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Characterization of the biofilm forming ability of *Listeria monocytogenes* and evaluation of the TTC-assay as a method to assess the effect of disinfectants on biofilm bacteria

Karakterisering av biofilmdannende egenskaper hos *Listeria monocytogenes* og evaluering av TTC-metoden som et verktøy for å måle effekten av desinfeksjonsmidler på bakterier i biofilm

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Food Science – Food safety, -quality and hygiene

Preface

This master thesis was carried out at the Section for Food Safety, Antimicrobial Resistance and Zoonoses at the Norwegian Veterinary Institute (NVI). The study was a part of the project InhibioList at the NVI. The thesis is a part of the teaching program Food Science, at the Faculty of Chemistry, Biotechnology and Food Science at the Norwegian University of Life Sciences (NMBU).

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Anna Eline Engum Bruvoll

Abstract

Food-borne diseases are a problem throughout the world, and knowledge about when and where the food is contaminated in the processing line is important to reduce the risk of illnesses. By the use of molecular typing methods as pulsed-field gel electrophoresis (PFGE), it is possible to find relations between isolates from humans and isolates from food. *Listeria monocytogenes* (*L. monocytogenes*) is a pathogenic bacterium that is frequently isolated from fish processing facilities. It tolerates a wide range of growth conditions, which makes it a potential problem in refrigerated ready-to-eat (RTE) foods. Bacteria can persist in food environments in the shape of biofilm, and be a source of contamination.

A total of 84 isolates of *L. monocytogenes* sampled from seven different fish processing facilities, were characterized with regard to biofilm forming ability and PFGE type. The biofilm forming ability was investigated by growing biofilm in microtiter plates, using two different growth media (LB and LB without NaCl), and incubating at 37 °C, 20 °C and 12 °C. Biofilm bacteria was quantified by using crystal violet-staining (CV assay). To investigate the susceptibility of biofilms to disinfectants, the TTC assay was evaluated as a method to investigate eradication of biofilm bacteria after treatment with disinfectants. Minimal inhibitory concentrations of six disinfectants were determined using the TTC assay and compared to the colony forming unit (CFU) count and the CV assay.

The results showed that biofilm formation occurred only in LB-medium. The biofilm forming ability varied between isolates, even with the same PFGE type. There was a significant difference between the biofilm forming ability of serogroup IIa and IVb at all temperatures. The TTC assay indicated eradication of biofilm bacteria by all disinfectants, but these results were not supported by the CFU count and the CV assay. The CV assay indicated that biofilm was not removed by the disinfectants. The CFU counts showed that all disinfectants reduced the number of bacteria in the biofilms, but only two disinfectants, Aco hygiene des QA and Novadan disinfect Maxi, killed all cells after treatment with the concentration and time recommended by the supplier.

The results from this study indicate that biofilm forming ability varies between isolates with similar and different PFGE-types. The combined results from the validation of the TTC assay indicates that it overestimates the efficiency of disinfectants on biofilm bacteria.

Norwegian abstract

Næringsmiddelbåren sykdom er et vidt utbredt problem, og kunnskap om hvordan og hvor næringsmidler kontamineres i produksjonskjeden er viktig med hensyn til å redusere risiko for sykdom. Ved bruk av molekylære typings-metoder slik som pulsfelt-gelelektroforese (PFGE) kan det knyttes sammenhenger mellom bakterier isolert fra mennesker og næringsmidler. *Listeria monocytogenes* (*L. monocytogenes*) er en patogen bakterie som ofte blir isolert fra fiskeforedlingsanlegg. Den tolererer et vidt spekter av vekstbetingelser, noe som gjør den til et potensielt problem i kjølelagret, spiseklar mat. Ved å etablere seg i produksjonslokaler i form av biofilm, kan den være en potensiell smittekilde.

I denne studien ble 84 isolat av *L. monocytogenes* samlet fra syv ulike fiskeforedlingsanlegg, karakterisert med hensyn til biofilmdannende egenskaper og PFGE-type. De biofilmdannende egenskapene ble undersøkt ved å dyrke biofilm i mikrotiter-plater ved bruk av to ulike vekstmedium (LB og LB uten NaCl), og inkubering ved 37 °C, 20 °C og 12 °C. Bakterier i biofilm ble kvantifisert ved krystallfiolett-farging. For å undersøke toleransen biofilm har til desinfeksjonsmidler ble TTC-metoden benyttet. Det ble evaluert hvor god metoden var til å måle reduksjon av bakterier i biofilm, etter behandling med desinfeksjonsmidler. Minste hemmende konsentrasjon ble bestemt for seks ulike desinfeksjonsmidler ved bruk av TTC-metoden. Resultatene ble sammenlignet med resultater fra telling av kolonidannende enheter (CFU), og kvantifisering av bakterier i biofilm.

Biofilmdannelse fant bare sted i vekstmediumet LB. Biofilmdannelse varierte mellom isolatene, både for like og ulike PFGE-typer. Det var en signifikant forskjell mellom biofilmdannende egenskaper for serogruppe IIa og IVb ved alle temperaturer. TTC-metoden indikerte at alle desinfeksjonsmidlene drepte bakteriene, men disse resultatene samsvarte ikke med resultatene fra telling av CFU og kvantifisering av biofilm. Fra kvantifiseringen av biofilm ble det indikert at desinfeksjonsmidlene ikke fjerner biofilmen. Resultater fra CFU-telling viste at alle midler reduserte bakterietallet, men bare to midler, Aco hygiene des QA og Novadan disinfect Maxi drepte alle celler etter behandling med desinfeksjonsmidler brukt etter anbefalt konsentrasjon og tid fra leverandør.

Resultatene fra studien indikerer at biofilmdannende egenskaper er varierende mellom stammer av like og ulike PFGE-typer. Fra valideringen av TTC-metoden, viser de samlede resultatene at metoden overestimerer effekten desinfeksjonsmidler har på bakterier i biofilm.

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1. Introduction

Food-borne diseases are a problem throughout the world. These diseases could be presented as mild self-limiting gastroenteritis, or serious and fatal illnesses (Adams & Moss 2008). Elderly, pregnant or people with impaired immune system are most susceptible for disease. Contamination of food products can occur in any part of the processing line, from raw material to the consumers table. For public health and economical aspects, it is important to increase knowledge about where and when food is contaminated, and how preventive measures should be carried out.

1.1 *Listeria monocytogenes*

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive, facultative anaerobe and non-spore forming bacteria (Jadhav et al. 2012). The genus *Listeria* consists of 15 species, but only *L. monocytogenes* is considered an important human pathogen (Granum 2015). It is found widespread in the nature and can be isolated from soil, sewage and water. The bacteria are tolerant to salt up to a 10 % NaCl, can grow in a pH-range from 6-9, and in a temperature range of 0-45 °C (Adams & Moss 2008). This tolerance to a wide range of growth conditions along with its ability to grow in vacuum-packed and modified atmosphere-packed products makes the bacteria a potential problem in refrigerated ready-to-eat (RTE) food products that does not require treatment by consumer. The bacteria has been isolated from a wide range of food products, from dairy and meat products to vegetables (Ferreira et al. 2014).

L. monocytogenes is the causative agent of the disease listeriosis, which could either be an invasive disease, or febrile gastroenteritis. Although the infectious dose is unknown, it is believed to be high due to findings of bacterial numbers in excess of 10^3 cfu/g^{-1} in products associated with outbreaks (Granum 2015). Everyone can be infected by *L. monocytogenes*, but listeriosis is mainly reported in people with impaired immune system, elderly or pregnant women (Adams & Moss 2008). The disease varies in its manifestation. One type causes diarrhea, abdominal pain, fever and vomiting. The invasive type can manifest as a mild, flu-like illness, or as a more serious form as meningitis, meningoencephalitis or septicemia. For pregnant women obtaining the bacteria, the mother can go unaffected or have an influenza-like illness, but if a transplacental foetal infection occurs, there is a risk of abortion and stillbirth. In Norway, several cases with listeriosis is reported every year (Folkehelseinstituttet

2017). In 2013, there was an outbreak involving three patients. By the use of the subtyping method multiple-locus variable number tandem repeat analysis (VNTR) analysis (MLVA), the source of contamination was found to be vacuum packed fermented fish known as "Rakfisk".

1.1.1 Listeria monocytogenes in the food industry

L. monocytogenes have been detected in many different RTE products, and has frequently been isolated from fish processing facilities, both from raw materials and equipment used during food processing, and from the environment (Dauphin et al. 2001; Rørvik et al. 1995). Specific strains could persist in the food processing environments over time, and thus be potential contaminators for food during processing or after processing. Persistence of bacteria could be due to harborage sites and niches where *L. monocytogenes* is protected from cleaning and disinfection routines. A project at the food research institute Nofima in Norway screened a salmon slaughtering plant for prevalence of *L. monocytogenes* (Forskrift om næringsmiddelhygiene). They found that the salmon was contaminated early in the slaughtering process, and suggested this might be a possible contamination route when delivered as a raw material to other food processing plants.

If there is a risk of growth by *L. monocytogenes* in food product the producer has to screen for the prevalence of the bacteria in the processing environments and equipment. Different criteria for prevalence of *L. monocytogenes* are defined for ready-to-eat products depending on whether they are produced for patients with special nutritional needs and infants (absence of bacteria in 25 g product), or for people outside these groups (<100 cfu/g product) (Forskrift om næringsmiddelhygiene). If the limits are exceeded, the products must be recalled, and preventive measures has to be taken to make sure contamination is not repeated.

1.2 Biofilm

Biofilms have been defined as complex communities of microorganisms that are attached to a surface and enclosed in self-produced extracellular polymeric substances (EPS) (Shi & Zhu 2009). EPS is mostly composed of proteins, polysaccharides and DNA (Colagiorgi et al. 2016). Biofilm formation is a mechanism bacteria has for survival in unfavorable

environment. They are found widespread in nature as single or multi-species structures (Srey et al. 2013). Biofilms are frequently found in food processing environments where they allow bacteria to persist. If these biofilms contain pathogenic bacteria it could lead to contamination of food products and food-borne outbreaks of disease.

1.2.1 Development of biofilm

Development of biofilm can be considered as a process in several steps (figure 1). The first step involves adhesion of bacterial cells to a surface, which is reversible (Srey et al. 2013). This could be done in an active or passive manner depending on the bacterial cells´ physicochemical properties (e.g. flagella, fimbria or pili)(Van Houdt & Michiels 2010). Flagella is a helical structure ranging out from the cytoplasm and cell wall, which is important for motility. It has shown to be important for enhanced surface attachment for different bacteria; *Escherichia coli* (*E. coli*), *L. monocytogenes* and *Yersinia enterocolitica* (*Y. enterocolitica*). Pili and fimbriae are also structures found in different bacteria that enhance surface attachment and biofilm forming (Shi & Zhu 2009). Attachment of bacteria are also affected the properties of the surface, e.g. type of material, texture, hydrophobicity and temperature affects the adherence of bacteria (Di Bonaventura et al. 2008; Kadam et al. 2013).

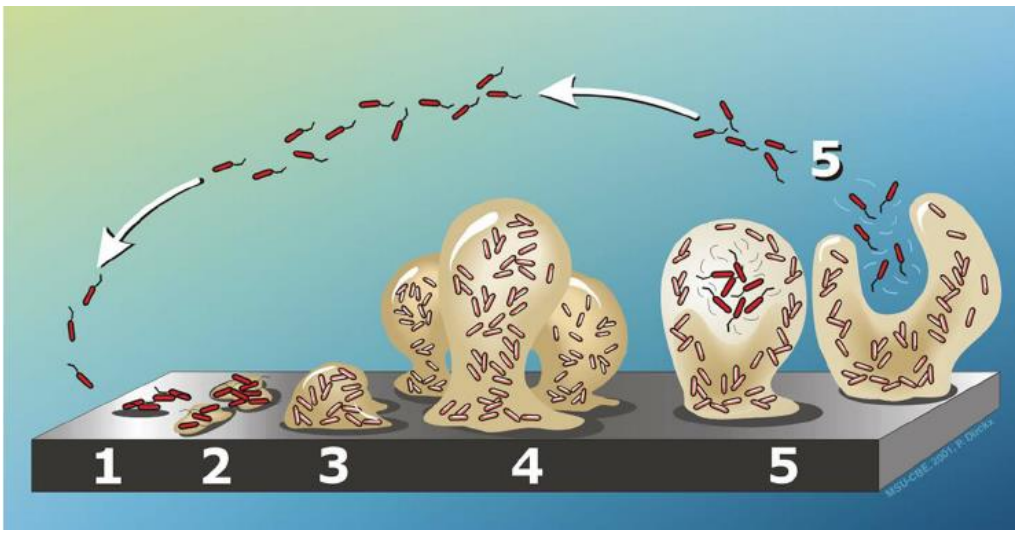


Figure 1. Process of biofilm formation. 1: Attachment of cells, 2: Cells proliferate and extrapolymeric substances (EPS) are formed, 3: A three-dimensional structure forms, 4: Maturation and 5: Detachment of cells with recolonization (Van Houdt & Michiels 2005).

Following the initial attachment to the surface the cells proliferate and produces EPS (Srey et al. 2013). The presence of EPS makes the bonding strengthen, and the attachment irreversible. At this point shear force or chemical agents is needed to remove the biofilm (Srey et al. 2013). The third step involves development of biofilm architecture. The three-dimensional structure that is formed makes a chemical heterogeneity in the biofilm (Bridier et al. 2011). On the outer layer of the biofilm, cells have access to nutrients and oxygen, while the internal cells are in a nutrient-poor environment with higher concentrations of waste products. Maturation follows as the fourth step, in which an organized structure is developed (Srey et al. 2013). Intercellular communication known as quorum sensing, permits the bacterial cells to monitor and produce chemical signals (autoinducers) which make them respond to population densities by expressing genes (Watson et al. 2014a). The mature biofilm can contain water channels that can facilitate transport of nutrients and waste products. In the mature biofilm, cells can detach and return to their planktonic state, finding new places for attachment and biofilm development.

1.2.2 *Listeria monocytogenes* and its biofilm forming ability

L. monocytogenes is capable of producing biofilm on different materials used in the food industry (Di Bonaventura et al. 2008). Studies of the biofilm forming ability have shown that the bacteria adhere better to hydrophilic material such as stainless steel and glass when compared to hydrophobic surfaces such as polystyrene (Bonsaglia et al. 2014; Di Bonaventura et al. 2008). Adhered cells of *L. monocytogenes* have been shown to change from rod to coccoid shaped, and grow more slowly than planktonic cells (Colagiorgi et al. 2016).

Development of the biofilm structure is influenced by several factors such as strain, surface, temperature and medium (Di Bonaventura et al. 2008; Harvey et al. 2007). The components in the extracellular matrix of *L. monocytogenes* biofilms have been widely studied to understand more of the biofilm forming ability of the bacteria. As stated earlier, the EPS mainly consists of polysaccharides, proteins and DNA. Polysaccharides are major components of the biofilm matrix, and the polysaccharide teichoic acid (TA) have been related to the biofilm forming ability because it is found both in the cell wall and in the EPS (Colagiorgi et al. 2016). Different proteins in the EPS are suggested to have a role in the cells attachment to surfaces, and enhancing biofilm formation. Biofilm-associated protein (BapL) and flagellin (FlaA) have been hypothesized as having a role in the initial attachment to surfaces (Lemon et al. 2007).

