CFU/mL) [50].
Standard deviations (SD) were calculated using Excel for samples performed with biological duplicates.

5 Results

5.1 DNA extraction

Three DNA extraction methods were tested to find the best suited for the PMA-qPCR process. The Ct values for the three tested DNA extracted methods are shown in table 3. The boiling lysis did not return any C_t values besides from the dead 10^7 CFU/mL PMA-treated sample, where a C_t value was observed. For the DNeasy Blood & Tissue Kit, the C_t was undetermined for the living 10^7 sample, which was expected to give a value. Additionally, a C_t value was observed for the dead 10^7 PMA treated sample, which was unexpected. For the stool kit, C_t values were observed for all the samples except the dead PMA-treated samples, where no C_t was expected. Overall, the C_t values for the Blood & Tissue Kit were lower compared to the Fast DNA Stool Mini Kit, indicating a higher concentration of the target DNA. The IAC C_t values (HEX signal) were acceptable for all samples, the results are not included.

Table 3: *The values in the table are C_t values for the three tested DNA extraction methods (QIAmp Fast DNA Stool Mini Kit, DNeasy Blood & Tissue Kit, and Boiling lysis). The samples are dilutions of C. jejuni, 10^7 and 10^6 CFU/mL, viable cells and dead (heat-inactivated) cells both treated with PMA and non-PMA treated. 'Undetermined' means the signal did not surpass the threshold within the cycles, not returning a value.*

Sample (CFU/mL)	PMA	Stool Mini Kit	Blood & Tissue Kit	Boiling lysis
Viable 10^7	-	16.37	Undetermined	Undetermined
Viable 10^6	-	20.13	17.08	Undetermined
Viable 10^7	+	17.90	15.94	Undetermined
Viable 10^6	+	21.35	18.26	Undetermined
Dead 10^7	-	21.33	13.67	Undetermined
Dead 10^6	-	24.35	17.00	Undetermined
Dead 10^7	+	Undetermined	26.41	38.64
Dead 10^6	+	Undetermined	Undetermined	Undetermined

Based on this result, the QIAmp Fast DNA Stool Mini Kit was selected for DNA extraction further in the project.

5.2 PMA efficiency and standard curve

The efficiency and effect of PMA were investigated on a 10-fold dilution series of *C. jejuni*. An overview of the dilutions and the samples can be seen in table 4, indicating if PMA was

applied. The C_t values are included in the table, revealing that PMA successfully inhibited the DNA amplification of the dead (heat-inactivated) -PMA cells (D10-x PMA), which is evident from the fact that all C_t values were undetermined. C_t values were determined for the dead cells non-PMA treated for the 10^5-10^7 CFU/mL. From the dead cells, 10^6 and 10^8 CFU/mL were plated to test if the inactivation was obtained. No growth was observed on the plates. For both non-heat treated +PMA and -PMA, C_t values were determined for samples 10^4-10^7 CFU/mL. No C_t values were determined for samples below, 10^4 CFU/mL. The negative control, *E. coli,* returned undetermined C_t, together with the process control for the DNA extraction. Furthermore, the C_t values for the IAC, all returned acceptable, ensuring a reliable qPCR run. The amplification plot for the IAC runs (HEX signal) can be seen in appendix 10.2.

Table 4: *A 10-fold dilution series of heat-inactivated (dead) and non-heat treated (viable) C. jejuni cells treated with PMA and non-treated with corresponding C_t value from qPCR. 'Undetermined' means the signal did not surpass the threshold within the cycle. A negative control (E. coli) and process control that contains no DNA is included.*

Sample name	CFU/mL	Heat-treated	PMA	C_t value
D10-2	10^7	+	-	20.6
D10-3	10^6	+	-	23.7
D10-4	10^5	+	-	26.1
D10-5	10^4	+	-	Undetermined
D10-6	10^3	+	-	Undetermined
D10-7	10^2	+	-	Undetermined
D10-8	10^1	+	-	Undetermined
D10-2 PMA	10^7	+	+	Undetermined
D10-3 PMA	10^6	+	+	Undetermined
D10-4 PMA	10^5	+	+	Undetermined
D10-5 PMA	10^4	+	+	Undetermined
D10-6 PMA	10^3	+	+	Undetermined
D10-7 PMA	10^2	+	+	Undetermined
D10-8 PMA	10^1	+	+	Undetermined
L10-2	10^7	-	-	15.0
L10-3	10^6	-	-	16.8
L10-4	10^5	-	-	21.8
L10-5	10^4	-	-	24.4
L10-6	10^3	-	-	Undetermined
L10-7	10^2	-	-	Undetermined
L10-8	10^1	-	-	Undetermined
L10-2 PMA	10^7	-	+	15.3
L10-3 PMA	10^6	-	+	19.8
L10-4 PMA	10^5	-	+	21.0
L10-5 PMA	10^4	-	+	26.5
L10-6 PMA	10^3	-	+	Undetermined
L10-7 PMA	10^2	-	+	Undetermined
L10-8 PMA	10^1	-	+	Undetermined
E. coli 10-3	10^6	-	-	Undetermined
Process control	None	None	-	Undetermined

The dCt was calculated (table 5) using equation 1 for the non-heat treated samples to determine if PMA affected the amplification of the DNA. The dCt for the non-heat treated samples should be 0 (±1) [37], where two of the samples were not within the range, both had a dCt above 1.

Table 5: *The calculated dCt for the non-heat treated samples that returned a C_t value from the qPCR analysis. The dCt should be 0 (±1).*

Sample	CFU/mL	C_t (+PMA)	C_t (-PMA)	dCt
L10-2	10^7	15.3	15.0	0.3
L10-3	10^6	19.8	16.8	3.0
L10-4	10^5	21.0	21.8	-0.8
L10-5	10^4	26.5	24.4	2.1

Through this study, the standard curve samples were run through the qPCR analysis three times. The C_t values for the 10-fold dilution series of the non-heat treated +PMA *C. jejuni* cells (considered viable) were plotted against Log CFU/mL. CFU/mL was calculated from plate counts of 10^2-10^4 CFU/mL, followed by log transformation. A linear regression was obtained from the standard curve, and the efficiency was calculated. Following, one of the standard curves was selected. For the standard curve (figure 11), the efficiency was calculated using equation 2. The standard curve has an efficiency of 93.2%, which was considered acceptable. The R^2 value of the curve was 0.949. The LOD and LOQ, were both detected to be 2.39 Log CFU/mL, as the lowest estimated concentration of *C. jejuni* that could be detected and quantified using qPCR from serial dilutions [50].
The additional standard curves can be seen in appendix 10.1, where 21A has an efficiency of 76% and R^2 of 0.92 and 21B has an efficiency of 84.2% and R^2 of 0.97.

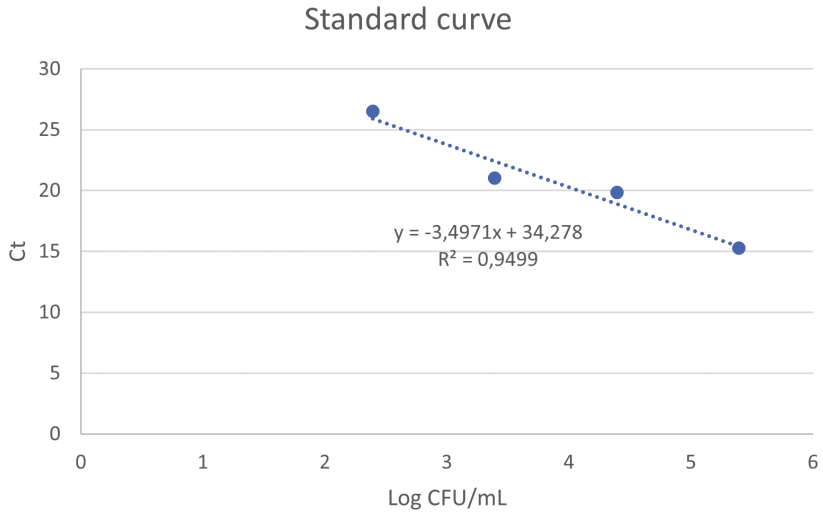

Figure 11: *The standard curve was made from calculated Log CFU/mL against the C_t values from the non-heat treated +PMA 10-fold dilution series samples of C. jejuni.*

Figure 12 illustrates the amplification plots for the qPCR standard curve analysis. It can be observed that there are exponential phases at the non-heat treated -PMA cells DNA (figure 12A), at the non-heat-treated +PMA (figure 12B), and the heat-inactivated -PMA cells DNA

(figure 12C), which was expected. No exponential phases are observed for the heat-inactivated +PMA cells DNA (figure 12D), which indicates inhibition of amplification by PMA. For the non-heat treated -PMA (figure 12A) and +PMA (figure 12B), it can be observed that the amplification of the 10^4 CFU/mL was reduced for the +PMA sample compared to the -PMA. Furthermore, non-heat treated +PMA amplifications cross the threshold at later cycles compared to the non-heat treated -PMA, indicating inhibition of the DNA of the non-heat treated +PMA (viable) cells in the samples. The amplification was not observed for any samples below 10^4 CFU/mL, for both non-heat-treated -PMA (figure 12A) and +PMA (figure 12B).

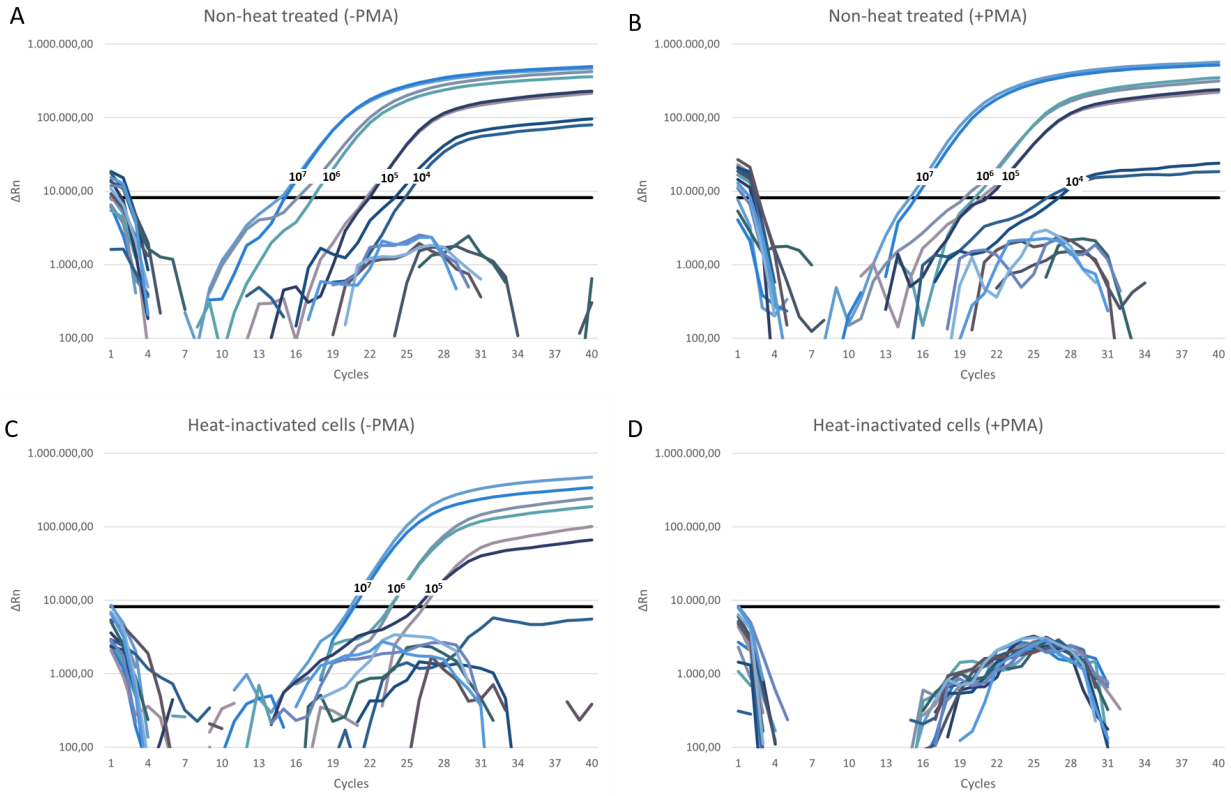

Figure 12: *Amplification plots for the standard curve (FAM signal), all samples were run in duplicates. The black horizontal line represents the threshold. A: amplification plot of the non-heat treated -PMA cells DNA. B: amplification plot of the non-heat treated +PMA cells DNA. C: amplification plot of the heat-inactivated -PMA cells DNA. D: amplification plot of the heat-inactivated +PMA cells DNA.*