Guilbaud et al suggested that flagella also could be important for biofilm structure, due to observation of a unstructured biofilm made by strains lacking flagella (2015). The extracellular DNA (eDNA) found in the matrix is important for holding the structure with polysaccharides and proteins. It also serves as an energy and nutrition source, and may have a role in the initiating attachment of cells. Studies of the architecture of listerial biofilm have found that *L. monocytogenes* forms a honeycomb-like structure, though flat unstructured mono- and multilayers have also been described for *L. monocytogenes* biofilm (dos Reis-Teixeira et al. 2017; Guilbaud et al. 2015).

1.2.3 Biofilm control strategies in the food industry

The prevalence of biofilm increases the risk of food contamination, hence prevention of biofilm formation would be optimal. Biofilm bacteria has been shown to be difficult to eradicate due to resistance of disinfectants. The age of biofilm, stress responses or the presence of dormant cells is some of the factors that have been related to the increased resistance (Van Houdt & Michiels 2010). Impaired uptake or active transporters for pumping out disinfectants are common resistance mechanisms (Aase et al. 2000). For example, it has been proposed that bacteria could express efflux pumps that lead to increased resistance to antimicrobial compounds. The activity of efflux pumps in *L. monocytogenes* strains can lead to increased tolerance to QAC (Moretro et al. 2017). In food processing environments, bacteria could be exposed to sub-lethal concentrations of disinfectants in case of poor rinsing after being applied in the food processing environment. This could possible lead to adaptive responses leading to increased tolerance for the disinfectants (Lundén et al. 2003; Moretro et al. 2017).

Removing the biofilm in the food industry is difficult, which makes it important to develop good routines to control biofilm. In addition to killing the biofilm bacteria, removal of the polymer matrix of surfaces is important to prevent reattachment of cells (Bredholt et al. 1999).

1.2.3.1 Cleaning

Regular and thorough cleaning is important to remove food components that may lead to bacterial attachment and proliferation (Srey et al. 2013). To obtain a good cleaning result, it is important that the right cleaning agent is chosen. For the disinfectants to have maximum effect it is important that the surfaces are clean (Van Houdt & Michiels 2010). The cleaning

result is dependent on the process parameters time, temperature and mechanical and chemical forces applied. These parameters are affected by each other, e.g. increasing the concentration of a cleaning agent would decrease the needed application time.

1.2.3.2 Sanitary design

L. monocytogenes could persist in growth niches in the food-processing environment (Ferreira et al. 2014). This involves sites where cleaning and disinfection routines are inaccessible, e.g. drains, equipment and cracks in surfaces. *L. monocytogenes* have several times been detected in food environments after cleaning (Gudbjörnsdóttir et al. 2004; Heir & Langsrud 2014).

Sanitary design involves planning the processing environments to make cleaning effective and efficient, so that survival and growth of bacteria is inhibited (Taskforce 2013). Food contact surfaces should be free of sharp corners and crevices. Disassembly of equipment should be easy, to make cleaning and inspection routines easy. Surfaces and equipment should be self-draining, since water and organic matter retention could lead to areas where bacteria could thrive (Carpentier & Cerf 2011).

1.2.3.3 Chemical control

The most common control strategy involves chemical control (Srey et al. 2013). It is the use of antimicrobial products to kill or reduce micro-organisms and prevent growth on surfaces before production restarts (Simões et al. 2010). Quaternary ammonium compounds (QAC), chlorine compounds, peroxides and iodine products are groups with disinfectants commonly used in the food industry (Granum 2011). The efficacy of the disinfectants is influenced by a number of factors such as time, concentration, temperature and water hardness. For example, the effect of peroxide based disinfectants decrease with decreasing temperature. Disinfectants are also affected by the presence of organic material; proteins, fat and carbohydrates (Srey et al. 2013). For example, hypochlorite is easily inactivated by proteins (Granum 2011).

1.3 Methods to detect biofilm forming ability of bacteria

A variety of methods have been developed to investigate the biofilm forming ability of bacteria. Some of these involves growing biofilms by using microtiter plates, stainless steel coupons and petri dishes (Folsom et al. 2006; Harvey et al. 2007; Peeters et al. 2008a). Growing biofilm in microtiter plates allows for a large amount of data to be analyzed in one

assay (Tsukatani et al. 2008). Methods to detect biofilm forming ability can be classified in assays for quantification of living and dead cells and assays for detecting viable cells (Peeters et al. 2008a).

1.3.1 Methods to detect viable cells in biofilm

1.3.1.1 Assays with tetrazolium salts

Tetrazolium salts have been widely used as a tool to indicate metabolically active cells (Berridge et al. 2005). Triphenyl tetrazolium chloride (TTC) is a tetrazolium salt that has been used as a dye in cell-based applications. When TTC is reduced, a tetrazole ring is disrupted and the insoluble formazan product triphenyl formazan (TPF) are produced, giving color that could range from weakly red to a very bright red color. The formazans that are reduced are directly proportional to the metabolically active cells (Moussa et al. 2013).

In this study, the TTC assay was developed as a method to measure the eradication of bacteria in biofilm after treatment with disinfectant. The method was validated by using a colony forming unit count (CFU) and crystal violet staining (CV assay). The CFU count was used to indicate the killing effect of the disinfectants. The CV assay would state whether the reduction in metabolically active cells were due to killing of bacteria, or removal of the biomass.

1.3.1.2 Enumerating biofilm cells

The bacterial cells in a biofilm can be estimated by using plate count. Different approaches to dislodge biofilm cells can be used. Pommenidou et al. used cotton swab to dislodge cells from biofilm in microtiter wells and on stainless steel coupons. Others have used inoculating loops in microtiter wells (Romanova et al. 2007). Biofilm bacteria has been shown to be difficult to detach from surfaces (Bredholt et al. 1999). The bacteria in the biofilm forms microcolonies or clusters, which could give an underestimation of cells due to of formation of single colonies when plated. For counting, it is necessary that each colony is the result of a single cell.

1.3.2 Quantification of biomass

1.3.2.1 Crystal Violet assay

For quantification of the biomass in biofilm, CV is a widely used dye. Negatively charged molecules in the biofilm are stained, and excess CV is washed off (Pantanella et al. 2013). A solvent (e.g. ethanol or acetic acid) is used to dissolve the CV. The amount of dye solubilized is proportional to the size of the biofilm. It stains living and dead cells in the biofilm, is easy to apply and allows a rapid interpretation of the results.

1.4 Subtyping *Listeria monocytogenes*

Food-borne outbreaks could range from involving a few to many patients either localized in a small area or being geographically dispersed. This makes detecting the source of outbreak difficult require precise methods to differentiate between serotypes and strains as causative agents. Molecular methods are continuously being improved, and they differ in their discriminatory ability. A subtyping method should ideally be specific, sensitive, fast and reproducible (Jadhav et al. 2012).

1.4.1 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is a subtyping method that has been considered as gold standard because of its high discriminatory power. It has been discussed as having low reproducibility. It has been established by several researchers that PFGE typing is more discriminatory than for example serotyping (Fugett et al. 2007)

PFGE can be used to determine the size of large genomic DNA. The method is based on the use of restriction enzymes, and for *L. monocytogenes* the enzymes *Asc*I *and Apa*I are most commonly used (Jadhav et al. 2012). Genomic DNA is digested in large fragments from 40 and 600 kb. The DNA is embedded in agarose plugs, and the electric pulse field gives band patterns that could be differentiated into different pulsotypes. Combining PFGE with other molecular methods makes it possible to identify more subtypes (Zunabovic et al. 2011). It also gives the opportunity to map the prevalence of certain strains and serotypes, finding a connection between production facilities and persistent strains.

1.4.2 Serotyping

Serotyping is a subtyping method that classifies micro-organisms according to somatic and flagellar antigen presented on the cell surface (Granum 2015). Serotyping methods have been considered as rapid methods suitable for first-level characterization, but because of low discriminatory power it should be paired with other molecular methods such as PFGE (Nyarko & Donnelly 2015). 13 serotypes have been described for *L.monocytogenes*, where three (1/2a, 1/2b and 4b) has been reported as causative agents for 95 % of cases of human listeriosis (Granum 2015). Serotype 1/2c is also commonly isolated from food and animal sources (Adams & Moss 2008). Most common serotypes found in the food processing environments are 1/2 a, 1/2b, 1/2c, 4a and 4b (Jadhav et al. 2012).

1.4.3 Multiplex PCR

Multiplex PCR is a method that can be used for rapid separation of *L.monocytogenes* strains (Kérouanton et al. 2009). In contrast to serotyping, the specific serotypes are not detected. The strains are classified in serogroups (IIa, IIb, IIc, Iva and IVb) based on the presence of specific genes. The serotypes commonly found in food processing environments belong to the following serogroups; IIa: 1/2a, IIb: 1/2b, IIc: 1/2c and IVb: 4a and 4b. Compared to serotyping, serogrouping by multiplex PCR have been considered better due to enhanced reproducibility, and it is less cost-effective and time-consuming (Doumith et al. 2004; Kérouanton et al. 2009).

1.5 Aim of the study

The main aim of this study was to characterize *L. monocytogenes* strains isolated from fish processing facilities in Norway regarding biofilm forming ability, pulsed-field gel electrophoresis (PFGE) and susceptibility to disinfectants. This was divided in two sub aims:

I) Determine the biofilm forming ability of isolates from fish processing facilities in Norway, and find out if there is a relationship between PFGE-type and biofilm forming ability.

II) Determine how well the TTC-assay measures eradication of bacteria in the biofilm after treatment with disinfectants, and if possible, use the method to determine the efficiency of the disinfectants.

2. Materials and methods

2.1 Materials

Growth media with no specified producer was made in the media production at the Norwegian Veterinary Institute. Recipes are presented in Appendix 1.

2.2 *Listeria monocytogenes* isolates

In this study, 84 isolates of *L. monocytogenes* obtained from fish processing facilities in Norway for the Norwegian Veterinary Institute were used (table 1). The environment of the facilities were sampled for routine hygiene controls, and *L. monocytogenes* isolates were collected for the project InhibioList. The Institute received the isolates in transport tubes. At arrival the isolates were cultivated on blood agar, incubated overnight at 37 °C and frozen in Heart Infusion Broth (HIB) with 15 % glycerol at -80 °C. Isolates used in this study were cultivated on blood agar at 37 °C and refrozen in HIB at -20 °C. The isolates were classified in serogroups according to results obtained with multiplex PCR performed in advance of this study.

For experiments, bacteria were inoculated directly from the frozen -80 °C stocks into 200 µl Tryptic Soy Broth (TSB), in sterile flat bottomed polystyrene 96-well cell culture microtiter plates with lid (Thermo Fisher Scientific, Nunclon Delta Surface). The isolates were set up in triplicate and incubated under static conditions at 37 °C overnight. All plates were incubated in boxes with wet paper and the lid halfway on to create a humid environment and minimize evaporation, while allowing circulation of air.

Table 1 part 1. Seven facilities (A-H) participated in this study, with a total of 84 isolates of *Listeria monocytogenes (L.monocytogenes)*. Serogroup results were obtained from multiplex PCR previous to the study.

Isolate	**Serogroup**	**Facility**	**Sampled (month/year)**	**Isolate**	**Serogroup**	**Facility**	**Sampled (month/year)**
1	IIa	A	07/16	***22***	IIa	A	07/16
2	IIa	A	07/16	***23***	IIa	A	07/16
3	IIa	A	07/16	***24***	IIa	A	07/16
4	IIa	A	07/16	***25***	IIa	A	07/16
5	IIa	A	07/16	***26***	IIa	A	08/16
6	IIa	A	07/16	***27***	IIa	A	08/16
7	IIa	A	07/16	***28***	IIa	A	08/16
8	IIa	A	07/16	***29***	IIa	A	08/16
9	IIa	A	07/16	***30***	IIa	A	08/16
10	IIa	A	07/16	***31***	IVb	A	08/16
11	IIa	A	07/16	***32***	IIa	A	08/16
12	IIa	A	07/16	***33***	IIa	A	08/16
13	IIa	A	07/16	***34***	IIa	A	06/16
14	IIa	A	07/16	***35***	IIa	A	06/16
15	IIa	A	07/16	***36***	IIa	A	06/16
16	IIa	A	07/16	***37***	IIa	A	06/16
17	IIa	A	07/16	***38***	IVb	A	06/16
18	IIa	A	07/16	***39***	IIa	A	06/16
19	IIa	A	07/16	***40***	IIa	D	05/14
20	IIa	A	07/16	***41***	IVb	D	05/14
21	IIa	A	07/16	***42***	IIa	D	10/14

Table 1 part 2. Seven facilities (A-H) participated in this study, with a total of 84 isolates of *Listeria monocytogenes (L.monocytogenes)*. Serogroup results were obtained from multiplex PCR previous to the study.

Isolate	**Serogroup**	**Facility**	**Sampled (month/year)**	**Isolate**	**Serogroup**	**Facility**	**Sampled (month/year)**
43	IIa	D	08/14	***64***	IVb	G	-/15
44	IIa	G	11/14	***65***	IVb	G	-/15
45	IIa	G	11/14	***66***	IIa	G	10/15
46	IVb	G	11/14	***67***	IIa	G	10/15
47	IVb	G	11/14	***68***	IIa	G	10/15
48	IIa	G	01/15	***69***	IIa	D	-/15
49	IIa	G	01/15	***70***	IIa	C	01/15
50	IIa	E	11/13	***71***	IVb	C	07/15
51	IIa	G	12/14	***72***	IIa	C	11/15
52	IIa	C	02/08	***73***	IIa	H	09/15
53	IIa	C	05/08	***74***	IIa	G	01/16
54	IIa	C	05/08	***75***	IIa	H	10/15
55	IVb	C	05/08	***76***	IIa	H	11/15
56	IVb	C	06/08	***77***	IIa	D	05/16
57	IVb	C	11/13	***78***	IIa	D	06/16
58	IIa	C	05/12	***79***	IIa	D	09/16
59	IVb	C	06/08	***80***	IIa	D	08/16
60	IVb	C	04/14	***81***	IIa	G	09/16
61	-	F	-	***82***	IIa	D	08/16
62	IIa	F	-/15	***83***	IIa	G	06/16
63	IVb	G	-/15	***84***	IIa	D	09/16

2.3 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is a typing method which separates high molecular weight DNA molecules (Watson et al. 2014b). The electric field apply pulses oriented orthogonally to each other, and makes the DNA molecule reorient through the gel. The 84 *L. monocytogenes* isolates used in this study were typed by engineers at the Norwegian Veterinary Institute in advance of this study according to a protocol by Roussel et al. modified in house (2014). The modified protocol is presented in appendix 2.

Seven isolates (13, 20, 29, 43, 45, 53 and 73) were re-typed in this study according to the same protocol. Briefly, bacterial cultures were incorporated in SSP-gel plugs (20 % SDS, 1.2 % SeaKem Gold agarose, 20 mg/ml Proteinase K). The bacteria in the plugs were lysed by lysisbuffer (1M Tris pH 8, 250 mM EDTA pH 8, 10 % L-lauryl sarcosine, MilliQ-water, 20 mg/ml Proteinase K), then plugs were washed in MilliQ-water (MQ-water) and TE-buffer followed by DNA digestion with restriction enzymes. The restriction enzymes recognizes target sequences and cut at specific positions.

The isolates were separately digested for 4 h at 37 ºC and 4h at 30 ºC with the restriction enzymes *Asc*I and *Apa*I. The same procedure was used for *L. monocytogenes* H9819, as was used as a reference strain. *Salmonella breanderup* H9812 was used as a size marker and the DNA was digested for 4h at 37 ºC using the restriction enzyme *Xba*I. After incubating, the enzyme solution was carefully replaced with 0.5 x TBE-buffer by pipetting. The plugs were stored in 0.5xTBE-buffer overnight at 4 ºC.

A 1 % SKG-agarose gel with 30 wells was prepared the same day as the electrophoresis, and the plugs were carefully placed in the wells with a small inoculation loop. Size marker *S. breanderup* H9812 were placed in every sixth well including the first and last. The wells were sealed with 0.8 % SKG agarose gel. PFGE was carried out for 20-21 h with the following settings: 6 volts/cm, the included angle was =120°, initial switch time was = 4 sec, and the final switch time was = 40 sec. After the electrophoresis, the gel was stained with GelRed™ for 30 min, visualized in GelDoc and analyzed in the software platform BioNumerics 6.6. Similarity between isolates were band based using the Dice correlation coefficient, 1 % band tolerance. The clustering algorithm, unweighted pair group method using arithmetic averages (UPGMA) was used, and dendrograms were constructed for the isolates in facility A, and for the isolates in facility C-H.

2.4 Biofilm assay

The biofilm forming ability of the 84 *L. monocytogenes* isolates was assessed in 96-well cell culture microtiter plates. Crystal violet (CV) staining was used to quantify the biofilm forming bacteria.

Biofilm was grown by inoculating 5 µl bacterial suspension from the overnight culture in triplicate on 3 plates filled with 200 µl Luria Berthoni (LB) medium and 3 with 200 µl LB w/o NaCl. One plate with each medium was incubated at 37 °C, 20 °C and 12 °C for 24 ± 1 hour, 48 ± 1 hour and 1 week (168 ± 1 hour) respectively. Wells H7-H12 served as negative control containing medium only.

Following incubation, optical density (OD) at 595 nm was measured in a microtiter plate spectrophotometer (Labsystems Multiskan MS or Tecan Sunrise). The microtiter plates were emptied of planktonic cells, and tapped on paper to remove excess liquid. The plates were washed twice by adding 200 µl tap water in each well, empty out the water and tap the plates on paper. After washing, 200 µl 0.1 % CV was added in each well and the microtiter plates were incubated for 20-25 min in room temperature. The microtiter plates were emptied of CV, and washed three times with tap water as previously described. The third washing step was carried out with 240 µl tap water to remove all excess CV. The biofilm could be observed as a purple circle at the bottom of the well. The CV in each well was dissolved in 200 µl ethanol:aceton (70:30). Absorbance was measured at 595 nm after 10 min of incubating at room temperature.

The data were corrected for the negative control (medium only), and normalized after the median value in each experiment. Every experiment was carried out in three independent replicates. To find differences between biofilm forming ability for serogroup IIa and IVb, a Students t-test assuming equal variance was performed in Microsoft Excel 2010.

2.4.1 Standard curve for crystal violet

A standard curve for CV was made to determine the concentrations where differences could be measured. The standard curve was made with ethanol:aceton (70:30). Eleven serial twofold dilution was made with 1 % CV and ethanol:aceton (70:30). Ethanol:aceton (70:30) was used as control. 200 µl of each dilution was added in triplicate to a microtiter plate and absorbance at 595 nm were measured in a microtiter plate spectrophotometer (Tecan Sunrise).

2.5 TTC assay as a method for detecting viable cells in biofilm after treatment with disinfectant

A method was developed using the tetrazolium salt 2,3,5 triphenyl-tetra-zolium chloride (TTC) for measuring the eradication of bacteria in biofilm after treatment with disinfectants. Metabolically active cells reduce TTC to 1,3,5-triphenylformazan (TPF) which can be seen as a red color. Within certain limits, there is a linear relationship between the color intensity and the amount of TTC that has been converted. The susceptibility bacteria had to disinfectants were screened using the TTC assay. Then, the efficiency of the disinfectants were validated using CFU count and the CV assay.

2.5.1 Standard curve for TTC

A standard curve for TTC was made to determine the concentrations where differences could be measured. The standard curve was made with ethanol:aceton (70:30). In an Eppendorf tube, a solution was made with 900 µl 1 % TTC, 900 µl LB and 200 µl Dhithiothreitol (DTT). The tube was incubated at room temperature overnight. The following day, conversion of TTC to TPF could be observed visually as a red color. The tube was centrifuged at 16 000 rcf for 4-5 min in an Eppendorf centrifuge (Beckman CS-15R centrifuge). The supernatant was pipetted off, and 2 ml ethanol:aceton (70:30) was added. Eleven serial twofold dilution was made with ethanol:aceton (70:30). Ethanol:aceton (70:30) was used as control. 200 µl of each dilution was added in triplicate to a microtiter plate and absorbance at 450 nm was measured in a microtiter plate spectrophotometer (Tecan Sunrise). The same procedure was performed using sterile MQ-water instead of ethanol:aceton (70:30).

2.5.2 TTC assay

To use the TTC assay it was necessary to find the minimum concentration of disinfectant where growth of L. monocytogenes was not inhibited. A microtiter plate with 200 µl LB broth and 5 µl bacterial suspension from an overnight culture was prepared and incubated for 37 °C for 24 ± 1 hour. Following incubation, the microtiter plates were rinsed twice as described in chapter 2.4, with 200 µl sterile MQ-water. Six different disinfectants commonly used in the fish industry was tested with the lowest recommended use from the suppliers. Dilutions with the disinfectants were prepared applied the same day. A stock solution with 1 or 0.5 % disinfectant was made in 30 ml sterile MQ-water. 10 serial twofold dilutions were made by

taking 15 ml from stock solution and adding it in 15 ml MQ-water. Sterile MQ-water was used as control. This experiment was carried out with two isolates (45 and 73) that had shown good biofilm production in the first experiment characterizing biofilm forming ability.

The disinfectant was added in one column, one concentration at a time. After incubating (1 or 5 min) it was pipetted off, the wells washed with 200 µl sterile MQ-water, and 200 µl Dey Engley Neutralizing Broth was immediately added to neutralize the antimicrobial effect. The neutralizing broth was emptied out after 5 min, and 200 µl MQ-water was added in all wells. When all concentrations of the disinfectant had been applied, the plates were emptied and tapped on paper to remove excess liquid. A 0.1 % solution of (TTC) (Sigma-Aldrich) was prepared with LB broth and filtered in a Minisart Syringe filter (0.2 µl). 200 µl of this solution was added to each of 96 wells. The plates were incubated at 37 °C for 24 ± 1 h.

Following incubation, absorbance was measured at 450 nm and 595 nm. The microtiter plates were emptied and washed one time with 220 µl tap water. Excess water was removed by tapping the microtiter plates on paper, and 200 µl ethanol:aceton (70:30) was added to dissolve the color. The plates were incubated at room temperature for 15 min before absorbance was measured at 450 nm. Data were corrected for a negative control (medium only).

2.5.3 Validation of the TTC assay

To validate the assay, 5 different concentrations were chosen for each disinfectant based on the results from the previous experiment (table 2). Because it was of interest to determine how well the TTC-assay showed eradication of biofilm bacteria after treatment with disinfectants, concentrations were chosen where metabolically active cells had been indicated, and concentrations where they were not indicated. This included three concentrations where there were no red color and no conversion of TTC, one concentration where red color had been observed and a control with sterile MQ-water. Biofilm of isolate 73 were grown in three microtiter plates, using the same procedure as in chapter 2.4. Absorbance at 595 nm was measured before treatment. The microtiter plates were treated with disinfectant and neutralizing broth as described in 2.5.1. Uninoculated wells underwent the same treatment, and were used as negative controls for each concentration of disinfectant. Each well in the first plate was added 200 µl of a filtered 0.1 % solution of TTC diluted in LB. The plate was

incubated at 37 °C. To validate the method the second and third plates were used for CV assay and colony forming unit count (CFU).

Table 2. Six disinfectants commonly used in the fish industry were screened for minimal inhibitory concentration (MIC) on *Listeria monocytogenes* (*L. monocytogenes*) biofilm. Biofilm were grown in microtiter plates, treated with disinfectants, and incubated at 37 °C for 24 h. Viable cells reduced TTC to TPF, which was seen as a red color. Numbers in red indicate the lowest concentrations and the concentrations were red color was observed. Concentrations (%) and time tested (min) are shown.

Disinfectant	**Concentrations used (%)**					**Time (min)**
Aco hygiene Des QA (QA)	0	0.0078	0.0150	0.0310	1	5
Aco Hygiene Des PE (PE)	0	0.0625	0.1250	0.250	0.5	1
Oxy Des (OX)	0	0.0156	0.0313	0.125	0.5	1
Perfectoxid (PF)	0	0.0156	0.0313	0.0625	0.5	1
Novadan Disinfect Maxi (MX)	0	0.0039	0.0078	0.0156	1	5
Sodium Hypochlorite (SD)	0	0.0039	0.0078	0.0156	0.5	1

2.5.3.1 CV assay

The microtiter plate was stained with 0.1 % CV to indicate amount of biofilm after being treated with a disinfectant. The same procedure as described in chapter 2.4 was used. Absorbance was measured at 595 nm, and corrected for a negative control.

2.5.3.2 Colony forming unit count

200 µl LB was added the first well in a triplicate treated with one concentration of disinfectant. The cells in the biofilm were dislodged by scraping the bottom for 20-30 seconds with a sterile mini Cell Scraper (Biotium). The suspension was mixed with a pipette, and pipetted to the next well. When cells from all three wells had been mixed, the suspension was transferred from the third well to an Eppendorftube and vortexed for approximately 10s. The suspension was transferred to a new microtiter plate and a dilution series was prepared (1:10 in a total volume of 200 µl LB), 6-7 dilutions in total. 5 µl from each dilution was drop plated in triplicate on a blood agar plate. All 6-7 dilutions were plated on the same plate (figure 2). The number of viable bacteria per biofilm was calculated.

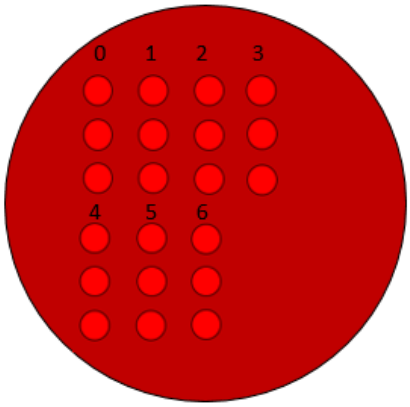


Figure 2. Illustration of a blood agar plate seeded with 5 µl bacterial culture. The stock solution (0), and dilutions 1-6 were seeded on the plate in triplicate, and the mean value of each triplicate was calculated.

3. Results

3.1 Characterization of *Listeria monocytogenes* with regard to PFGE type

3.1.1 PFGE re-typing

The 84 isolates of *Listeria monocytogenes* (*L. monocytogenes*) used in this study was PFGE typed by engineers at the Veterinary Institute using restriction enzymes *Asc*I and *Apa*I to digest bacterial DNA. The data were analyzed in the software platform BioNumerics 6.6. Isolate 13, 20, 29, 43, 45, 53 and 73 were re-typed for this study. PFGE pattern is presented in figure 3.

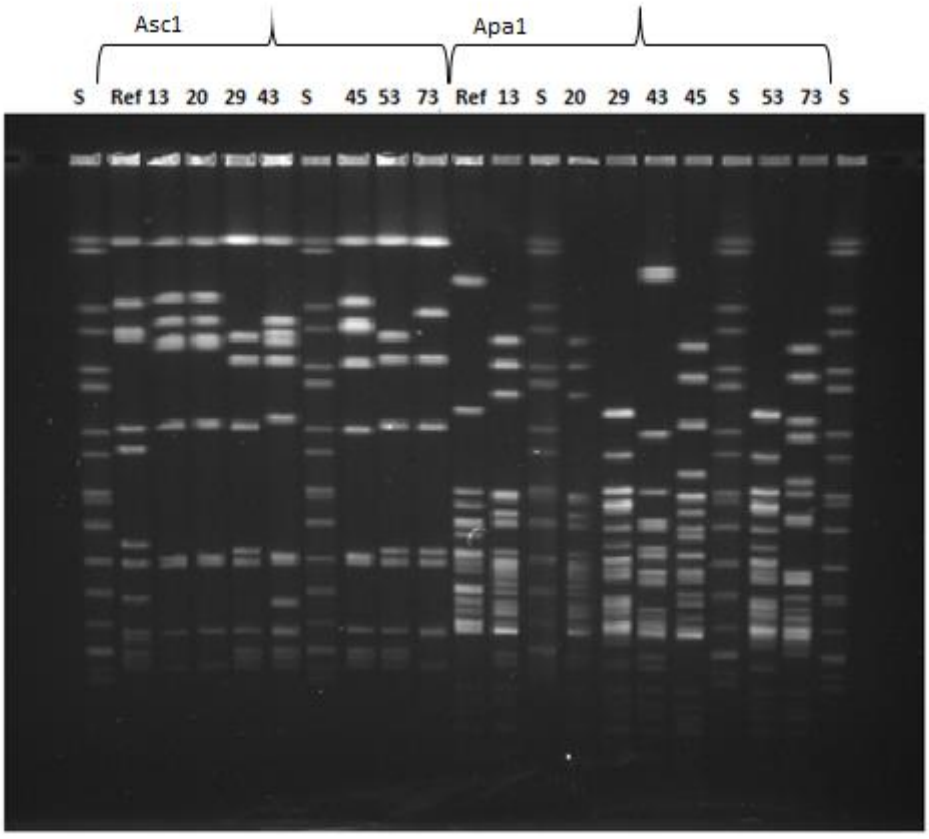


Figure 3. PFGE pattern for selected isolates of *Listeria monocytogenes* (*L. monocytogenes*) (13, 20, 29, 43, 45, 53, 73). *Listeria* strain H9812 (Ref) was used as a reference strain. DNA was digested with restriction enzymes *Asc*I and *Apa*I. *Salmonella breanderup* (S) was used as a size marker. The DNA was digested with the enzyme *Xba*I.