5.3 LIVE/DEAD *Bac*Light

Ten-fold dilution series of *C. jejuni* was investigated by the LIVE/DEAD *Bac*Light method. Figure 13 shows fluorescence microscopy images of one 96-well plate containing stained *C. jejuni* cells, using a LIVE/DEAD *Bac*Light Viability kit. Figure 13A is taken with the FITC filter, figure 13B is taken with the Cy5 filter, and figure 13C is an overlay of two images with each filter where the contrast/intensity was increased. The positions of the samples were shown in an illustration in figure 7. The assay consisted of three groups of *C. jejuni* cells:

viable (blue square), heat-inactivated (red square), and a mix of viable and heat-inactivated (50:50) (purple square). The described groups were stained with a solution (1:2) of SYTO 9 and PI. Three controls were constructed: a viable cell control (VC), stained with SYTO 9, a heat-inactivated control (DC), stained with PI, and a non-dye control (10-fold dilution series). The VC and DC were non-diluted *C. jejuni* cells (10^9 CFU/mL). The composition of cells is the same in both figure 13A, B, and C. Nothing was added to well E09.
Green fluorescence emitted by the SYTO 9 dye can be observed in figure 13A. Green fluorescence was prominently visible in the first column of the viable and heat-inactivated cells and in the first column of the mixed cells. The VC exhibited the most intense green fluorescence. A weak green fluorescence in the second column (10^{-1}) was be observed for the heat-inactivated cells. Red fluorescence emitted by the PI dye can be seen in figure 13B. Red fluorescence was prominently visible in the first column of the viable and heat-inactivated cells. Where the fluorescence appeared more intense at the viable cells. A weaker red fluorescence appeared in the first column of the mixed cells, compared to the viable and heat-inactivated. Red fluorescent was observed for the DC, but did not appear as intense as the VC with the FITC filter (figure 13A). Both SYTO 9 and PI can be seen in figure 13C. Both green and red fluorescence was observed at the NDC, which was not observed in figure 13A and B. Red fluorescence was observed in the VC where PI was not added. Green fluorescence was observed in the DC, where SYTO 9 was not added. A mix of red and green fluorescence was observed in the first column (blue and red squares), and at the first column of the mixed cells (purple square), which is consistent with the observations from figure 13A and B. Prominently red fluorescence was observed in the second column (10^{-1}), especially at the heat-inactivated cells. Green and red fluorescence was observed in columns 10^{-2}-10^{-4}, for all cell groups (figure 13D), whereas no fluorescence was observed in figure 13A and B.

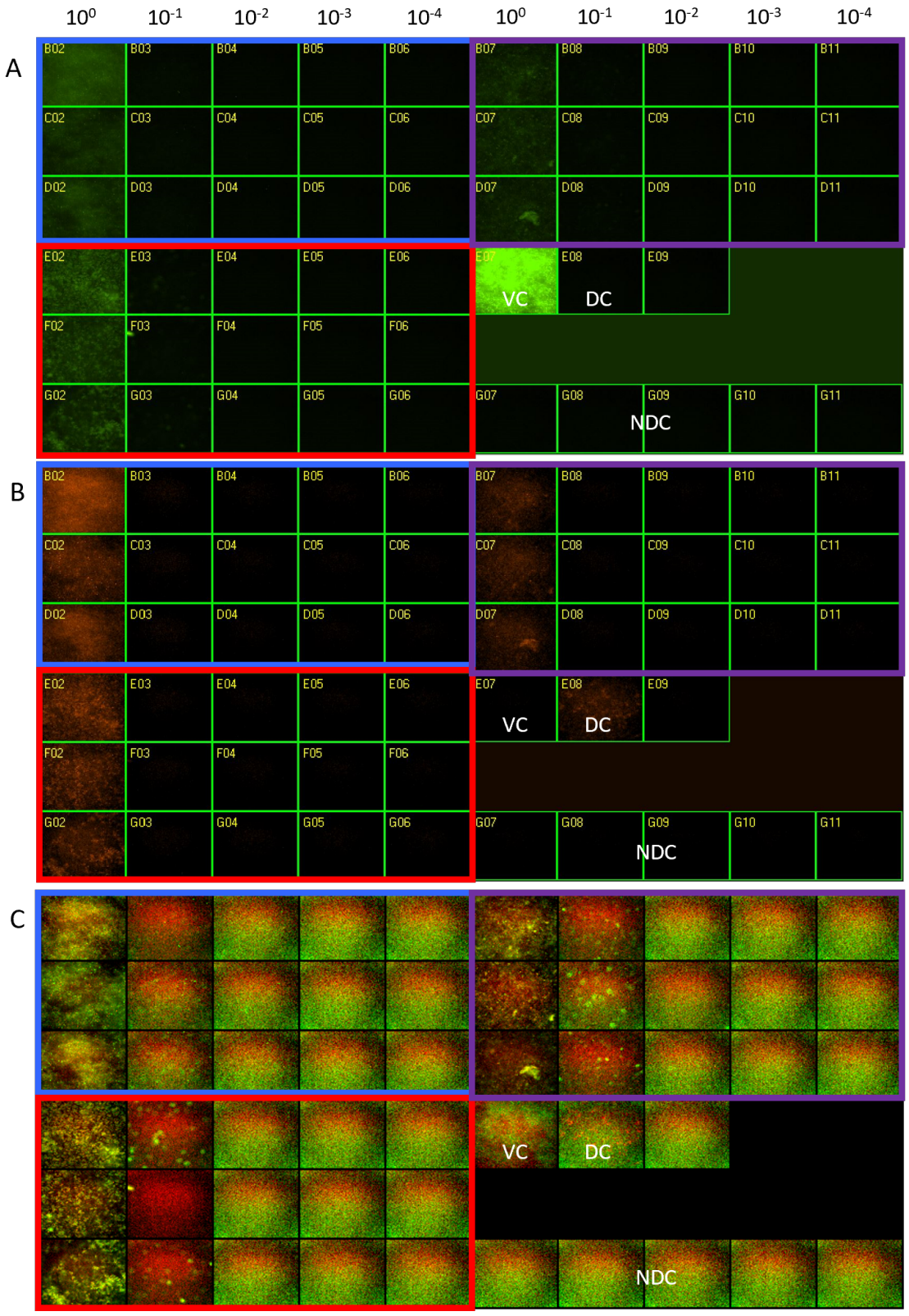

Figure 13: *Fluorescence microscopy images of 10-fold dilution series of C. jejuni, stained with SYTO 9 and PI. A: emission of SYTO 9 (FITC filter), B: emission of PI (Cy5 filter), C: emission of SYTO 9 and PI (FITC and Cy5 image overlay). Blue: viable cells, red: heat-inactivated (dead) cells, purple; viable and dead cells (50:50), VC: viable cell control, DC: dead cell control, NDC: Non-dye control.*

5.4 Environmental stressors inducing the VBNC state in *C. jejuni*

The C_t values from the qPCR analysis were converted to Log CFU/mL by using the standard curve from section 5.2.

5.4.1 Desiccation of *C. jejuni*

5.4.1.1 Desiccation trial of *C. jejuni*

This study has investigated the potential induction of the VBNC state in *C. jejuni* through desiccation.

As a preliminary experiment, 1 mL of saline was left on an empty Petri dish at room temperature. After 4 days, the plate was dried out, and salt crystals were left on the plate. Therefore desiccation with *C. jejuni* solutions were performed within this time frame.

A desiccation assay was conducted as a trial to investigate if *C. jejuni* would survive desiccation and investigate if the assay would generate results from the qPCR analysis. The PMA-qPCR estimated Log CFU/mL are shown in figure 14. From the figure, the presence of both viable (+PMA) and non-viable cells was observed, with a majority of non-viable cells. Viable cells were observed for the sample of 1 mL with water present (incubation with a glass beaker with water). This was not observed for the 1 mL sample without the water present. Viable cells were detected in the sample with a 500µL volume and no water present. No viable cells were observed for the 500µL sample where water was present. The control was the bacterial stock solution, which was kept at 4 ℃ (±3 ℃) for 48 hours, where it was observed that almost all the detected cells were viable. No growth was observed for any of the desiccated samples.

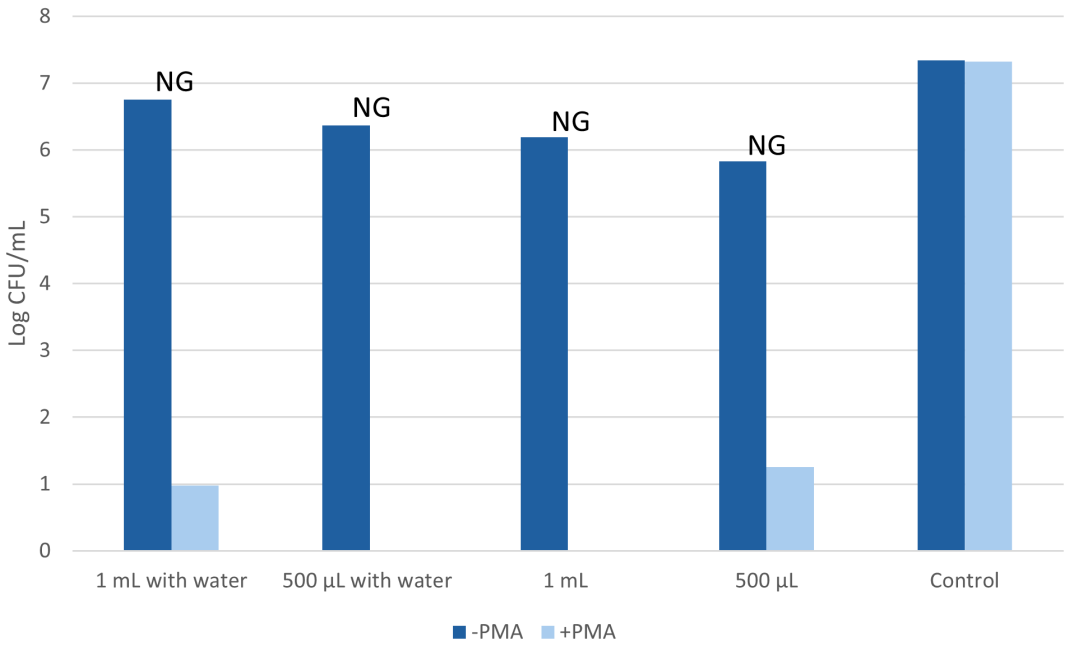

Figure 14: *Desiccation assay. Log CFU/mL estimated by PMA-qPCR of the tested samples. Sample names correspond to the different volumes added to the plates, with and without the glass beaker with water. -PMA (dark blue), +PMA (light blue). NG means no growth was observed after plating on BA.*

5.4.1.2 Desiccation of *C. jejuni* with chicken juice

To further investigate the effect of desiccation on *C. jejuni* an assay was conducted with the addition of chicken juice to examine the potential influence of a nutrient source during desiccation. During the assay, the average humidity recorded was 60.7 %RH, which was within the established acceptable range (49-70%) for this experiment. However, the measurements (figure 15) revealed that the humidity levels were inconsistent, failing to replicate the conditions at the stable. An increase to 100 % RH followed by a sudden and steep decline was observed (figure 15). Subsequently, the RH stabilized at ∼30 %RH, which was not within the range. The sudden increase, followed by a steep decline, was due to the lid of the jar by mistake was closed and, when noticed, opened again and closed on half.