A cluster analysis of re-typed isolates and isolates typed by engineers both show five different fingerprint types (figure 4). The similarity scale on figure (a) indicate that isolate 20 and 13 are about 96 % similar, in contrast to about 85 % similarity in figure (b). The band pattern is similar, but bands for isolate 20 in figure (a) are very weak. The isolates were in general grouped similarly between the analysis, with the exception of isolate 43, were two bands are differentiated to the far left in figure (b).

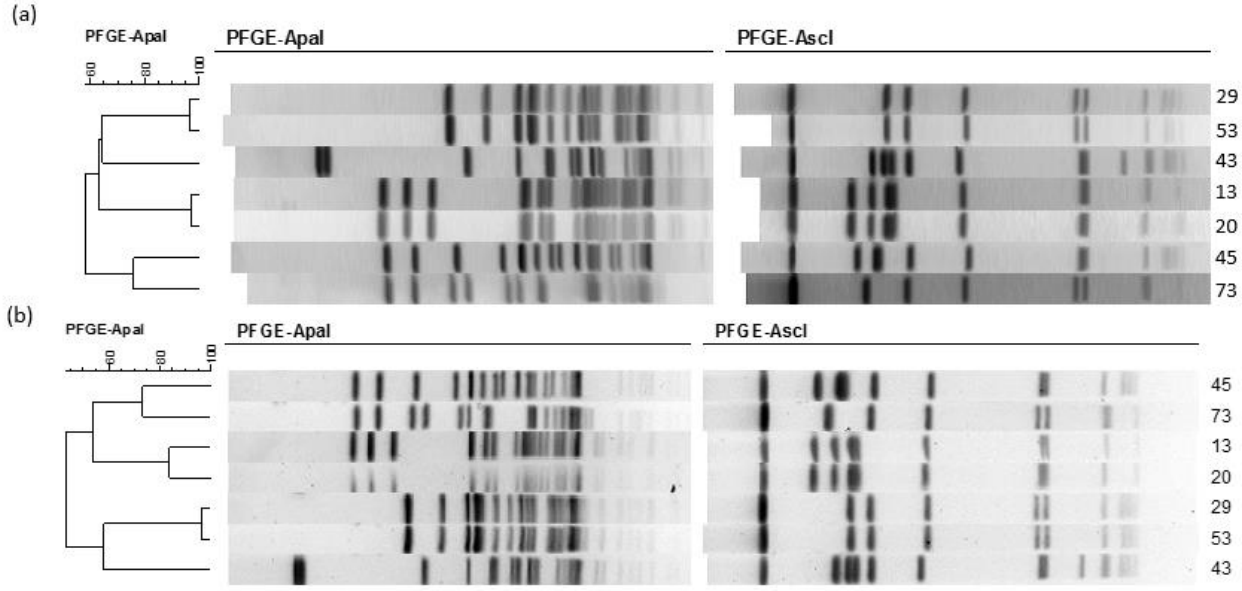


Figure 4. Isolates 13, 20, 29, 43, 45, 53 and 73 (isolate number indicated to the right) from (a): PFGE-typing performed in advance of this study, and (b): re-typing. DNA were digested with restriction enzymes *Apa*I and *Asc*I. Data were analyzed in BioNumerics 6.6, and a dendrogram was made with regard to restriction enzyme *Apa*I. Similarity scale is indicated by the scale bar at the top of the dendrogram.

3.1.2 Comparative cluster analysis

The biofilm forming ability of the 84 isolates were assessed in two growth media, LB and LB w/o NaCl after incubating at three different temperatures, 37 °C, 20 °C and 12 °C for 24 ± 1h, 48 ± 1h and 1 week (168 ± 1h), respectively. The results obtained from the biofilm assays are assembled with the results from PFGE typing. The data are divided in two groups (A and C-H) based on the facilities they were isolated from. A comparative cluster analysis was performed with regard to restriction enzyme *Apa*I. Serogroup results were obtained from multiplex PCR conducted in advance of this study. Isolates from facility A and C-H are presented in a cluster analysis with results from the biofilm assay.

Of a total of 84 isolates, 14 were classified in molecular serogroup IVb, the rest in serogroup IIa. One isolate (61) could not be identified. From facility A, five different fingerprint types were obtained from both restriction enzymes, with the biggest group containing 29 isolates (figure 5). Isolates from facility A were sampled in a period of 3 months.

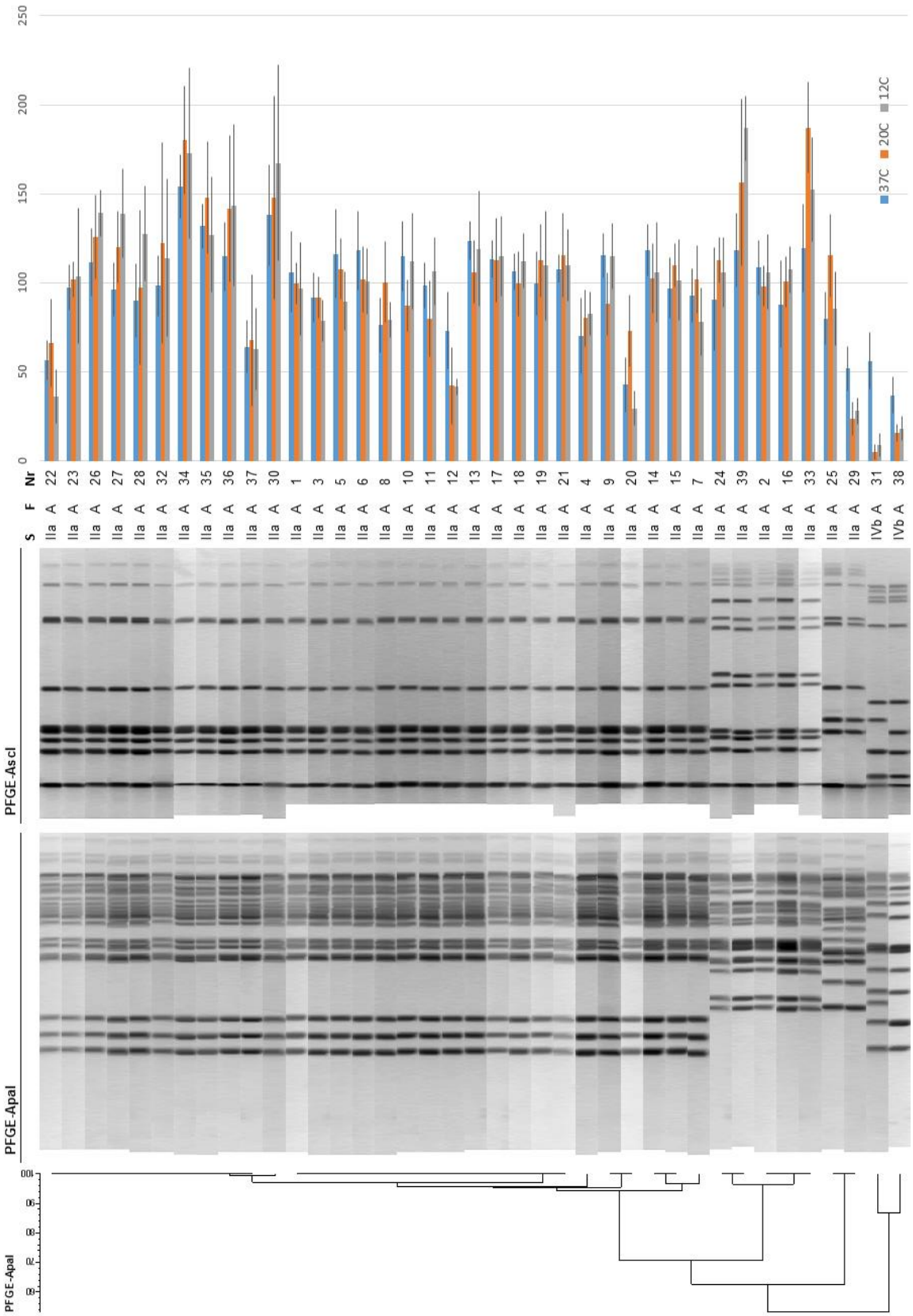


Figure 5. Comparative cluster analysis and biofilm forming ability at 37 °C for Listeria monocytogenes strains 1-39 isolated from facility A. DNA were digested with restriction enzymes *Apa*I and *Asc*I. Data were analyzed in BioNumerics 6.6. Similarity is indicated by the scale bar at the top of the dendrogram. Abbreviations: St; serogroup, F; facility, Nr; isolate. Biofilm was grown on microtiter plates in LB and assessed by staining with 0.1 % crystal violet. Absorbance was measured at 595 nm and was corrected with the mean value of a negative control (medium alone). Bars represent normalized mean value of three independent experiments, each with three technical replicates. Standard deviation is shown.

From facility C-H, 38 fingerprint types were obtained with restriction enzyme *Apa*I and 37 with *Asc*I (figure 6). The biggest group with similar types contained 3 isolates, all obtained from facility D. In general, there was a bigger range in the date of sampling for each facility compared to in facility A. Isolate 57 and 60 from facility C have the same fingerprint and occurred over a 6 months period. Isolate 40, 77 and 78 from facility D are the same type, and were isolated at 05/14, 05/16 and 06/16 respectively. Isolate 72 and 79 are according to the dendrogram 100 % similar, but different band patterns can be observed. The same can be observed for isolate 50 and 52.

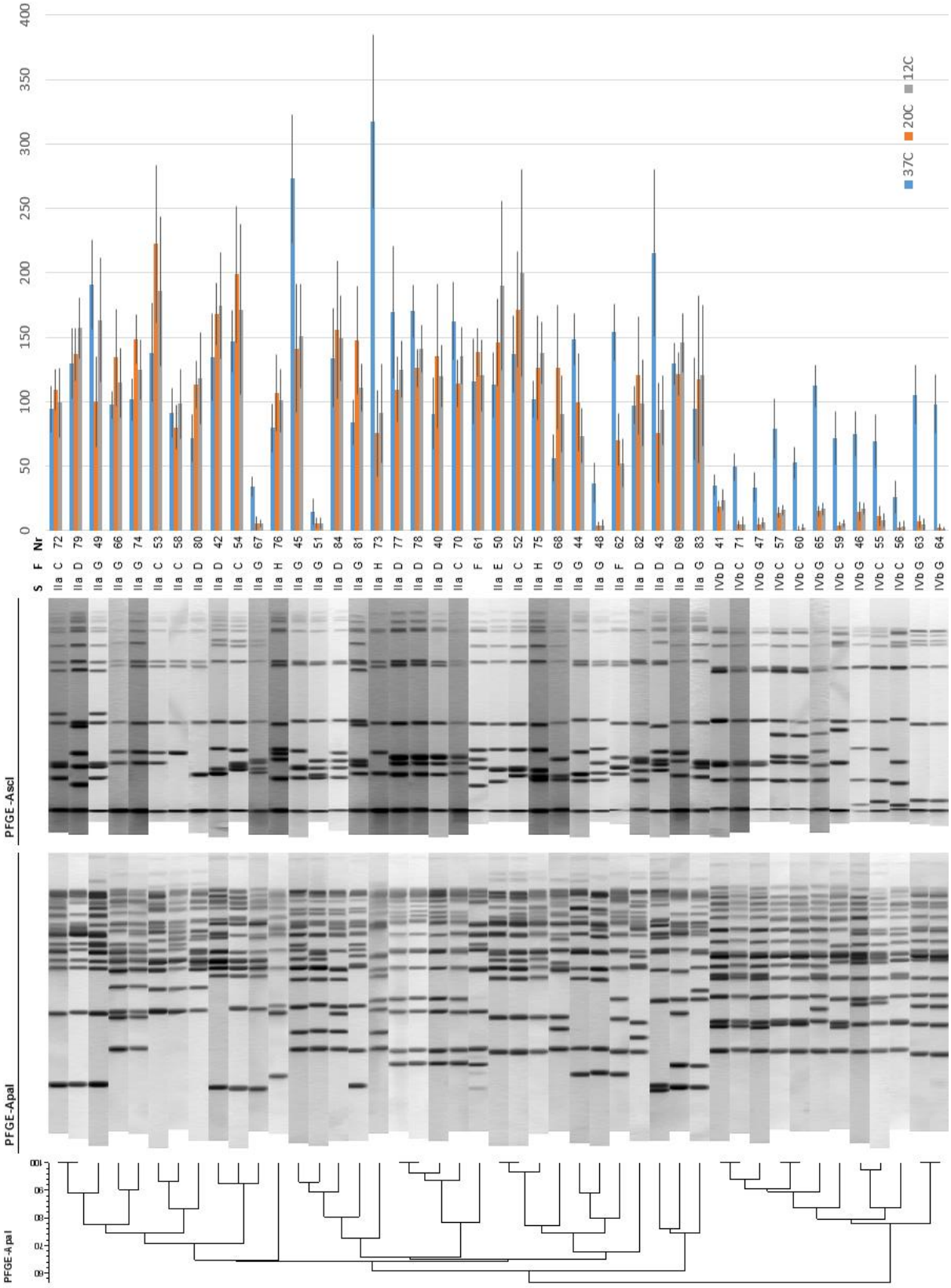


Figure 6. Comparative cluster analysis and biofilm forming ability at 37 °C for *Listeria monocytogenes* (*L.mono*cytogenes) strains 40-84 isolated in facility C-H. Similarity is indicated by the scale bar at the top of the dendrogram. DNA were digested with restriction enzymes *Apa*I and *Asc*I. Data were analyzed in BioNumerics 6.6. Abbreviations: St; serogroup, F; facility and Nr; isolate. Biofilm was grown in triplicates on microtiter plates in LB and assessed by staining with 0.1 % crystal violet dissolved in ethanol:aceton. Absorbance was measured at 595 nm and was corrected with the mean value of a negative control (medium alone). Bars represent normalized mean value of three independent experiments, each with three technical replicates. Standard deviation is shown.

3.2 Characterization of *Listeria monocytogenes* with regard to biofilm forming ability

3.2.1 Biofilm assay

The relative growth patterns of the isolates in LB w/o NaCl were similar the growth patterns in LB, but the values for biofilm were low (absorbance at 595 nm <100) and the isolates were not considered as biofilm formers. The results are not presented.

3.2.1.1 Facility A

The lowest average growth for all isolates in facility A was observed at 37 °C for 24 h with isolate 38 (median value in a typical experiment was 0.150). Higher growth was seen at 20 °C for 48 h and 12 °C for 1 wee (figure 7). There was little variation in growth between isolates. Median value for growth in a typical experiment at 20 °C was 0.249, and at 12 °C; 0.240.

Results from the biofilm assay show that the biofilm forming ability varied between isolates with different PFGE-type, and for isolates of the same type. Isolate 30 and 37 show the same fingerprint obtained from both restriction enzymes. Isolate 37 had lower biofilm forming ability in contrast to isolate 30. Isolate 31 and 38 were poor biofilm formers at all temperatures. Most of the isolate had better biofilm forming ability at 20 °C and 12 °C. At 37 °C, 20 °C and 12 °C the range of biofilm formation in a typical experiment was between 0.022 to 0.522, -0.009 to 0.657 and -0.002 to 0.483, respectively. While the median values in a typical experiment at 37 °C, 20 °C and 12 °C was 0.186, 0.213 and 0.149.