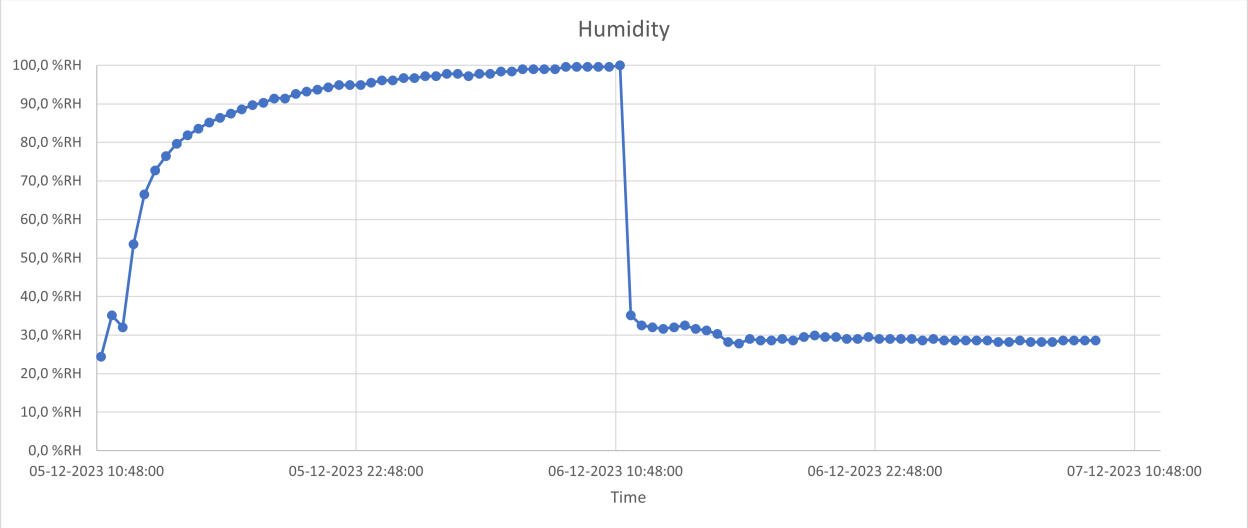

Figure 15: *Humudity graph over the desiccation trial. The x-axis shows the time over 48 hours, the y-axis shows %RH.*

Figure 16 shows the calculated Log CFU/mL results from the assay; the sample names correspond to the solution they were incubated in. The addition of chicken juice seemed to influence the viability of *C. jejuni* through desiccation, as a higher number of viable cells (+PMA) were detected compared to the solution without chicken juice. In the samples containing 0.9% NaCl and 5% chicken juice, the proportion of viable cells was observed to be 2.8 (SD=0.5) Log CFU/mL compared to 5.4 (SD=0.3) Log CFU/mL of all cells (-PMA). Whereas the proportion of viable cells in the sample containing only *C. jejuni* and 0.9 NaCl had 2.1 (SD=0.1) Log CFU/mL compared to 5.3 (SD=0.2) Log CFU/mL of all cells. No clear reduction in viable cells was observed from the controls, except from the control containing chicken juice, where it was noticeable that more cells were found viable compared to all cells. No growth was observed on the plates from the desiccation samples, besides one of the samples containing chicken juice (duplicates) where colonies were observed. Growth was observed for the controls. Contamination was noticed on plates with samples from the 0.9% NaCl with 5% chicken juice. White colonies were seen on the BA plates after incubation together with colonies looking like *C. jejuni*. The contamination was investigated with the help from laboratory technicians who performed Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) on the different colonies on the plates. The colonies consisted of *C. jejuni* and an undetectable bacterium. It was assumed that the contamination did not affect the further analysis. Pictures of the plates are shown in the appendix 10.4.

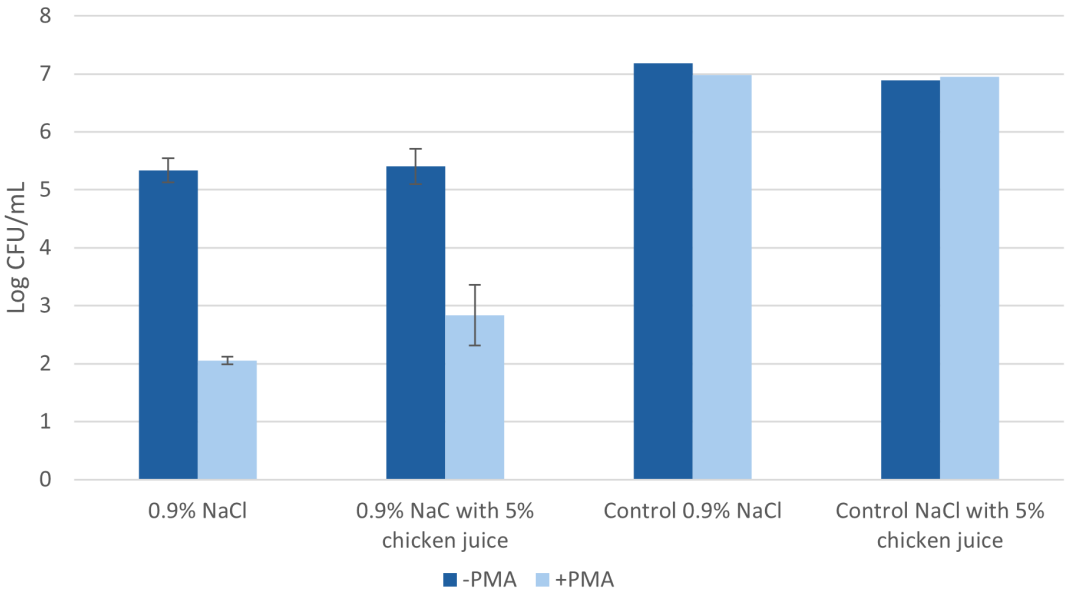

Figure 16: *Desiccation assay of C. jejuni. Log CFU/mL estimated by PMA-qPCR in 0.9% NaCl (saline) with and without chicken juice. -PMA (dark blue), +PMA (light blue). The error bars show SD.*

5.4.2 Osmotic stress of *C. jejuni*

This study has investigated the potential induction of the VBNC state in *C. jejuni* by osmotic stress. An assay with *C. jejuni* incubated in 7% NaCl, tap water, 0.9% NaCl, and 0.9% NaCl with chicken juice was performed. Figure 17, shows the calculated Log CFU/mL for the viable (+PMA) cells over time for the four different solutions. A reduction in the viable cells within the time frame (0-168 h) was observed for all the tested solutions. For 7% NaCl, a slight reduction of viable cells was observed between 0 to 5 h, compared to the other solutions. No reduction of viable cells between 5 h to 24 h was observed. This was followed by reduction in viable cells (figure 17A). For tap water (figure 17B) there was observed a reduction of viable cells between 0 and 5 h. No difference in cell count was observed between 5 and 24 h. A noticeable increase in viable cells between 24 h to 48 h was observed, followed by a decrease. For 7% NaCl and tap water, no growth was observed after 7 days (168 h), indicating the presence of VBNC cells, as PMA-qPCR detected a signal. Pictures of the BA plates can be seen in appendix 10.6. For the 0.9% NaCl solution, an exponential reduction over time was observed (figure 17D). For the 0.9% NaCl with chicken juice solution, the reduction did not follow an exponential reduction (figure 17C). The cell count of viable cells decreased slowly compared to the 0.9% NaCl solution.

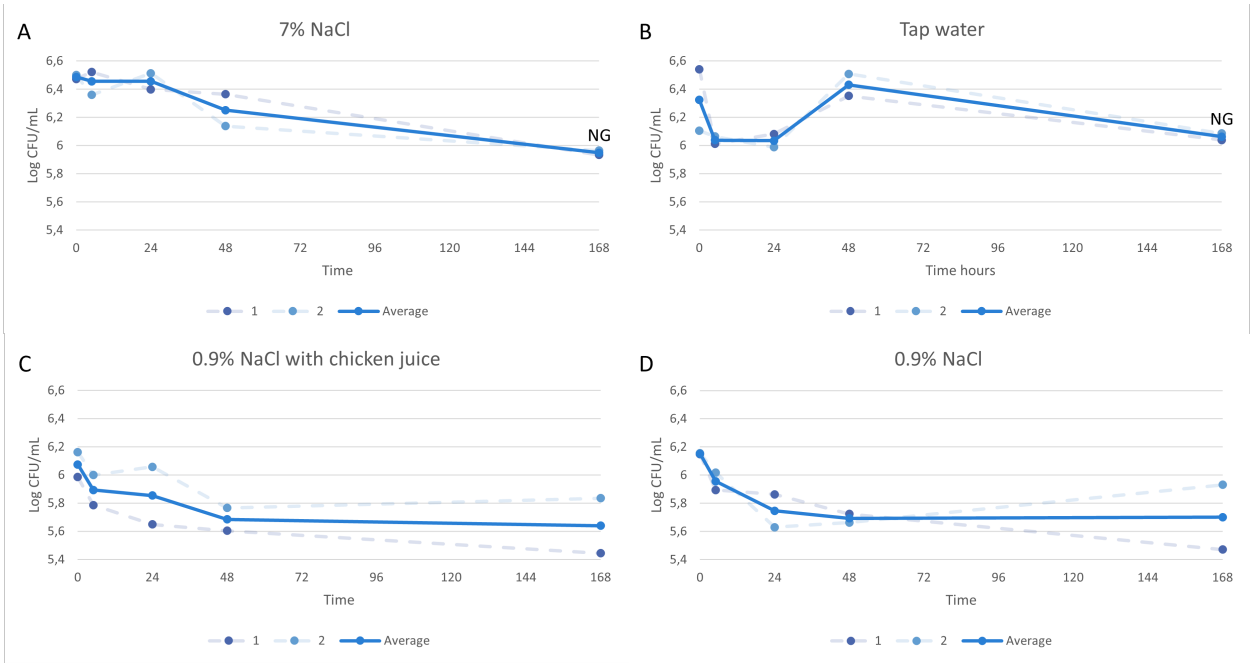

Figure 17: *Osmotic stress assay. LOG CFU/mL estimated by PMA-qPCR of +PMA cells (viable) over time for the four different solutions with C. jejuni. A: 7% NaCl, B: tap water, C: 0.9% NaCl with chicken juice, and D: 0.9% NaCl (saline). The dashed lines represent the biological duplicates, and the full line is the average of the duplicates. NG means no growth was observed on BA incubated at 41.5 ℃ at microaerobic conditions ON.*

Figure 18 illustrates the estimated Log CFU/mL for all cells (-PMA) compared to viable cells (+PMA)) at the different sampling times. For *C. jejuni* in 7% NaCl (figure 18A) and in tap water (figure 18B). The amount of all cells (viable and dead) was higher compared to the amount of viable cells at all sampling times for the tested solutions. The bar plots of all cells compared to viable cells for 0.9% NaCl and 0.9% NaCl with chicken juice can be seen in appendix 10.5.

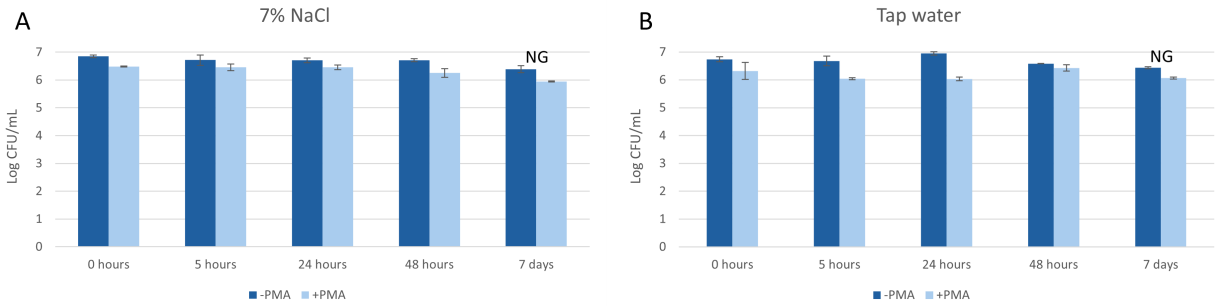

Figure 18: *Log CFU/mL estimated by PMA-qPCR at sampling times in A: 7% (w/v) NaCl, B: tap water. -PMA (dark blue), +PMA (light blue). NG means no growth was observed on BA incubated at 41.5 ℃ at microaerobic conditions ON. Error bars show SD.*

5.4.3 Nutrient deprivation

This study has investigated the potential induction of the VBNC state in *C. jejuni* by nutrient deprivation. *C. jejuni* was incubated in 0.9% NaCl and 0.9% NaCl with 5% chicken juice over a 14-day period. Figure 19, shows the estimated Log CFU/mL (+PMA and -PMA) for the solutions over time. The assay revealed a higher number of viable cells in the solution 0.9% NaCl with chicken juice compared to without chicken juice. A decrease in viable cells between day 2 to 7 was observed in the 0.9% NaCl sample (3.5 Log CFU/ml reduction). Subsequently, between day 7 to 14, the viable cells decreased slowly by ~0.7 Log CFU/mL. In contrast, the sample 0.9% NaCl with chicken juice decreased between day 2 to day 7 by 2.6 Log CFU/mL, followed by an increase between day 7 to day 14, with 0.2 Log CFU/mL. On day 14, 2.8 Log CFU/mL was detected in the 0.9% NaCl sample, whereas 4.3 Log CFU/mL was detected for the 0.9% NaCl with chicken juice (figure 19). No growth was observed on day 14 for both solutions (0.9% NaCl and 0.9% NaCl with chicken juice).

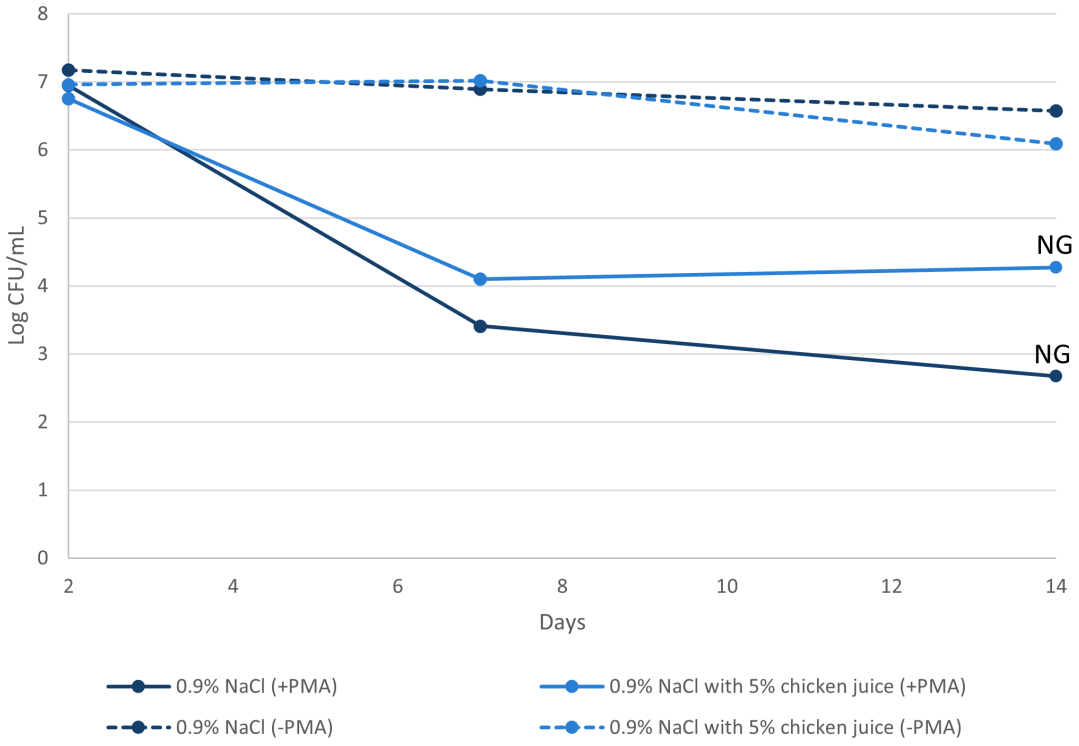

Figure 19: *Log CFU/mL of C. jejuni estimated by PMA-qPCR in 0.9% NaCl with and without chicken juice over 14 days (time). 0.9% NaCl +PMA (full line - dark blue), 0.9% NaCl -PMA (dashed line - dark blue), 0.9% NaCl with chicken juice +PMA (full line - light blue), 0.9% NaCl with chicken juice -PMA (dashed line - light blue). NG means no growth was observed on BA incubated at 41.5 ℃ at microaerobic conditions ON.*

The tubes with the bacterial solutions were left in the incubator at 25℃ for one month with no lid on to ensure atmospheric conditions. The liquid had evaporated, so it was not possible to take a sample after one month. In the tube with 0.9% NaCl and chicken juice, a yellow color was noticed on the side of the tube, in the liquid and yellow precipitate. In the tube

with 0.9% NaCl (saline) without chicken juice, a yellow precipitate was noticed, and a light yellow color of the liquid was observed. The tubes are shown in figure 20.

Figure 20: *Tubes from nutrient deprivation assay of C. jejuni after one month. The tube with 0.9% NaCl and 5% chicken juice is on the left, and the tube with 0.9% NaCl and no chicken juice is on the right.*

6 Discussion

6.1 DNA extraction

Three DNA extraction methods were evaluated for this study, with the aim of finding the most suitable method for PMA-qPCR analysis. The boiling lysis method did return undetermined as C_t values for almost all samples assessed. Besides, a C_t value was observed for one dead PMA-treated sample. Suggesting that PMA did not inhibit the amplification as expected [8]. Lv et al. 2020 [8] suggested that the DNA extraction method could influence the sensitivity and specificity of the qPCR. They successfully created similar standard curves using both a boiling method and an extraction kit. In contrast, in this study, the boiling lysis failed to produce C_t values for any of the other samples, which contrasts with the results observed in the Lv et al. 2020 study. For the Blood & Tissue Kit, the C_t value for one of the living samples was undetermined, where a value was expected. Additionally, the determined C_t values were lower compared to those from the Fast DNA Stool Mini Kit, indicating a higher amount of target DNA in the samples. Nevertheless, the QIAmp DNA stool mini kit was selected for further DNA extraction in the study. The kit was assessed to be most suitable for the study due to its faster extraction process compared to the Blood & Tissue Kit, and C_t values were obtained for samples as expected. The findings were based on a single trial with no duplicates (except for the Stool kit). The results may not deliver reliable insight into the methods. Further trials should be conducted to provide reliable evidence to conclude which DNA extraction method is best suited for facilitating the PMA-qPCR process within the aim of this study.

6.2 PMA-qPCR efficiency and standard curve

The standard curve was produced with a 10-fold dilution series of a pure culture of *C. jejuni*. Three standard curves were made over three trials with the same set of extracted samples. Both the efficiency and R^2 coefficient are important parameters for the reliability and performance of the qPCR analysis. The efficiency is considered a significant factor in relation to the quantification of DNA in the unknown sample and should be between 90-100%. The R^2 coefficient is a measure of the correlation between the C_t values and known Log CFU/mL and is acceptable if above 0.98 [50, 52]. For the three standard curves, the selected standard curve has an efficiency of 93.2% and R^2 at 0.95 (figure 11). The R^2 coefficient is below the acceptable, but the efficiency is within the expected range. In comparison, the parameters for the two other standard curves were both below the acceptable limits (appendix 10.1). Therefore, the standard curve with the highest efficiency was selected as the quantification of unknown samples was weighted highly in relation to the purpose of the rest of the study.

This study wanted to assess PMA-qPCR's ability to differentiate viable and heat-inactivated *C. jejuni* cells. This was conducted with a 10-fold dilution series with non-heat treated and heat-inactivated cells, where half was treated with 20µM PMA, and the other half was

diluted with saline to obtain equal volumes. The results showed that PMA successfully inhibited the amplification of DNA from heat-inactivated (dead) +PMA cells (table 4 and figure 12), as no samples reached the threshold. Compared to the heat-inactivated -PMA cells, where amplification was observed. Josefsen et al. 2010 [44] reported that the use of 23.81 µM (10 µg/mL) PMA achieved 100% inhibition of amplification by PMA treatment of heat-inactivated cells, as was also observed in this study. Moreover, from the results in this study, the amplification of DNA from non-heat treated +PMA samples was reduced (inhibited) compared to the non-heat treated -PMA samples. This was observed for the sample 10^4 CFU/mL, which emitted a lower fluorescence signal through the cycles, and the amplification reached the threshold at a later cycle. This may result from the PMA inhibiting damaged cells or the PMA concentration being excessive, delaying the amplification when compared to the non-heat treated -PMA samples. Two of the dCt values for the non-heat treated cells (considered viable) were determined to be above the expected (should be 0 ±1). Indicating that PMA affects the viable cells which will lead to an underestimation of the viable cells. Earlier studies have shown that excessive PMA concentrations can inhibit the amplification of viable cells, and therefore lead to an underestimation of viable cells [53]. The PMA concentration for this study was inspired by Josefsen et al. 2010 [44] and Lv et al. 2020 [8]. Lv et al. 2020 investigated different concentrations of PMA on *C. jejuni* and observed no significant differences in the amplification of dead cells treated with 20 µM or 50 µM PMA. They therefore chose 20 µM PMA, as it could effectively inhibit amplification of DNA from dead *C. jejuni* cells, without significantly affecting DNA amplification from the viable cells. As was observed in this study, Josefsen et al. 2010 [44] also noticed a reduction in the signal from qPCR amplification in PMA-treated samples compared to untreated. However, Nocker et al. 2006 [54] reported that viable cells with intact cell membranes did not take up PMA. It was only internalized by cells with compromised membranes. Their study involved testing different concentrations of PMA, including higher concentrations than the one used in this study. Suggesting that the viable cells in this study did not internalize PMA when having intact membranes.

Various studies claim that the combination of PMA and qPCR can detect viable cells, but Trinh et al. 2022 [55] describe three criteria to evaluate the viability of a bacterial pathogen: culturability, metabolic activity, and membrane integrity. Where the culturability cannot evaluate VBNC cells, the two other applications have evolved. Josefsen et al. 2010 [44] describe that the definition of viability can be distinguished in many ways, such as having an intact membrane, active metabolism, culturability, and the ability to sustain a proton gradient across the inner and outer cell regions. They suggest that the PMA-qPCR method may be supplemented with other compounds that can improve the distinguishing of viability. The addition of a supplement, such as a dye or glucose, for uptake, could evaluate an active metabolism. This approach might help to ensure the detection of only viable cells by preventing the detection of dead cells with intact membranes that otherwise may would be detected as viable [55].

For this study, the viability of cells was evaluated on the intact membrane as it is essential

for maintaining cellular integrity. It was assumed that PMA only penetrates when the cell has a compromised membrane, enabling differentiation of viable and non-viable *C. jejuni* [54]. This study differentiated the viable and heat-inactivated cells with the use of PMA-qPCR, although the PMA did not assess metabolic activity.