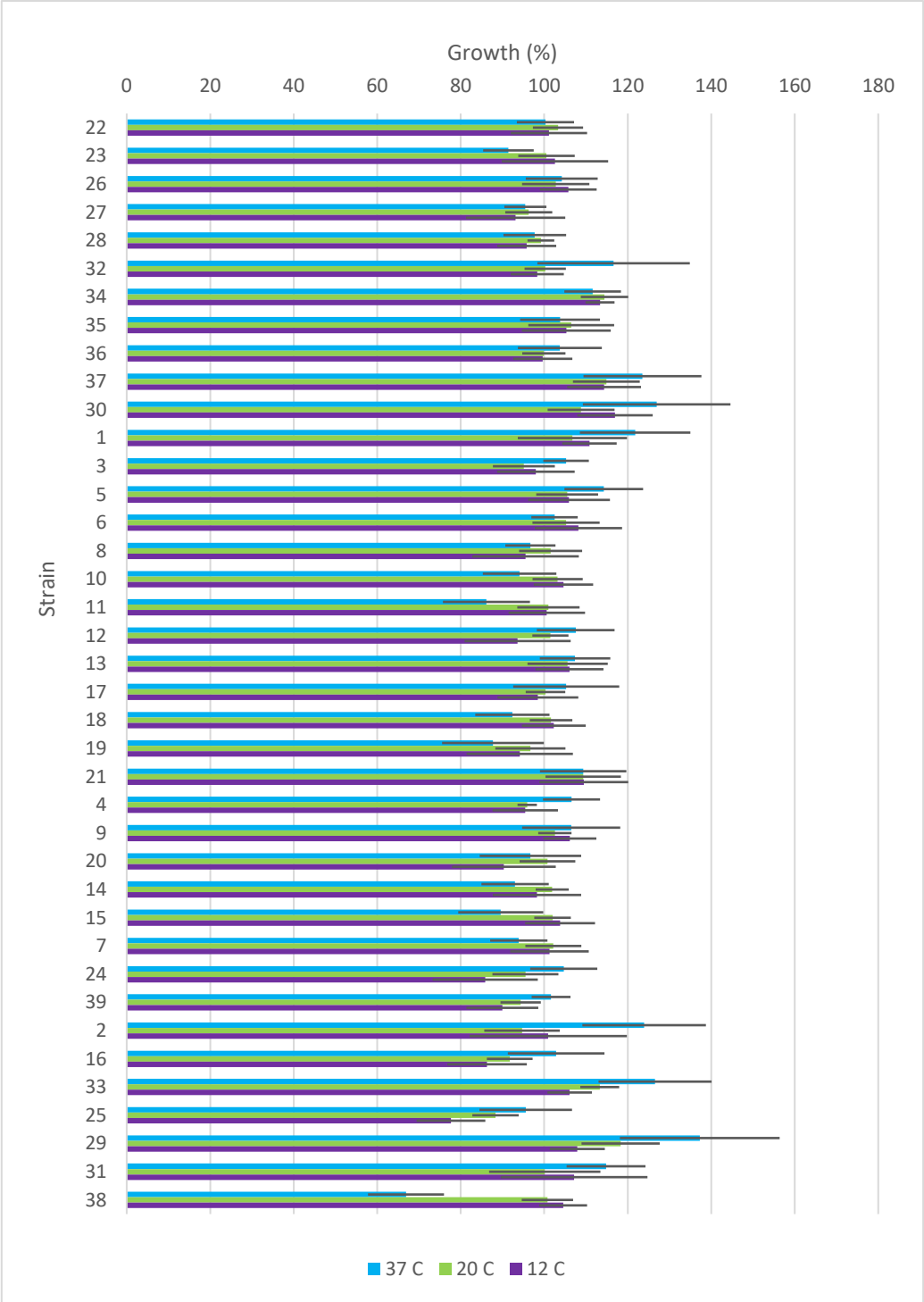


Figure 7. Growth of 39 isolates obtained from facility A, incubated in LB at 37 °C, 20 °C and 12 °C for 24 ± 1h, 48 ± 1h and 1 week (168 ± 1h). Bars represent normalized mean value of three independent experiments, each with three technical replicates. Optical density (OD) was measured at 595 nm and was corrected for the mean value of a negative control (medium alone). In each experiment, the values were normalized against the median value. Standard deviation is shown.

3.2.1.2 Facility C-H

Isolates from facility C-H had bigger variation in relative growth pattern (figure 8). A bigger variation in biofilm forming ability between isolates where also seen in facility C-H. As seen with isolates from facility A, most of the strains were better biofilm formers at temperature 20 °C and 12 °C. The isolates considered as best biofilm formers (45, 73 and 43) occurred only one time each, at different facilities. Nine isolates (67, 61, 48, 51, 62, 55, 41 and 47) formed very little or no biofilm at any of the temperatures tested. Isolate 47 and 56 had the lowest relative growth at 37 °C of isolate in these facilities, median value was 0.150 in a typical experiment.

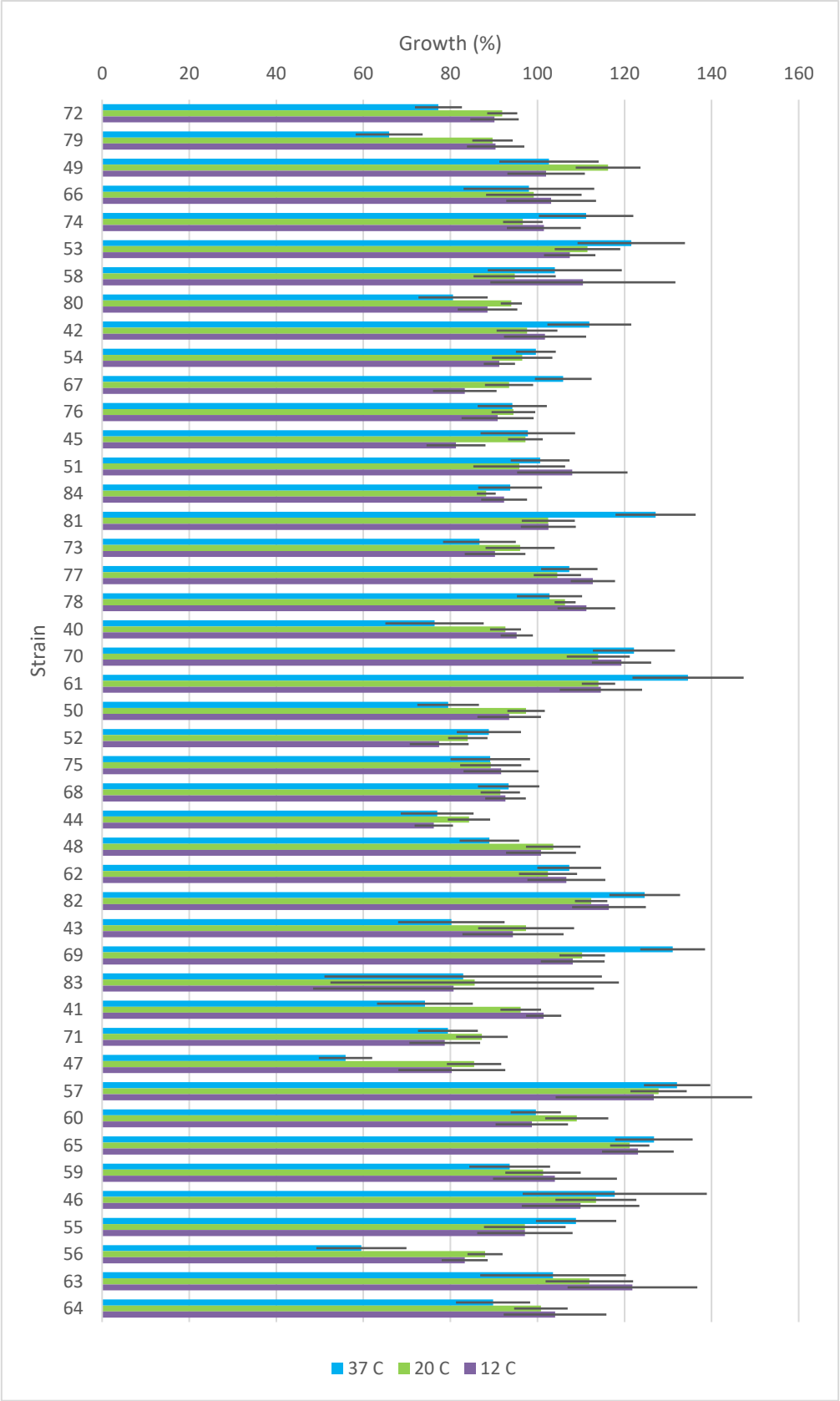


Figure 8. Growth of isolates 40-84 incubated in LB at 37 °C, 20 °C and 12 °C for 24 ± 1 h, 48 ± 1 h and 1 week (168 ± 1 h). Optical density (OD) was measured at 595 nm, and was corrected for the mean value of a negative control (medium alone). Bars represent normalized mean value of three independent experiments, each with three technical replicates. In each experiment, the values were normalized against the median value. Standard deviation is shown.

Serogroup IIa had better biofilm forming ability than serotype IVb (figure 4 and 5). Relative mean value for biofilm at 37 °C for serogroup IIa was 111.6 % compared to 64.4 % for serotype IVb. A significant difference in biofilm forming ability between the two groups was found at any temperature (table 3).

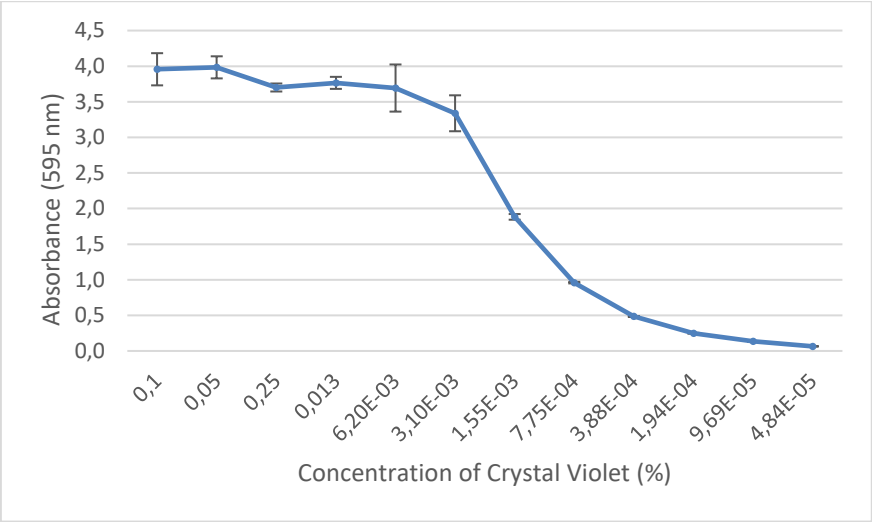
Table 3. Biofilm was grown in microtiter plates at temperature 37 °C, 20 °C and 12 °C for 24 ± 1h, 48 ± 1h and 1 week (168 ± 1h). Absorbance was measured at 595 nm and was corrected for the mean value of a negative control (medium only). In each of three independent experiments, values were normalized against the median value. A significant difference in biofilm forming ability was found between serogroup IIa (n=70) and IVb (n=14) at all temperatures.

Temperature (°C)	**Mean biofilm value (%)**		***P*-value**
	IIa	IVb	
37	111,6	64,4	0,00059
20	111,1	8,7	1,83E-14
12	110,8	9,6	3,01E-13

3.2.2 Standard curve for crystal violet

A standard curve with and crystal violet diluted in ethanol:aceton (70:30) was made (figure 9). This was used to determine concentrations of CV where differences in concentration could be measured. Figure (b) show that there is a linear relationship between CV-concentration and absorbance at 595 nm between concentration 1.84E-03 and 7.75E-04.

(a)



(b)

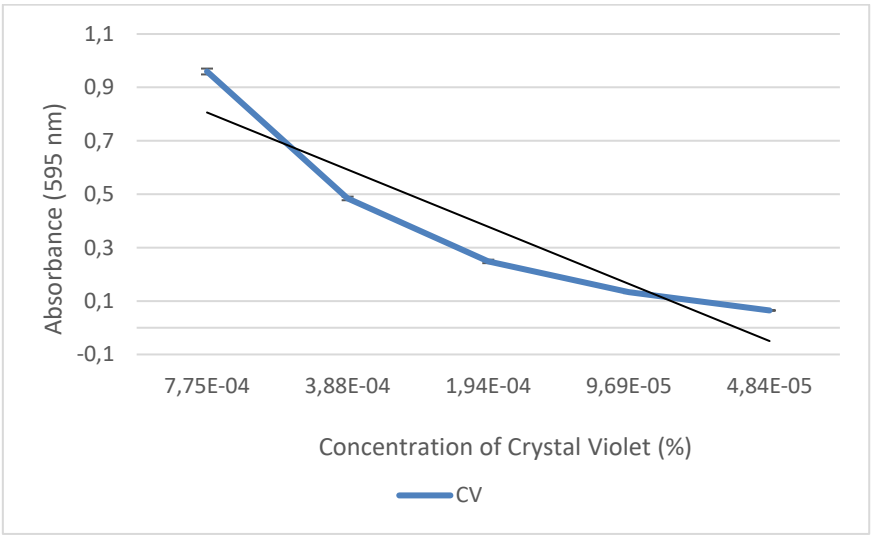


Figure 9. Standard curve (a) for Crystal Violet (CV) diluted in ethanol:aceton (70:30). Absorbance was measured at 595 nm and corrected for the mean value of a control with ethanol:aceton. The graph shows mean value of two independent replicates. Figure (b) shows relevant values for this study (0,065-0,960).

3.3 Use of the TTC assay to assess the ability of disinfectants to kill biofilm bacteria

3.3.1 TTC assay

The TTC assay was performed to screen the disinfectants for minimum inhibitory concentration, and find where viable cells were detected. The lowest recommended concentration and time of disinfectant recommended by the supplier was used.

Figure 10 show conversion of TTC to TPF after treatment with disinfectant Perfectoxid, isolate 45 in row A-C and 73 in row D-F. A gradually increasing red color could be observed from column 6-8.