6.3 LIVE/DEAD *Bac*Light

The LIVE/DEAD *Bac*Light assay was based on its potential, as through a previous literature study, was found to be a promising method for the detection of VBNC *C. jejuni* cells in combination with flow cytometry. The assay was a trial of the method, that aimed to assess its efficacy to differentiate viable and heat-inactivated *C. jejuni*. Previous studies have shown to be able to detect VBNC cells using LIVE/DEAD staining in combination with culturing dependent methods [26]. The observed result in this study was unexpected, as extensive green and red fluorescence was observed (figure 13C). This observation might be noise or background fluorescence as the intensity was increased. The extensive fluorescence makes distinguishing between viable and heat-inactivated cells difficult. The SYTO 9 (green) was expected to dye all cells, whether they were viable or dead, due to heat-inactivation. PI (red) penetrates the membrane of dead cells and has a stronger affinity for the nucleic acids than SYTO 9, displacing the SYTO 9 molecule [43]. Both green and red fluorescent were observed (figure 13C) in wells where viable cells were expected and in wells with only heat-inactivated cells. This could potentially lead to an overestimation of viable cells. Braun et al. 2019 [26] describe how it is almost impossible to prepare a live cell control that does not contain also dead cells. As a result, a small signal of dead cells will always be present, which will fluorescence red due to dual staining. Conversely, the heat-inactivated cells were not expected to exhibit green fluorescence, indicating the PI dye did not outcompete the SYTO 9 dye. If this is the case, it might be due to the ratio of the stains used. A 1:2 (SYTO 9:PI) ratio was chosen inspired by Magajna et al. 2015 [42], that successfully stained and discriminated *C. jejuni* cells in combination with confocal microscopy. However, our result may indicate that a higher ratio of PI or lower SYTO 9 is needed. The product information for the LIVE/DEAD *Bac*Light viability kit (Molecular Probes) [56] suggests a 1:1 ratio of the dyes. This would provide a good discrimination between viable and dead cells for most applications. Experimenting with the dye ratios to optimize staining for specific purposes is advisable. The result of this study was not as expected. Further research using this method would require a workload compared to the PMA-qPCR method that was established successfully. The LIVE/DEAD *Bac*Light assay was conducted as a single trial, therefore, repeating the assay would give valuable insight into whether the observed result was based on an error or if further improvements of the protocol are needed. Further testing and optimization of the LIVE/DEAD *Bac*Light protocol are necessary, as the technology and method were not firmly established in the laboratory.

6.4 Evaluation of environmental stressors inducing the VBNC state in *C. jejuni*

C. jejuni is exposed to various environmental stressors throughout the food industry. Desiccation and hyperosmolarity, among others, are strategies used in food processing to control foodborne pathogens [57]. Foodborne pathogens like *C. jejuni* have evolved survival strategies to persist and remain prevalent.
Upon a new flock of chickens is introduced to the stable, the area undergoes cleaning and washing, resulting in a moist environment, where the water is evaporating. If *C. jejuni* is present, it may end up in water droplets and subsequently desiccate. The influence of the desiccation on *C. jejuni* was therefore investigated.
A preliminary experiment was conducted where 1 mL saline was added to an empty Petri dish and left to see how long it would take to desiccate. Once the saline had completely evaporated, small salt crystals were observed on the plate. Illustrating the concentration of salt increases as the liquid evaporates. The gradual increase of salt concentration creates a hyperosmotic environment.

For the desiccation trial where different volumes were tested to desiccate with and without a glass beaker with water present, where viable cells were detected in only two samples. The desiccation was conducted at 41.5 ℃ at microaerobic conditions. The presence of water as a humidity source did not seem to have an effect, as viable cells were detected both with and without water present. However, the trial was only performed once and was set up as a trial to investigate whether it would provide any viable cell results. Therefore, no conclusions can be made about whether the presence of the water had an effect on the viability of the cells, as the humidity throughout the trial was not monitored. Additionally, no growth of colonies on BA was observed for any of the samples, except for the control which did not desiccate. Based on these results it seems that the desiccation likely induced the VBNC state in *C. jejuni*.

To further investigate the impact of desiccation of *C. jejuni*, a desiccation assay with chicken juice was designed. The chicken juice was added to simulate the organic material that might remain in the chicken stable after a potential wash/cleaning. The plates and controls were incubated at atmospheric conditions at 25 ℃, to simulate the conditions in the chicken stable. A datalogger was used to monitor the humidity throughout the assay. The logged % RH was unstable in the setup and failed to stay within the observed humidity range (49-70%) recorded at the chicken stable (appendix 10.3). Silva et al. 2011 [58] described that *Campylobacter* are sensitive to low humidity environments. Furthermore, De Cesare et al. 2003 [59], studied the survival and persistence of *C. jejuni* and *Salmonella* serotypes under organic loads (nutrient-rich) on food contact surfaces. They reported that the duration of the lag phase (the initial phase preceding bacterial growth, after adjustment to the environment) was affected by both the contact surface and the suspended medium. They also suspected that the rate of drying might have an impact on the phase. Where the drying rate is influenced

by temperature, air circulation, and relative humidity. They describe that the observation may indicate that the relative humidity, in combination with the temperature, affects the lag phase for bacterial growth of *C. jejuni*. This has also been suggested in a previous study where *C. jejuni* could be recovered from eggshell surfaces after an extended period of time when the relative humidity was increased, and the temperature was decreased compared to lower humidity and higher temperature [59]. Further investigations are needed to determine whether maintaining the humidity between 49-70% impacts the survival of *C. jejuni*, as previous research suggests relative humidity has an impact.

The desiccation assay with chicken juice showed that in the control with 5% chicken juice, a higher number of viable (+PMA) *C. jejuni* were detected compared to all cells (-PMA), where all cells are assumed to be a combination of viable and dead cells. This imbalance of viable and all cells might be due to uncertainty, such as human error or uncertainty of lab equipment. The addition of chicken juice seemed to positively affect the number of viable cells compared to the samples lacking chicken juice. After desiccation, the cells were retrieved, and plating was conducted. No growth was observed for the desiccated solutions without chicken juice, but a signal was still obtained from PMA-qPCR. The detected viable cells (+PMA) are assumed VBNC [8]. For the desiccated solution with 5% chicken juice, growth was observed for sample 2 (one of the duplicates), but not for sample 1. This indicates that the addition of chicken juice may extend the survival of *C. jejuni*, and more cells can maintain viability. This observation was supported by the higher number of viable cells detected by PMA-qPCR compared to the solution without chicken juice. Growth was observed after plating for the controls that were subjected to the same incubation conditions but were not subjected to desiccation. These observations indicate that desiccation for 48 h in saline with and without 5% chicken juice, induced the VBNC state in *C. jejuni*. A higher number of viable cells were detected by PMA-qPCR in the desiccation assay with chicken juice compared to the initial desiccation trial. The desiccation with chicken juice was conducted at 25 ℃ under atmospheric conditions, whereas the desiccation trial was at 41.5 ℃ under microaerobic conditions. This might indicate that survival from desiccation is affected by the temperature and oxygen level. These parameters were not investigated through this study, but from the results, a higher number of viable *C. jejuni* cells were observed when incubated at lower temperatures and atmospheric conditions. The desiccation of *C. jejuni* at different temperatures and oxygen levels must be investigated further to address the effect of the parameters fully.

As in the desiccation assay with chicken juice, the same pattern was observed for the nutrient deprivation assay. The presence of 5% chicken juice in the solution led to a higher detection of viable cells. A smaller decrease in viable cells between day 2 to day 7 was recorded for the solution with chicken juice compared with the solution without. The reduction of viable cells between the two samples (with and without chicken juice) is slight at first but, after 7 days, is more noticeable. Nonetheless, no samples were collected between day 2 and 7. This may

have missed the opportunity to monitor the development of the viable cells closer. A slight increase was observed in the solution with chicken juice between day 7 to day 14, though this might be due to an uncertainty in measurements. Therefore, it would be ideal and valuable to collect additional samples between days 7 and 14 to monitor development and identify the point where the cells enter the VBNC state. No growth after plating was observed for any of the solutions after 14 days, but viable cells were detected by PMA-qPCR, indicating VBNC *C. jejuni* cells.

When comparing the viable cells from the solutions with and without chicken juice, a difference of approximately 1.5 Log CFU/mL was detected for the viable *C. jejuni* after 14 days. This may suggest that the addition of chicken juice extended the survival of *C. jejuni* cells [48]. Birk et al. 2004 [48] suggested that chicken juice consists of protective compounds such as lipids and peptides, as they observed that it prolonged the viability of *C. jejuni* compared to conventional laboratory media. It is important to note that chicken juice is an organic substance obtained from thawed commercially frozen chicken; the composition of compounds and nutrients can vary, so it cannot be assumed that it consists of the same composition consistently [48].

After leaving the tubes with *C. jejuni* in saline and saline with 5% chicken juice for a month, a yellow layer was noticed on the sides of the tube within the tube with chicken juice (figure 20). Brown et al. 2014 [34] found that chicken juice enhances *C. jejuni*'s biofilm formation. They observed that the addition of 5% chicken juice to brucella broth increased the biofilm formation compared to brucella broth alone. This could explain the layer on the side of the tube, as a similar layer was not observed in the tube without chicken juice. The layer could also be due to the addition of an organic material. Furhter research is necessary to be able to confirm or not if biofilm was observed.

This study investigated the effects of a hyper-osmotic and hypo-osmotic environment on *C. jejuni* to assess whether these stressors could induce the VBNC state in *C. jejuni*. Previous studies have shown that osmotic stress caused by high concentrations of NaCl could induce the VBNC state in *C. jejuni*. Lv et al. 2020 [8], found that incubation of *C. jejuni* in 7% (w/v) NaCl lost the ability to culture completely after 24 h at 37 ℃. In this study, the ability of *C. jejuni* to grow on BA was lost after 7 days of incubation in 7% (w/v) NaCl at room temperature. *C. jejuni* may have entered the VBNC state earlier, but samples were not gathered between 48 hours and 7 days. The reduction of viable cells between 0 to 48 h was slower for the 7% NaCl solution, compared to 0.9% NaCl with and without chicken juice. This might be due to a kind of shock state, where *C. jejuni* adapts a survival mode when exposed to osmotic stress. Doyle et al. 1982 [29] found that *C. jejuni* survived longer when incubated in 4.5% (w/v) NaCl at 25 ℃ compared when incubated at 42 ℃, and even longer when incubated at 4 ℃. This aligns with the observations in this study that the point for induction of the VBNC state in *C. jejuni* was prolonged compared to the observations by Lv et al. 2020 [8]. This suggests that the induction of the VBNC state through osmotic stress in *C. jejuni* is temperature dependent. Furthermore, the results indicate that osmotic

stress does induce the VBNC state in *C. jejuni* as no growth was observed after 7 days in 7% (w/v) NaCl, but a signal was still obtained by PMA-qPCR. This could also be due to nutrient deprivation stress, as no nutrients were present in the solution. However, growth was observed for the 0.9% NaCl solution despite the lack of nutrients. In the nutrient deprivation assay, the VBNC state was observed after 14 days of incubation. This indicates that the inability to grow after 7 days in 7% (w/v) NaCl, could be due to the high concentration of NaCl and not the lack of nutrients, or possibly a combination of both factors.

It has been suggested that *C. jejuni* can survive in natural water, by entering the VBNC state [60]. The chickens at the broiler farm drink tap water in which *C. jejuni* may survive. It was investigated if *C. jejuni* could persist in tap water by entering the VBNC state. The composition of the tap water used in this study is not fully understood. It was tapped from the laboratory at DTU Lyngby and is, therefore, according to Lyngby-Taarbæk kommune ground water, classified as hard water. Meaning that it contains a high concentration of calcium and magnesium [61]. A noticeable unexpected increase of viable cells was detected after 48 hours from the tap water solutions. The increase was unexpected as nutrients and conditions were not provided to support growth. As the assay was performed with biological duplicates and both samples showed an increase, the assay should be repeated to monitor if the same trend is observed. Furthermore, tap water varies across regions in Denmark. Conducting the assay with tap water from a broiler farm would provide valuable insights, as it would simulate the original environment more accurately. Additionally, VBNC cells were determined after 7 days, similar to the findings with 7% (w/v) NaCl solution. Indicating that *C. jejuni* can survive in the tap water by entering the VBNC state.