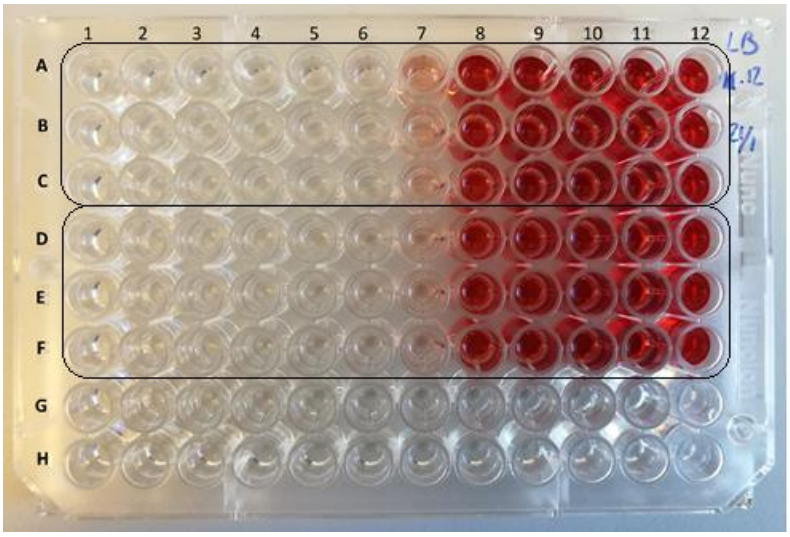
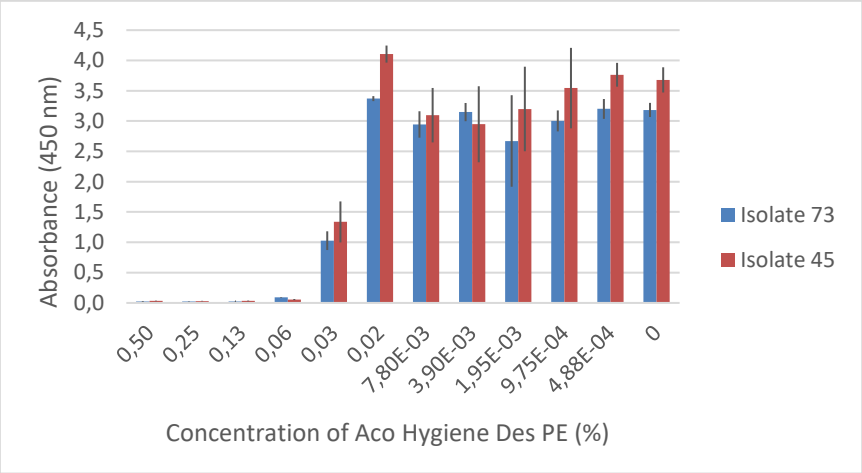


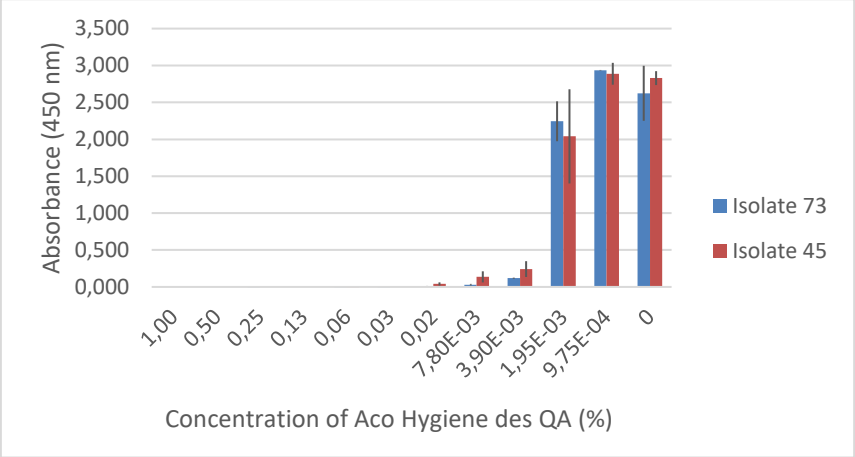
Figure 10. Conversion of 2.3.5.triphenyl-tetra zolium chloride (TTC) to TPF by viable cells. The microtiter plate was treated with desinfectant Perfectoxid and incubated with LB at 37 °C for 24 ± 1h. Isolate 45 was grown in row A-C and isolate 73 in row D-F. Column 1 was treated with the highest concentration of disinfectant (0.5 %), with 2-fold decreasing concentrations in each column from column 2-11. Column 12 was treated with sterile MilliQ-water. Absorbance was measured at 450 nm and corrected for mean value of a negative control.

Figure 11 shows the results from screening the MIC values for six disinfectants with isolate 45 and 73. All disinfectants varied in their effect on killing bacteria, but the effect was similar for both isolates.

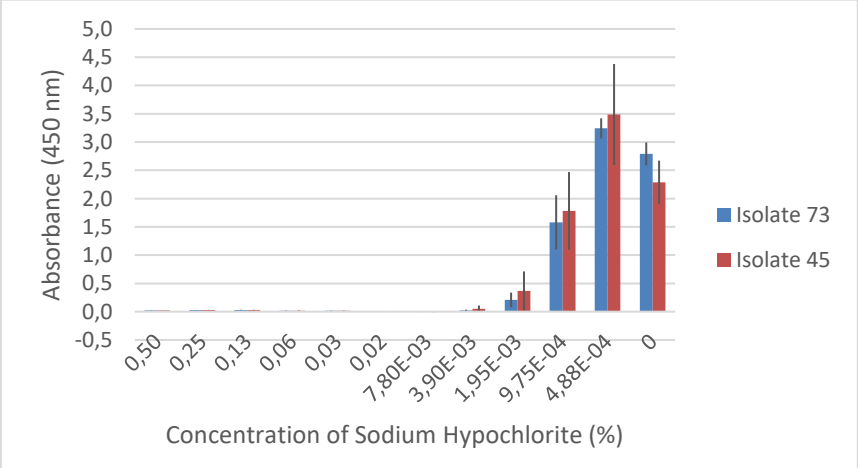
(a)



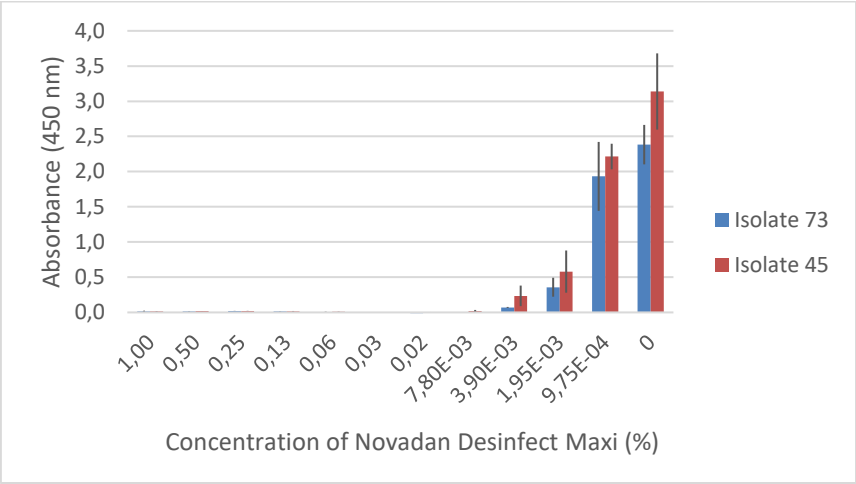
(b)



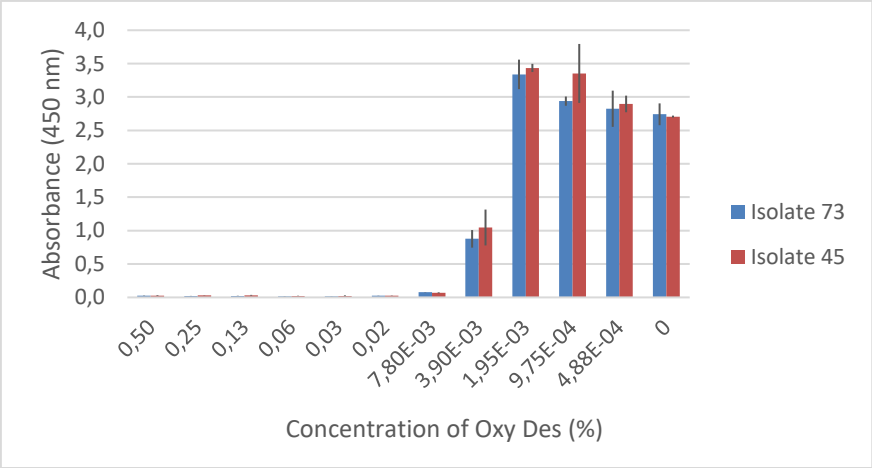
(c)



(d)



(e)



(f)

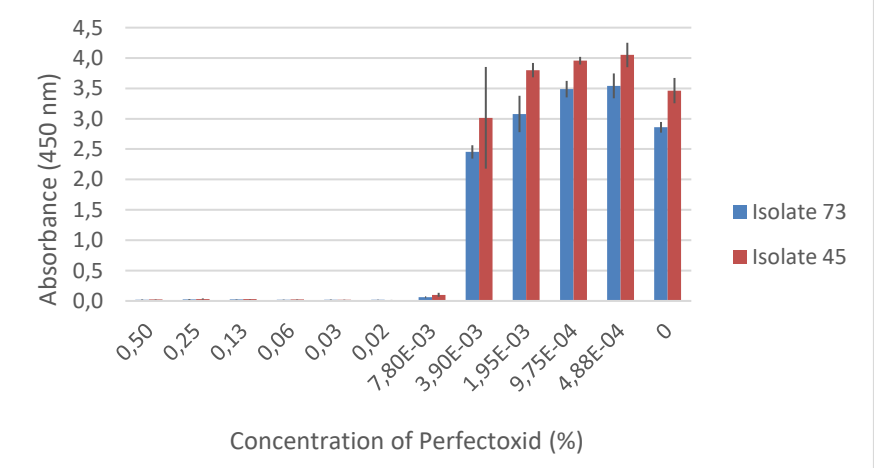


Figure 11. Minimum inhibitory Concentration (MIC) characterizing of disinfectants. A microtiterplate with isolate 73 and 45 was treated with different concentrations of disinfectant and a control with sterile MQ-water. a: Aco Hygiene Des PE, b: Aco Hygiene Des QA, c: Sodium Hypochlorite, d: Novadan disinfect Maxi, e: Oxy Des, f: Perfectoxid. To each well, 0.1 % TTC diluted in LB medium, was added and incubation was done at 37 ºC for 24 h. The amount of TTC converted to TPF was measured at 450 nm, after being dissolved in ethanol:aceton (70:30).

3.3.2 Standard curve for TTC

To determine concentrations of TPF after conversion from TTC, two standard curves were made: one with MQ-water and one with ethanol:aceton (figure 12). The highest absorbance was obtained dissolving TPF in ethanol:aceton (70:30), 5.917, while dissolving TPF using MQ-water, the highest absorbance obtained was 1.988. Differences in TPF concentration can with relatively high certainty be measured between 0.01 and 0.45 %; there is a linear relationship between TPF-concentration and absorbance at 450 nm between 0.01 and 0.45 %.

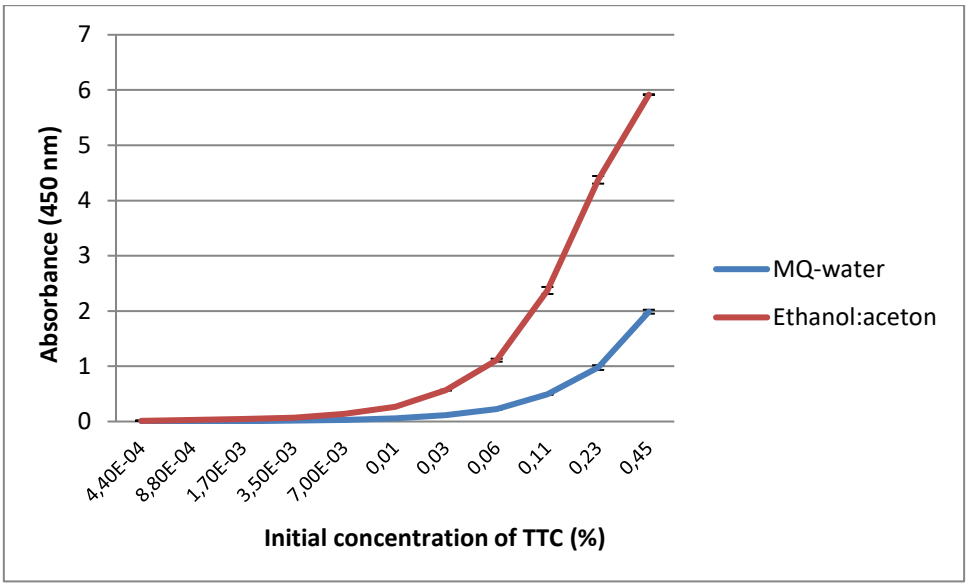


Figure 12. Standard curve for TTC was made in a microtiter plate by making a twofold 11-series dilution of TPF in MilliQ-water (MQ-water) and ethanol:aceton (70:30). The curve represent mean value of a three technical replicates corrected for the mean of a blank. Absorbance at 450 nm was measured.

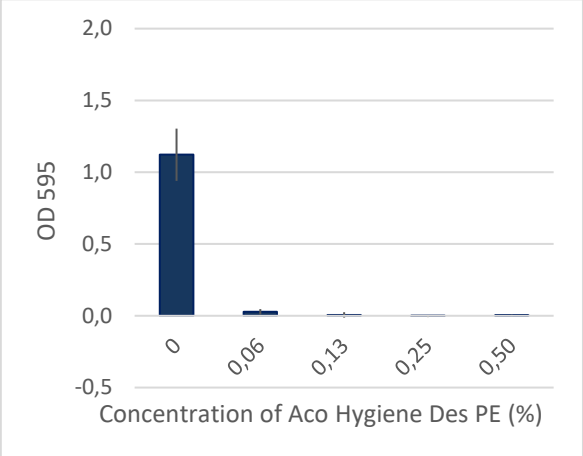
3.3.3 Validation of the TTC assay as a method to assess bacterial viability

Based on the results from the MIC characterization, 4 concentrations of each disinfectant were chosen. This included two concentrations with red color which would indicate viable cells, and two without. Sterile MQ-water was used as control. The TTC assay was performed using these concentrations, and to screen the effect of disinfectants the method was validated by performing a biofilm assay and a CFU count. The experiments were performed using isolate 73.

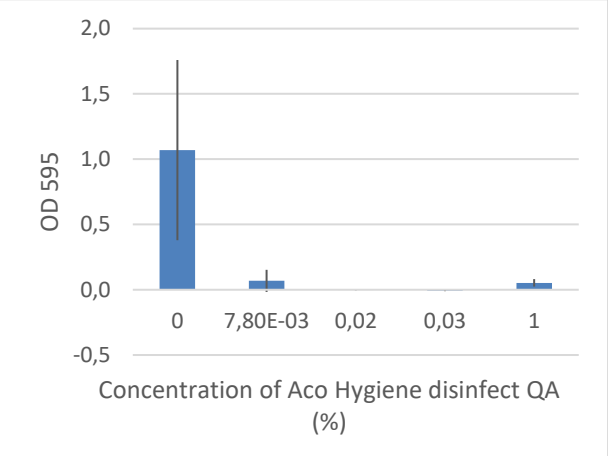
3.3.3.1 TTC assay

Following incubation with TTC and LB, the microtiter plates were measured at 595 nm to indicate possible growth (figure 13). A red color could be seen in the control column and the column treated with the lowest concentration of disinfectant, indicating viable cells. Results coincided with the initial characterization of MIC-value. Absorbance values in wells with no observed red color were low, indicating no growth.

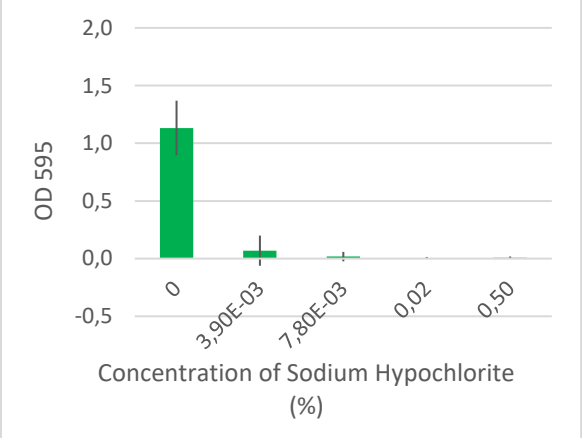
(a)



(b)



(c)



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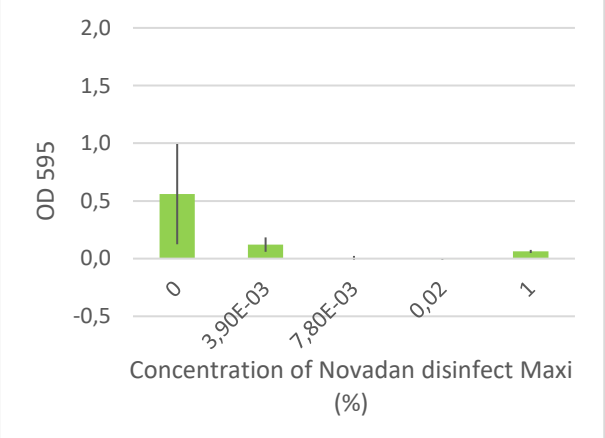
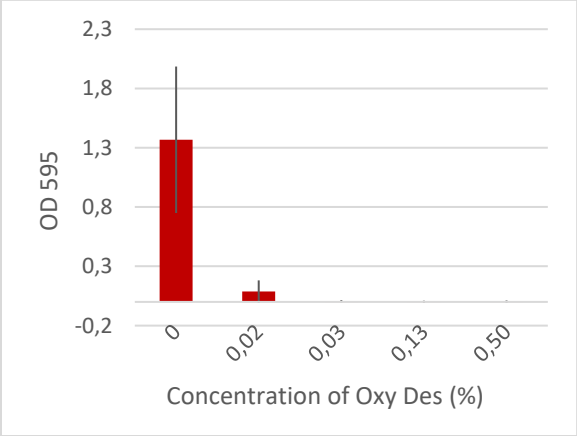


(e)



(f)

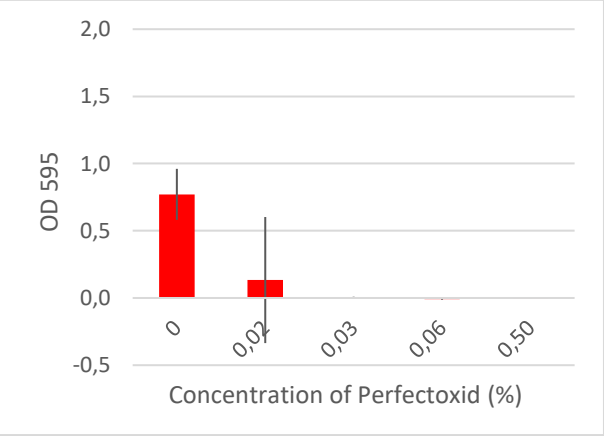


Figure 13. Growth of bacteria after incubation at 37 °C for 24 h. Optical density (OD) at 595 nm was measured. Biofilms were treated with a: Aco hygiene Des PE, b: Aco Hygiene Des QA, c: Sodium Hypochlorite, d: Novadan disinfect Maxi, e: Oxy Des and f: Perfectoxid. Bars represent mean value of two or more independent replicates.

After measuring OD 595, the plates were washed and TPF dissolved in ethanol:aceton (70:30). Absorbance was measured at 450 nm (figure 14). The amount of TPF varied between disinfectants.

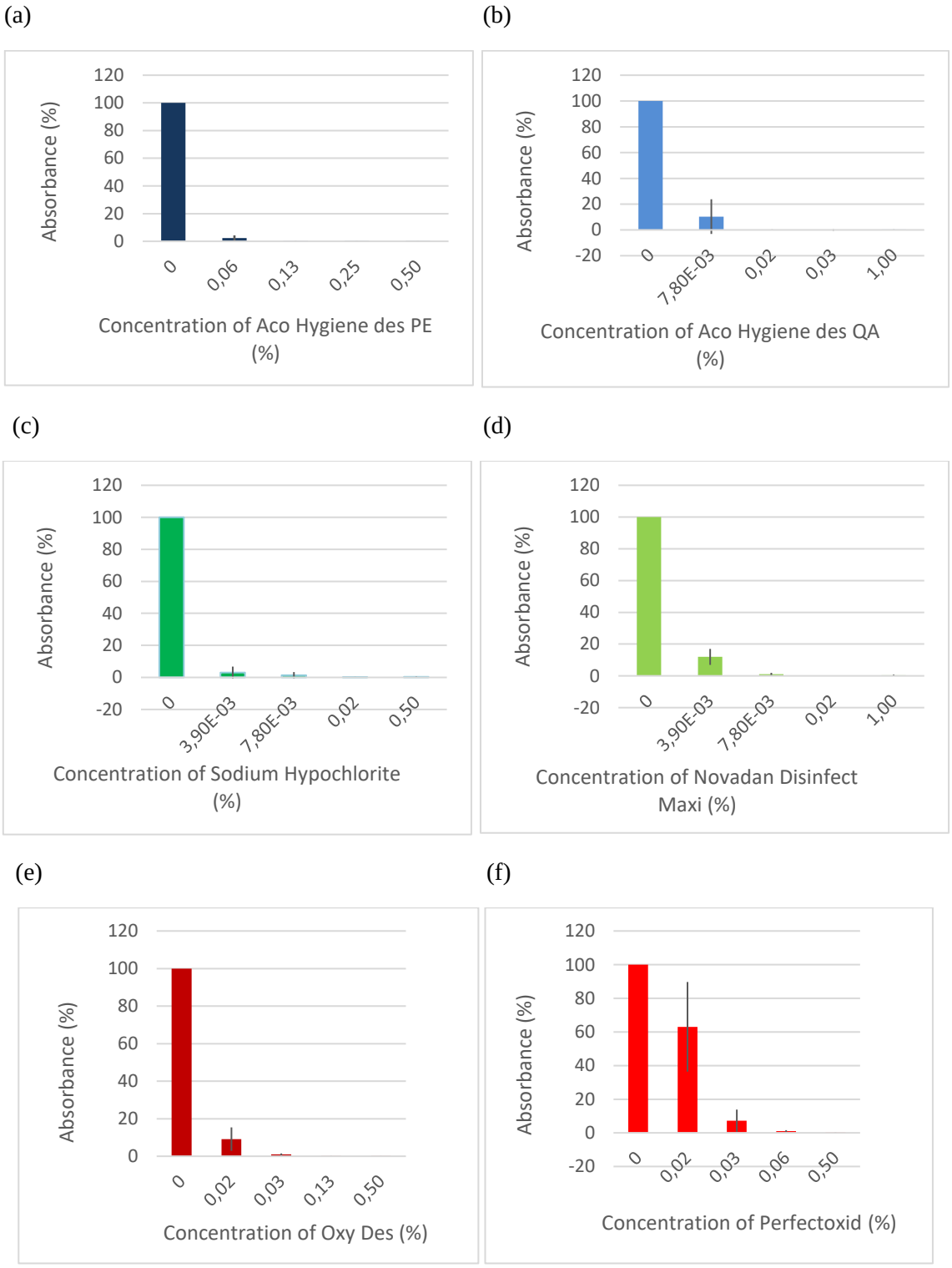
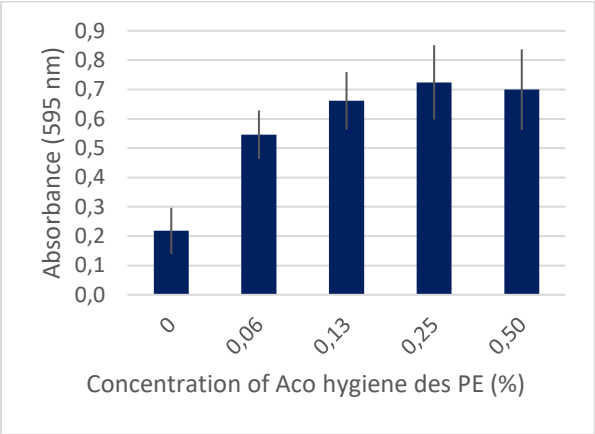


Figure 14. Amount of TPF converted by viable bacteria in biofilm after treatment with disinfectant a: Aco hygiene Des PE, b: Aco Hygiene Des QA, c: Sodium Hypochlorite, d; Novadan disinfect Maxi, d: Novadan disinfect Maxi, e: Oxy Des and f: Perfectoxid. Bars represent standardized mean value of two or more independent replicates. Absorbance was measured at 450 nm

3.3.3.2 Biofilm assay

Following treatment with disinfectant, one microtiter plate was assessed for biofilm with 0.1 % CV staining. The lowest amount of biofilm was observed in the control with MQ-water (PE, SH, OX and PF) or after treatment with the second lowest concentration (QA and MX), though there were little variation (figure 15). For all disinfectants, the amount of biofilm increased with increasing concentration of disinfectant. The highest biofilm value was seen after treatment with the highest concentration of disinfectant. Mean absorbance in the control column ranged from 0.109-0.218.

(a)



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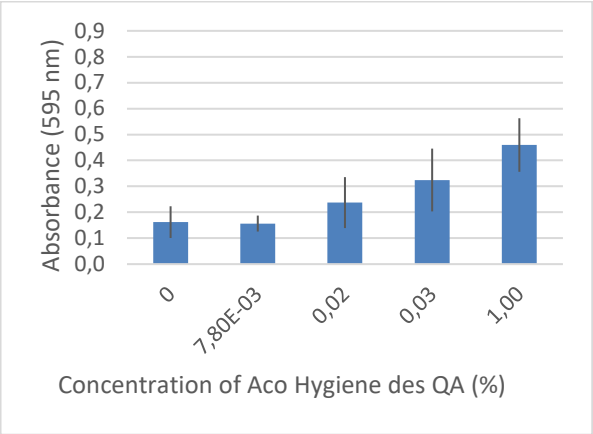

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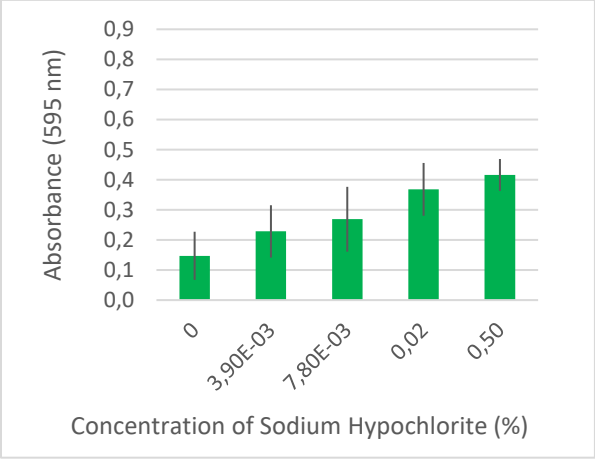

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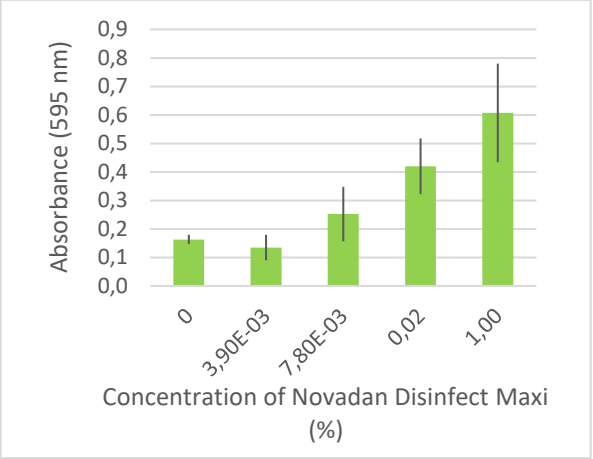

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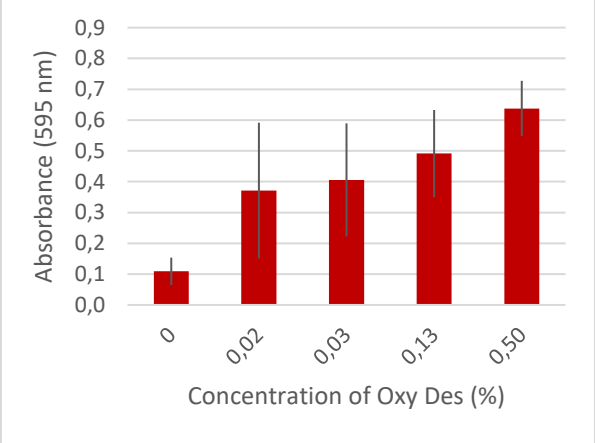


(f)

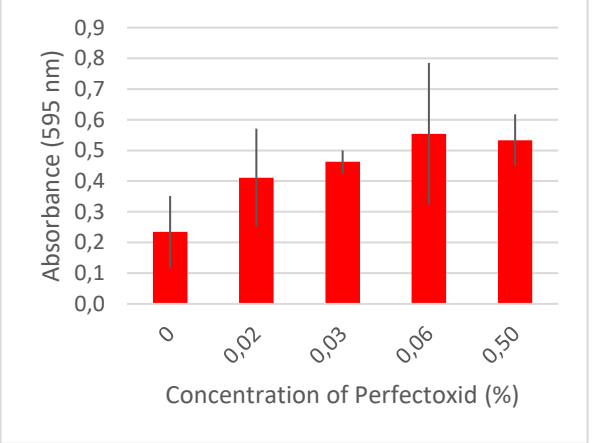


Figure 15. Biofilm was grown in LB in microtiter plates at 37 °C for 24 ± 1h. The plates were treated with disinfectants a: Aco hygiene des PE, b: Aco Hygiene des QA, c: Sodium Hypochlorite, d; Novadan disinfect Maxi, e: Oxy Des and f: Perfectoxid. Biofilm was assessed with 0.1 % CV staining, and was destained with ethanol:aceton (70:30). Absorbance was measured at 595 nm, and corrected for the mean of a negative control that had undergone the same treatment. Bars represent mean value of two or more independent replicates. Standard deviation is shown.