It is important to mention that the assays were conducted with pure cultures, which may not fully represent the findings when collecting samples from a broiler farm. Therefore, the *C. jejuni* strains found in the field may not respond to the environmental stressors as observed in this study. However, for the environmental stressors assessed in this study, it was observed that desiccation, osmotic stress, and nutrient deprivation indicate to induce the VBNC state in *C. jejuni*. Assuming the VBNC state is reached when PMA-treated cells showed a signal in the qPCR analysis and no growth was observed when cultured on BA ON at microaerobic conditions. Plate counts were not conducted when collecting samples and treating them with PMA. The accurate proportion of VBNC cells through the assays was therefore not determined which otherwise would have given valuable insights into the VBNC state over time.
VBNC cells were detected through this study, measured by PMA-qPCR in combination with plating where no growth of colonies was observed. The VBNC *C. jejuni* were assumed to be induced by the assayed environmental stressors. The induction of VBNC *C. jejuni* by the stressors indicate that *C. jejuni* can persist in the broiler farm by entering the VBNC state. This suggests when using culturing-dependent methods to detect *C. jejuni* in the food industry, the bacterium will avoid detection resulting in under-estimation. Moreover,

the VBNC *C. jejuni* may resuscitate and regain pathogenicity upon exposure to favorable conditions. In a previous study, Baffone et al. 2006 [62] observed that VBNC *C. jejuni* regained pathogenicity after in vivo passage in a mouse model. This observation suggests that if VBNC *C. jejuni* enter a human gut, the bacteria could possibly resuscitate and proliferate causing an intestinal infection [62].

7 Conclusion

This study assessed PMA-qPCR's ability to differentiate viable and heat-inactivated cells. It was found that the use of PMA made the differentiation possible, though PMA concentration optimization may be needed. Likewise, a standard curve for the PMA-qPCR protocol was established, enabling the quantification of viable cells and VBNC cells in an unknown sample in combination with culturing. The LIVE/DEAD *Bac*Light protocol, failed to differentiate viable and heat-inactivated cells. The SYTO 9 and PI ratio needs more experimental trials, together with more insight into the method and microscope.

VBNC *C. jejuni* cells were observed after desiccation with and without chicken juice, where the addition of chicken juice indicated higher viability of *C. jejuni*. The results from incubation of *C. jejuni* in osmotic stress environments (7% (w/v) NaCl and tap water) indicated to induce the VBNC state in *C. jejuni* within 7 days. *C. jejuni* may have entered the VBNC state earlier, but this was not observed due to sampling limitations. VBNC *C. jejuni* cells were observed after 14 days of incubation in a nutrient-deprived environment (0.9% NaCl), but also in 0.9% NaCl with 5% chicken juice. Enhanced viability was observed with the addition of chicken juice, suggesting chicken juice contains protective compounds. These findings indicate that *C. jejuni* can persist in a chicken broiler farm for several days by entering the VBNC state, but further testing is needed to confirm the assumption. PMA-qPCR successfully detected VBNC *C. jejuni* cells, presenting a promising method for detection in the food industry to avoid underestimation through the traditional culturing-dependent methods.

8 Future perspectives

A PMA-qPCR protocol was established for the detection of VBNC *C. jejuni*. Further optimization of the PMA concentration may be needed to enhance the efficiency further. VBNC *C. jejuni* were detected after exposure to environmental stressors. Prolonging the duration of the assays would be interesting and may enable the determination of *C. jejuni* persistence in the VBNC state. To deepen the understanding of the VBNC state in *C. jejuni*, future studies could investigate the ability to resuscitate in resuscitation models. This could give a further perspective if VBNC *C. jejuni* can regain pathogenicity after entering the VBNC state. This may also asses the viability of the cells, as cell viability can be defined in various ways. This study evaluated the viability of cells based on membrane integrity. Whereas, other studies have evaluated viability based on other factors, such as the ability to metabolize compounds. A further understanding of the viable/live aspect of cells could give valuable insights in relation to detecting viable bacteria. Moreover, conducting plate counts when collecting samples for PMA-qPCR would be valuable. This approach would enable quantification of the VBNC cell count, providing a more precise assessment of the proportion of cells that enter the VBNC state upon exposure to tested conditions over time. The LIVE/DEAD *Bac*Light assay, while promising, did not provide valuable results related to the assessment of differentiation of viable and heat-inactivated cells. Further testing and experimenting with the dye ratios is needed, to obtain a clear discrimination of *C. jejuni* cells and enable insight into the suitability of the LIVE/DEAD *Bac*Light method compared to the PMA-qPCR method.

While this study concentrated on pure cultures of a single strain of *C. jejuni*, previous research has shown strain-dependent variations in the VBNC state of *C. jejuni*. Therefore, examining different strains from the field (broiler farm) under environmental stressors would provide valuable insights into the stress response.

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10 Appendix

10.1 Standard curves

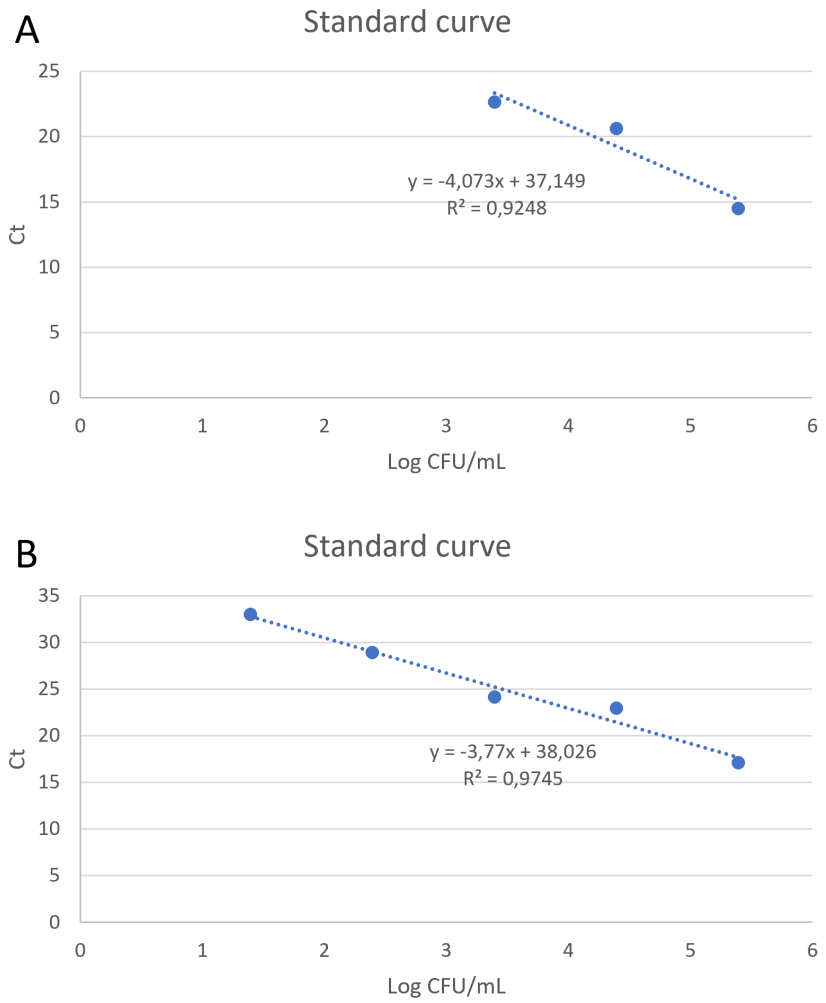


Figure 21: *Standard curves made from Ct values from the viable PMA-treated samples. A: Efficiency=76%. B: Efficiency=84.2%*

10.2 Amplification IAC

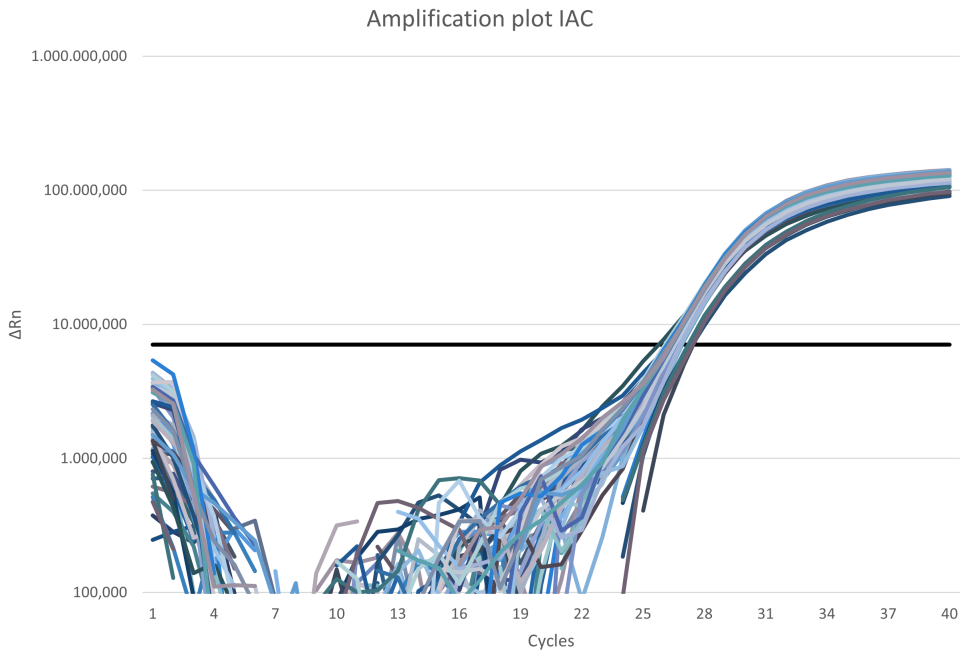


Figure 22: *IAC amplification plot (HEX signal), black line shows threshold*

10.3 Humidity chart from chicken farm

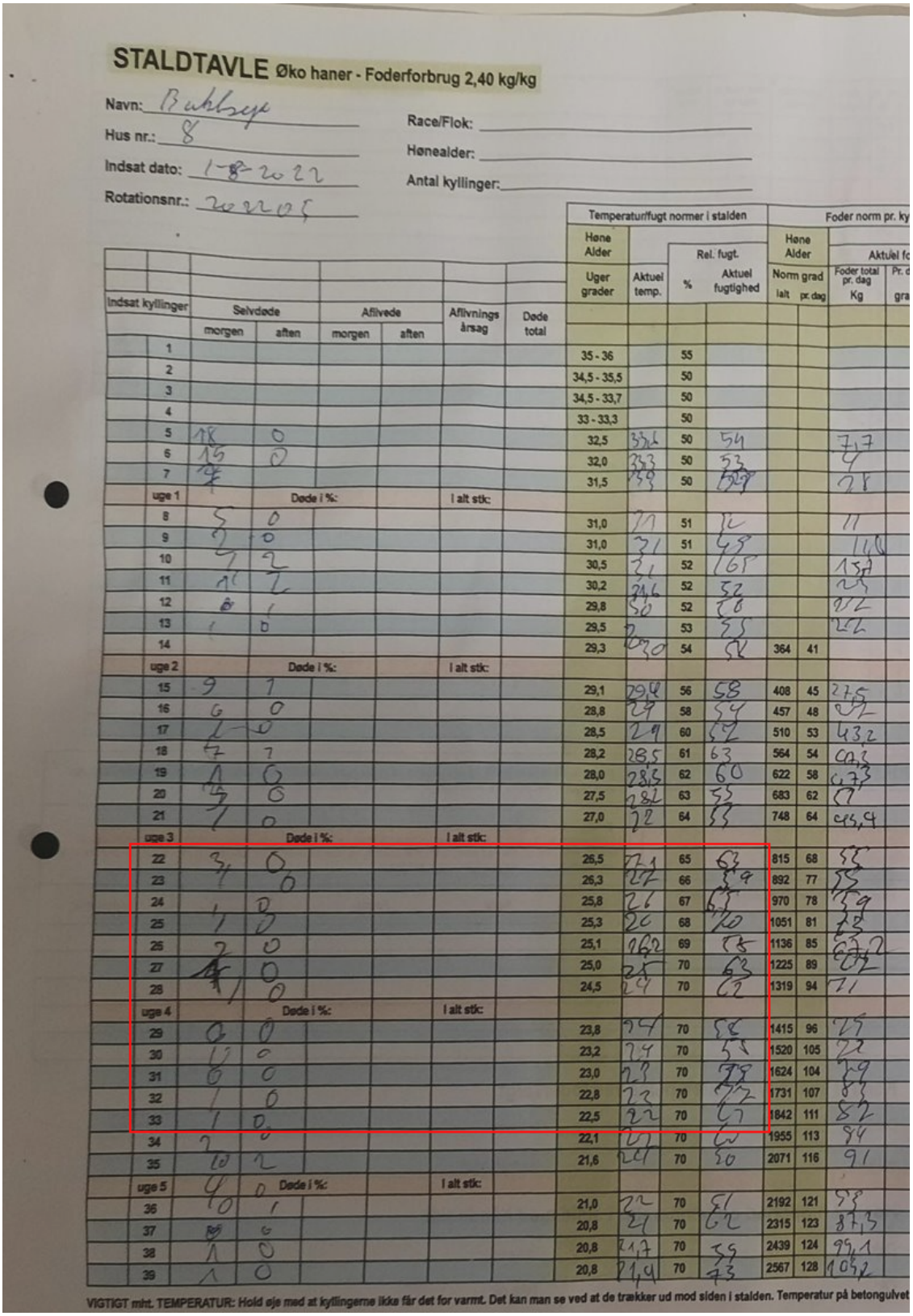


Figure 23: *chart from the chicken farm in Kolding showing the % relative humidity in stable and the actual measurements of the humidity within 5 weeks.*

10.4 Desiccation assay BA plates

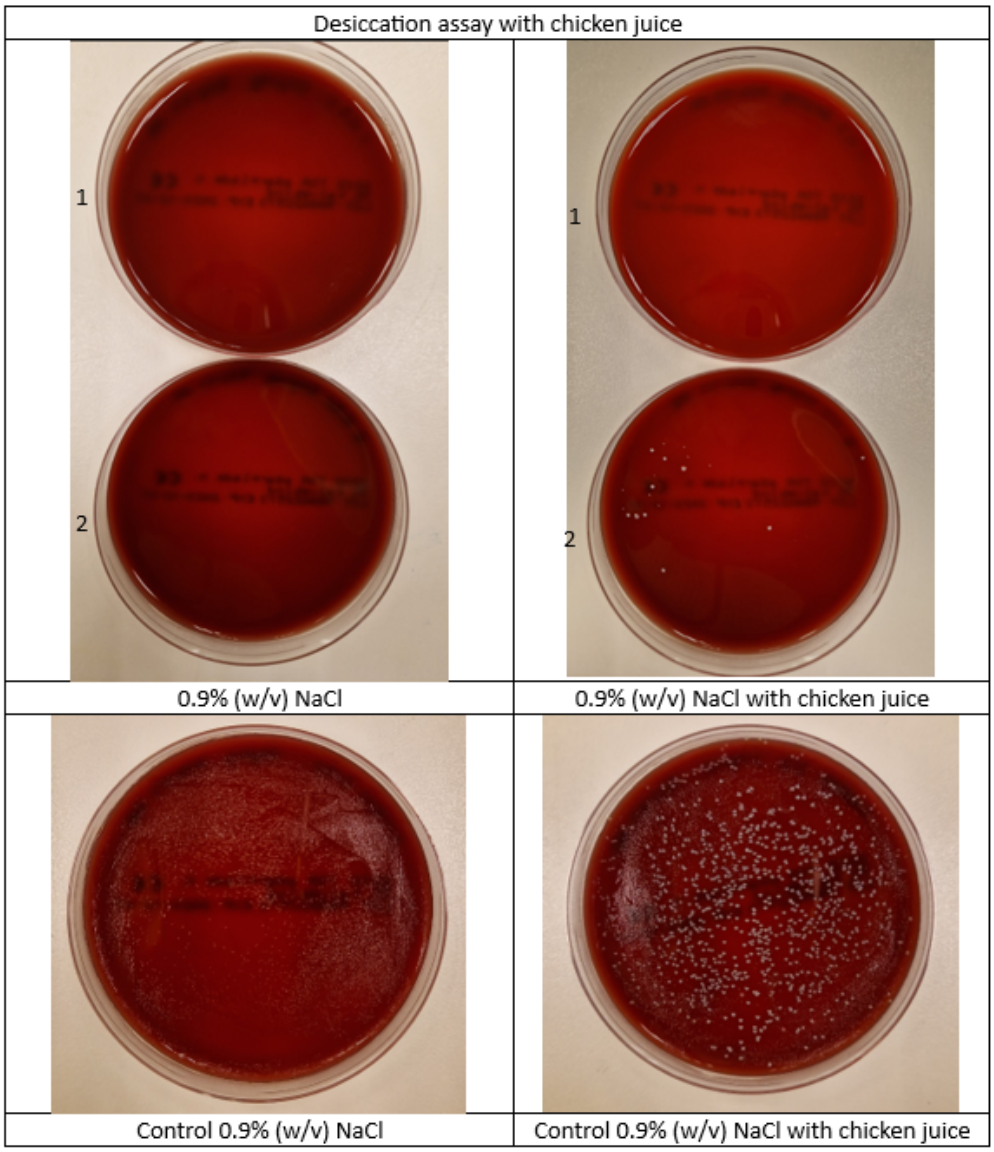
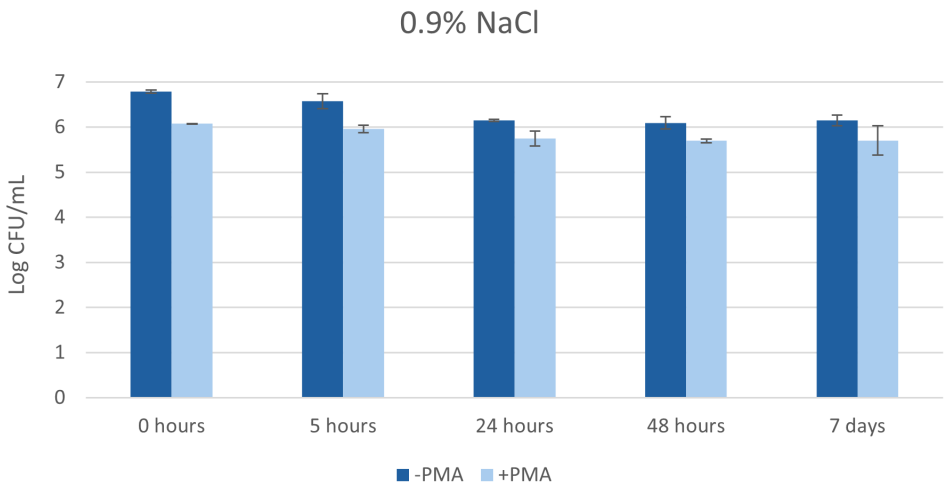
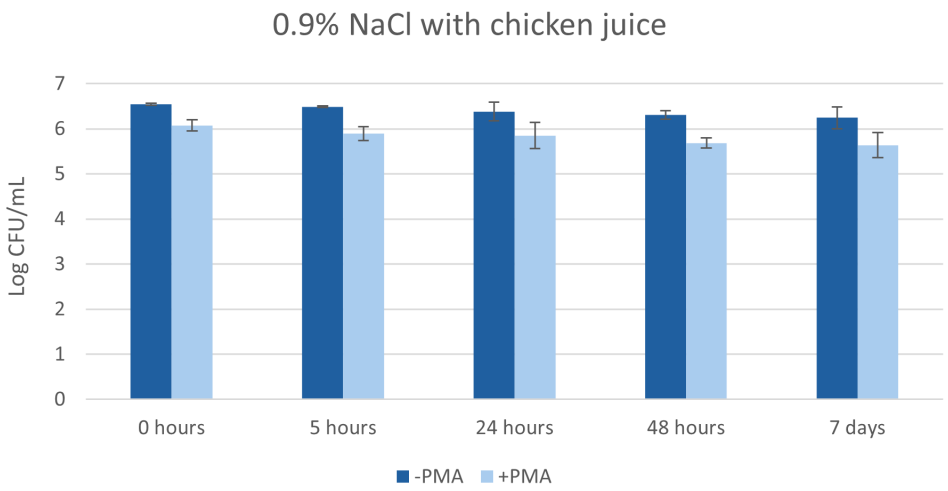


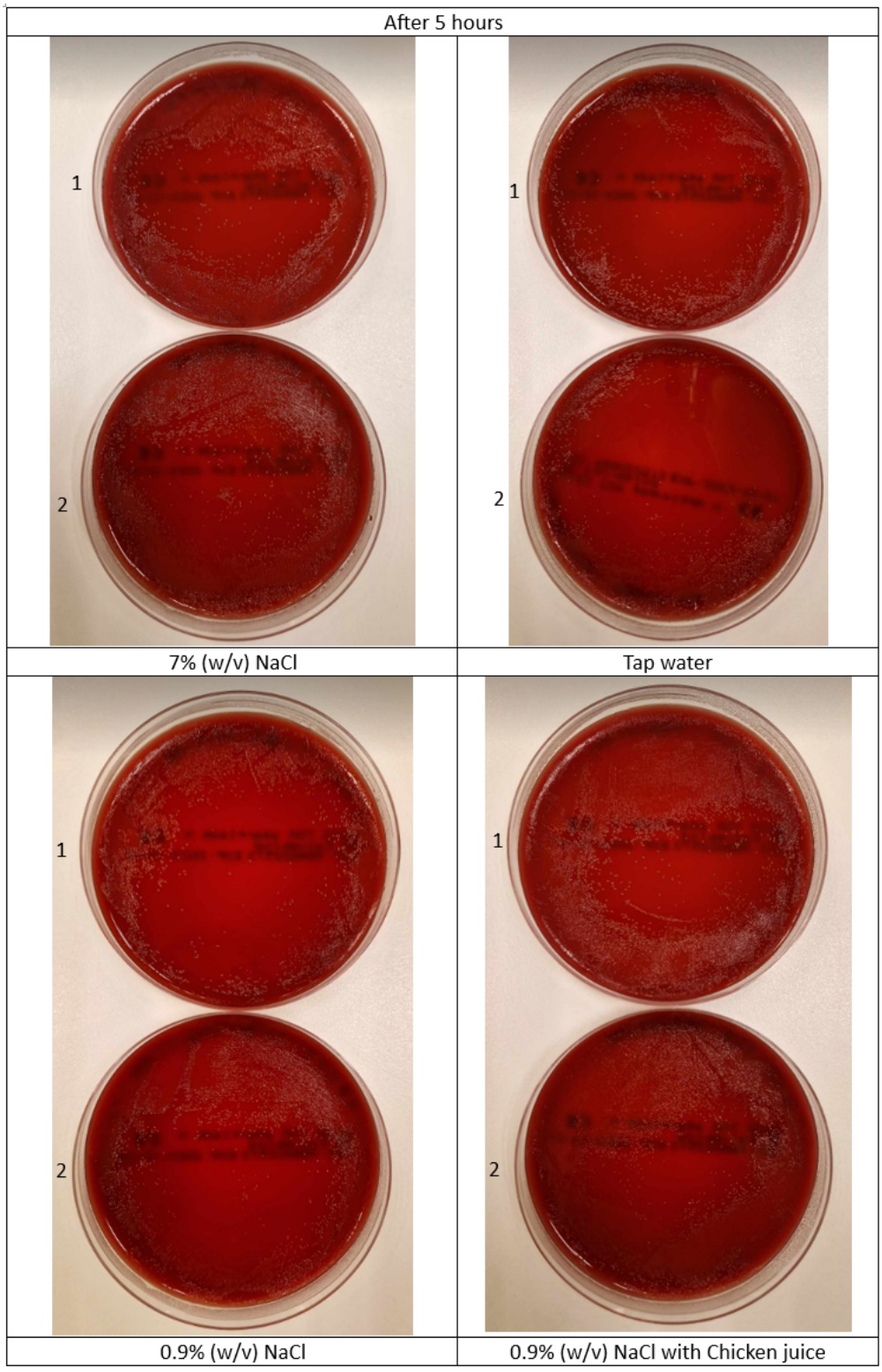
Figure 24: *BA plates from desiccation assay with chicken juice*

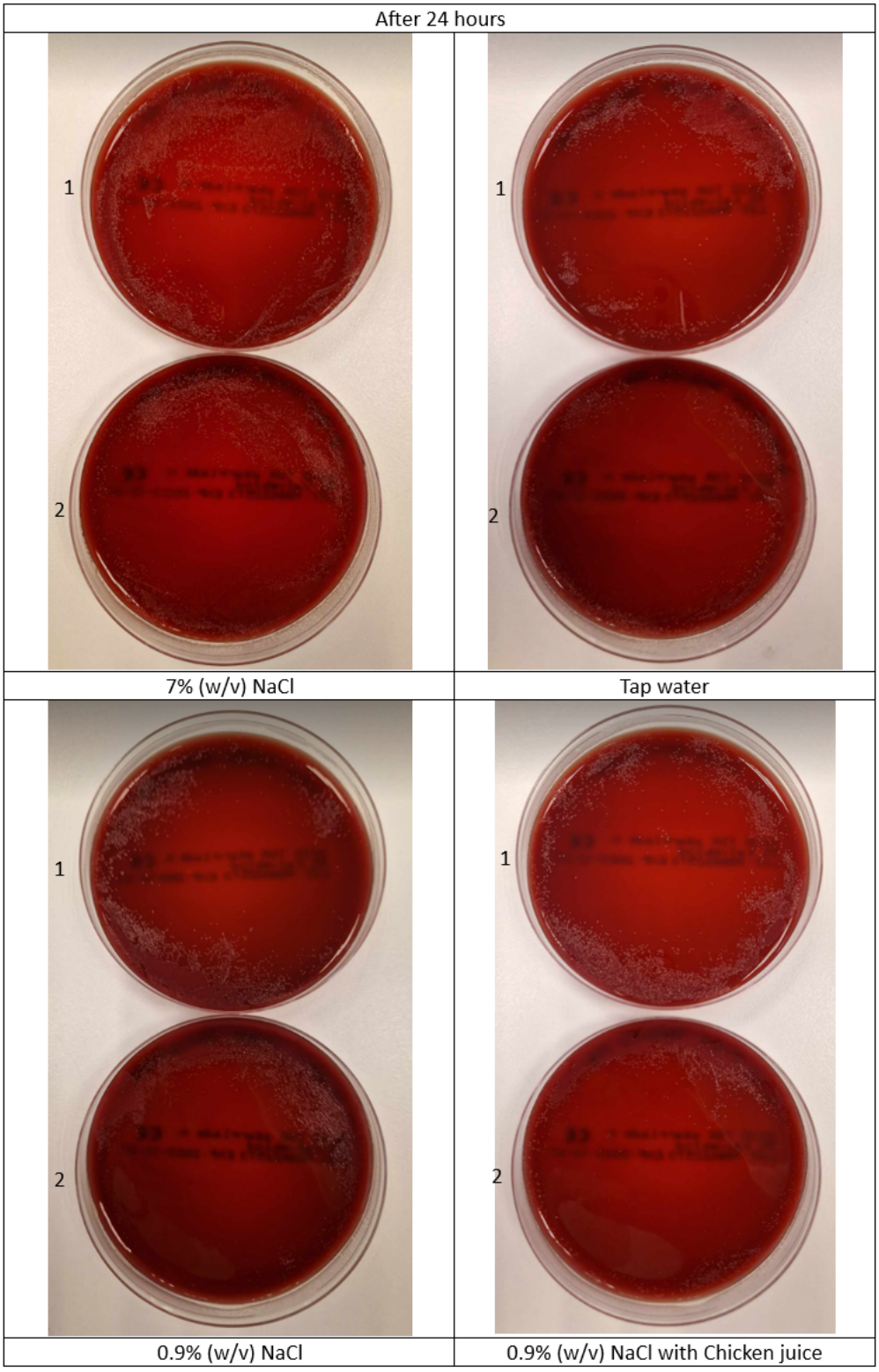
10.5 Osmotic stress bar plots

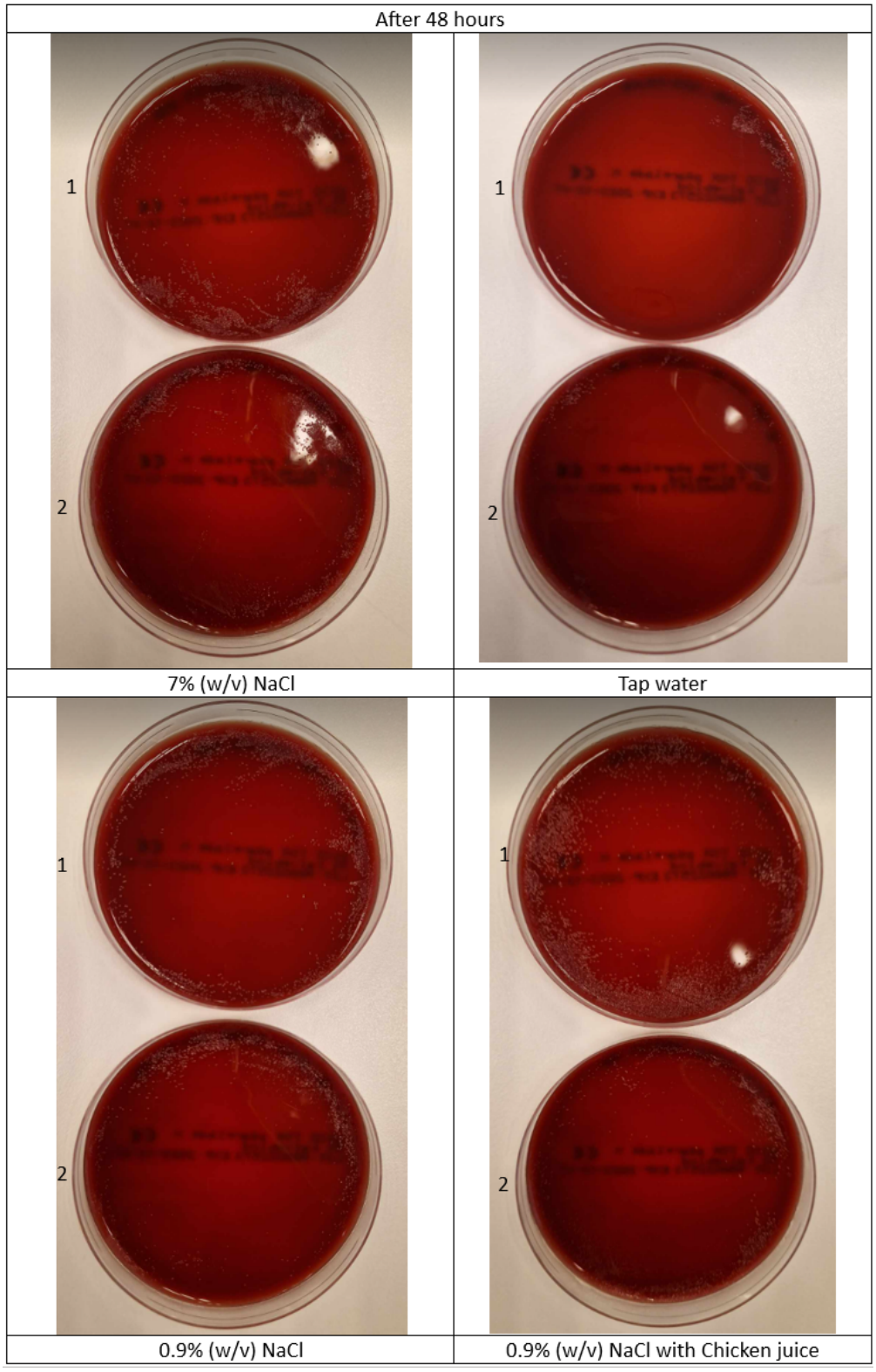


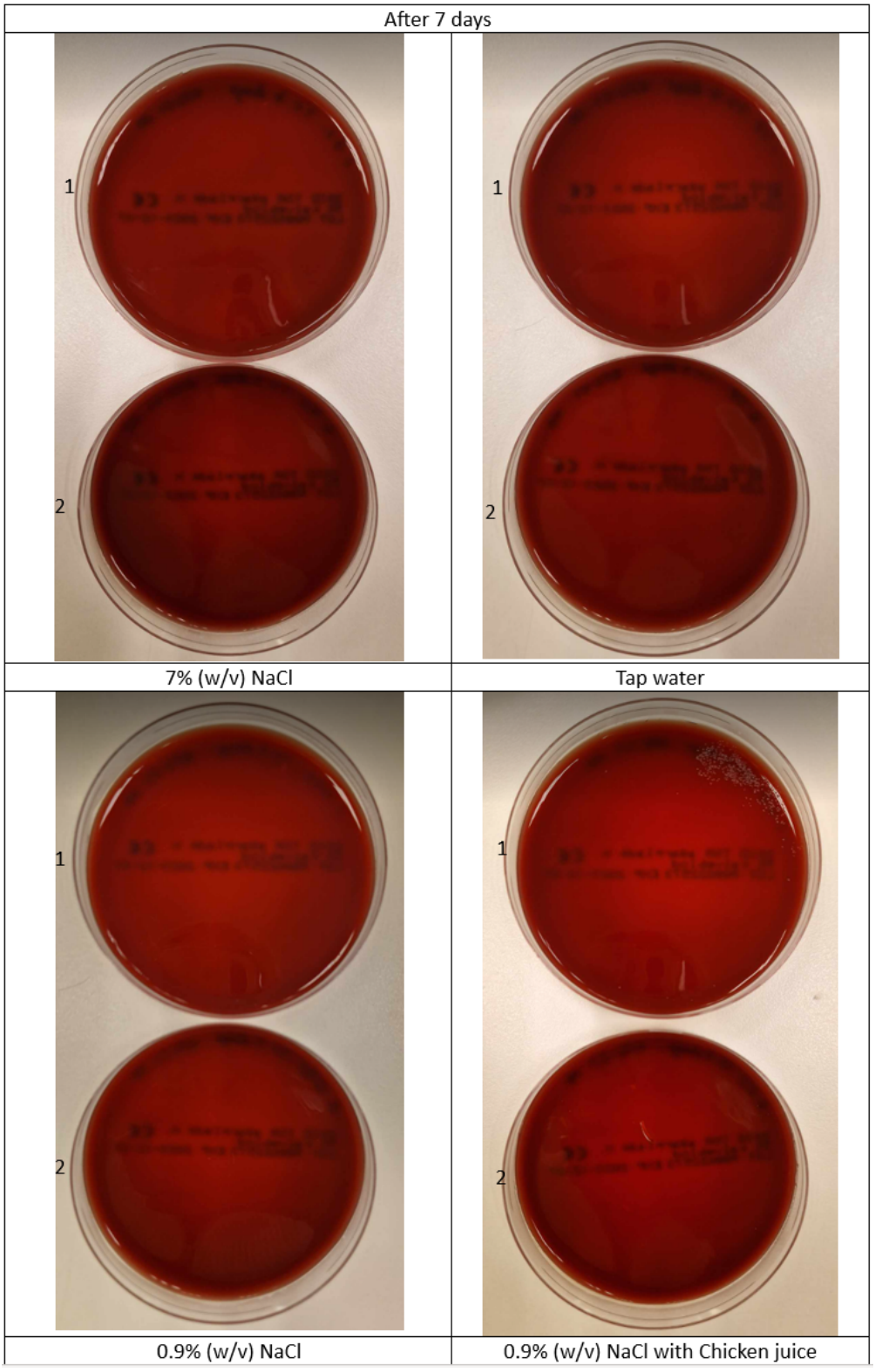


10.6 Osmotic stress BA plates




After 24 hours
1
2
7% (w/v) NaCl
1
2
Tap water
1
2
0.9% (w/v) NaCl
1
2
0.9% (w/v) NaCl with Chicken juice


After 48 hours
1
2
7% (w/v) NaCl
1
2
Tap water
1
2
0.9% (w/v) NaCl
1
2
0.9% (w/v) NaCl with Chicken juice


After 7 days
1
2
7% (w/v) NaCl
1
2
Tap water
1
2
0.9% (w/v) NaCl
1
2
0.9% (w/v) NaCl with Chicken juice