3.3.3.3 Colony forming unit count

Biofilm bacteria were dislodged from the wells with a cell scraper, and suspended in LB. Ten-fold serial dilutions were prepared, and five µl of the bacterial suspension were plated in triplicates on a blood agar plate and incubated at 37 °C. An example of a blood agar plate is presented in figure 16. Biofilm was treated with 0.0078 % QA. There were growth from 6 dilutions. The more diluted, the fewer bacteria can be seen.

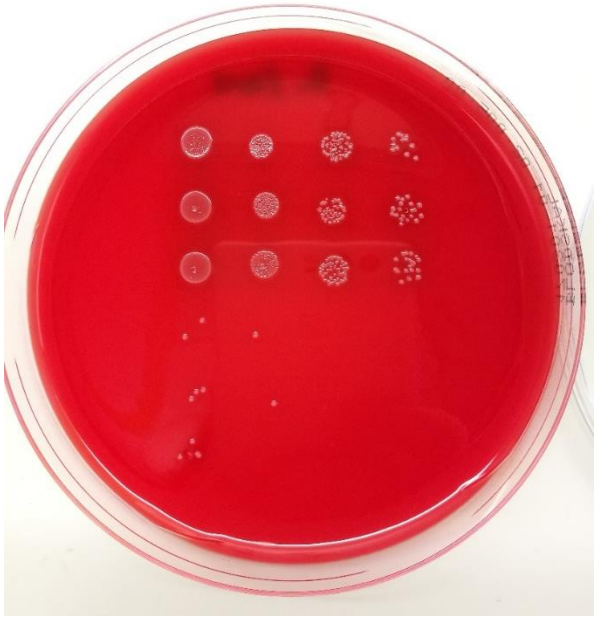
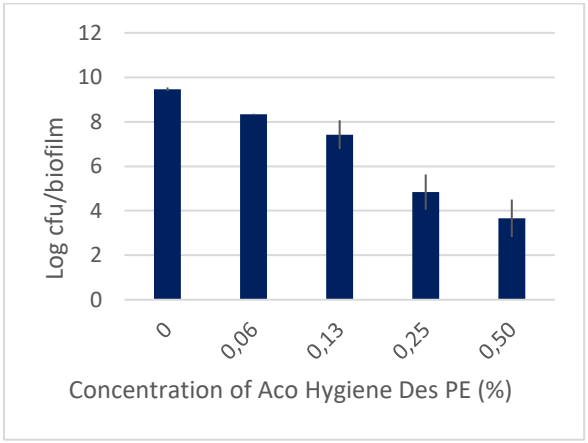


Figure 16. Blood agar plate seeded with 5 µl bacterial suspension in triplicate. Biofilm from three wells treated with 0.0078 % Aco hygiene disinfect QA were scraped with cell scraper and mixed in 200 µl LB. The suspension was diluted 1:10 in a total volume of 200 µl LB, 6 times.

Absorbance values from the TTC assay indicated viable cells in the control and the column treated with the lowest concentration of disinfectant. All disinfectants had an effect on reducing the amount of bacteria in the biofilm (figure 17). Agent QA and MX had no growth after treatment with the highest concentration (1 %) of disinfectant. The reduction in biofilm bacteria were 8.9 and 9.4 log cfu/biofilm. The reduction in biofilm bacteria for the other disinfectants ranged from 4.1-6.9 log cfu/biofilm. SH had the poorest effect overall, but were most effective at the second lowest concentration.

(a)



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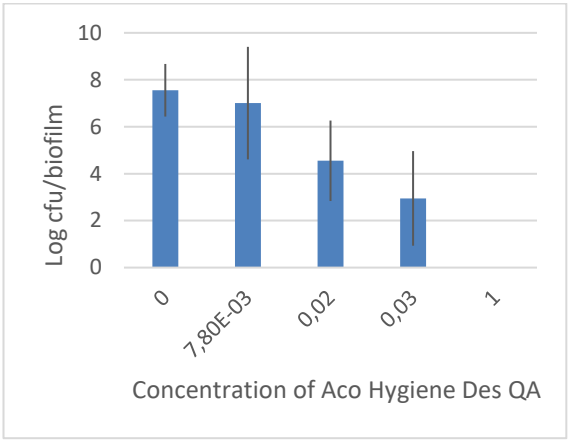

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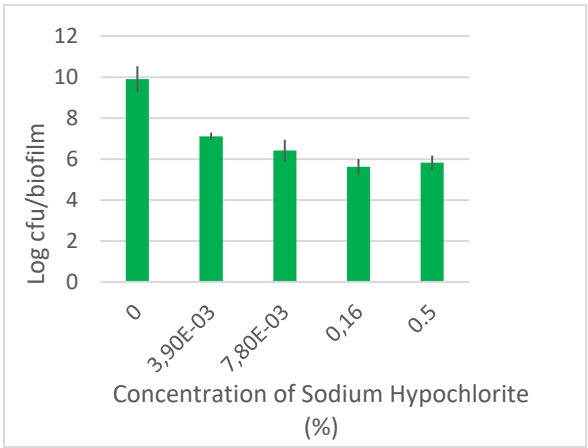

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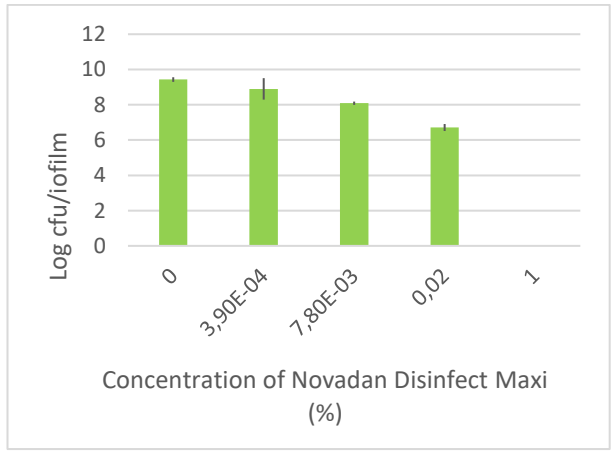

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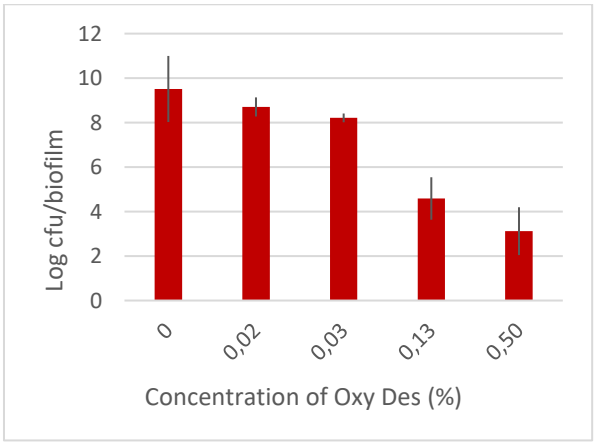

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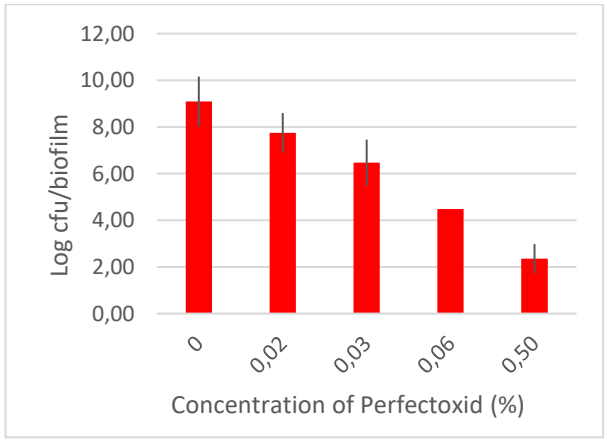

Figure 17. Biofilm were treated with a: Aco hygiene des PE, b: Aco Hygiene des QA, c: Sodium Hypochlorite, d; Novadan disinfect Maxi, e: Oxy Des and f: Perfectoxid. Bacteria were dislodged from the biofilm, made in a suspension with LB, plated on blood agar and incubated at 37 °C overnight. Bars represent mean value of two or more independent replicates. Figure (f), bar with concentration 0.06 represents 1 replicate.

4. Discussion

The purpose of this study was to characterize *L. monocytogenes* with regard to biofilm forming ability and compare it to the PFGE type, and to test the susceptibility of the biofilm bacteria to selected disinfectants commonly used in fish processing facilities. In the latter experiment it was of interest to establish a fast and simple method that can be used for reliable screening of the effects of disinfectants against biofilms. For this purpose, the TTC assay was established in the laboratory and experiments were done to determine how well the TTC assay measures viability of cells in the biofilm after treatment with disinfectants, and these results were compared to CFU counts. The disinfectants ability to remove biofilms were investigated with the CV assay.

4.1 Characterization of *L. monocytogenes*

4.1.1 Comparison of re-typed data and data from engineers

The 84 isolates of *L. monocytogenes* used in this study were typed in advance of this study by PFGE, and a selection of these isolates were re-typed in this study. The cluster analysis was made with regard to the restriction enzyme *Apa*I. Fugett et al. characterized isolates of *L. monocytogenes* from a variety of sources (humans, animals and foods) by using PFGE (2007). They found that combining two restriction enzymes was more discriminatory than using one, and reported that *Apa*I differentiated more types than *Asc*I. Based on this, all cluster analyses in this study were performed with regard to *Apa*I.

The band patterns were grouped similarly between the analyses. However, the cluster analysis from the re-typing of isolate 20 and 43 were judged as less similar by the software program BioNumerics. This could be due to the weak band pattern seen with isolate 43 from the re-typed data. When analyzing the data in BioNumerics, it was observed that the cluster analysis could estimate similarity between band patterns differently when the same isolates were analyzed. The weak band pattern seen with isolate 43 from the re-typing was most likely caused by damage to the gel plug when it was placed in the well with an inoculation loop.

4.1.2 PFGE-type

Typing patterns for the 84 isolates were divided in two groups (A and C-H) based on which facility they were isolated from. Fewer PFGE-types were found in facility A, than in facility C-H. The biggest group of similar PFGE-types in facility A contained 29 types, while in facility C-H only 3 similar PFGE types were sampled from the same facility (D). The PFGE data showed that there was only a slight difference between the number of PFGE-types differentiated by *Apa*I and *Asc*I. In facility C-H, 38 different PFGE-types were obtained for *Apa*I, and 37 for *Asc*I.

All isolates from facility A were sampled in a period of two months, but the majority of isolates with the same PFGE-type were sampled during the same month. This could indicate that the same isolate has been collected several times, or that it is a persistent strain. Persistent strains have been defined as strains isolated more than five times or over a time range of 3 months (Lundén et al. 2003). It is also defined as repeated isolation of the same strain on different dates (Ferreira et al. 2014). The isolates considered as best biofilm formers in the present study occurred only one time each.

4.1.3 Biofilm forming ability

In this study, the biofilm forming ability of the bacteria was investigated in two different growth media; LB and LB w/o NaCl at 37 °C, 20 °C and 12 °C. CV staining was used to quantify the biomass of the biofilm. The relative growth pattern for the 84 isolates used in this study was similar when incubated in LB and LB w/o NaCl. Although all of the tested isolates were able to form biofilms in LB medium, none of them was able to form biofilm in LB w/o NaCl. This is in agreement with the observation that biofilm formation by *L. monocytogenes* is enhanced when using growth media with 2 % and 5 % NaCl (Pan et al. 2010). However, it was unexpected that none of the isolates tested in this study were able to form biofilm in a low salt medium. Previous experiments in the laboratory have shown that a small percentage of *L. monocytogenes* isolates are better biofilm formers in low salt media than high salt media (Simm, personal communication). The relative growth pattern varied more for isolates at facility C-H than at facility A, and the same was observed for the biofilm values. In facility C-H more PFGE-types occurred than in facility A. This could explain the increased variation. The biofilm forming ability varied between isolates with different PFGE-type, but also for isolates having the same type. The variation of biofilm forming ability seen between strains

have been reported by several researchers (Borucki et al. 2003; Harvey et al. 2007; Kadam et al. 2013), and supports the results in this study. However, in the present study it must be considered that the isolates of the same PFGE-type could belong to different serogroups. This could possibly explain some of the differences in biofilm forming ability.

Most isolates formed more biofilm at 20 °C for 48 h, than at 37 °C for 24 h and 12 °C for 1 week. The isolates had, previous to this study, been classified in two different molecular serogroups; IIa and IVb, and from the biofilm assay, significant differences were found between the biofilm forming ability for these two groups at all temperatures. Isolates belonging to serogroup IIa had higher biofilm forming ability than isolates from serogroup IVb. Unbalanced datasets were used for the t-test, which could have had an effect on the P-value. Despite this, the low P-values obtained suggests that the observed difference between serogroups is probable. A study by Kadam et al. found that incubation temperature, growth media and serotype had a significant effect on the biofilm forming ability of *L. monocytogenes* (2013). They found that serotype 1/2a had better biofilm forming ability than serotype 4b growing in nutrient-rich medium, but in nutrient-poor medium type 1/2c showed better biofilm forming ability than serotype 1/2a and 4b. The findings stating that serotype 1/2a is better than serotype 4b is also indicated in the present study. However, only the serogroup in the current study is known. The fact that biofilm formation is affected by growth medium have also been found by others (Folsom et al. 2006; Harvey et al. 2007). In food processing environments, good growth conditions are highly available for bacteria, due to food spill and insufficient cleaning. Dependent on the nutrients available, different strains or serotypes could potentially be favored. In fish slaughtering facilities, juices from blood has been shown to favor growth of *Listeria* (Rørvik et al. 1995).

As stated in the introduction, isolates commonly involved in outbreaks with listeriosis are 1/2a, 1/2c and 4b (Granum 2015). The serotypes 1/2a and 3a are classified in serogroup IIa, and 4b, 4ab, 4d and 4e in serogroup IVb (Kérouanton et al. 2009). Because serotype 1/2a and 4b are more commonly isolated in food processing facilities, it is likely that the isolates in this study belong to these. Dauphin et al. sampled isolates from three cold-smoked salmon processing plants and found that 91 % of 82 isolates belonged to serotype 1/2a (2001). This supports the findings in the current study. Serotype 1/2a was also found to be the most frequent one in a study that sampled 311 isolates of *L. monocytogenes* in food environments

over 5 years (Nucera et al. 2010). It is interesting that the isolates most commonly found in food processing environments are most often related to outbreaks with listeriosis.

4.2 TTC assay as a method for detecting viable cells after treatment with disinfectants

4.2.1 Characterizing minimum inhibitory concentration using the TTC assay

In this study it was investigated if the TTC assay was a good method for measuring eradication of biofilm bacteria after treatment with disinfectants. Six different disinfectants were applied in a twofold decreasing concentration-series from a stock solution of 0.5 % or 1% for 1 and 5 min, which was the lowest recommended concentration and incubation time from the supplier. It was expected to observe an increasing red color with increasing metabolic activity. The conversion of TTC to TPF could be seen in the microtiter plates already a few hours after incubation start. According to the assay, disinfectants varied in the concentration where effective killing of biofilm bacteria was observed. A similar effect was seen for both isolate 45 and 73. To validate the assay, two concentrations of disinfectant in which viable cells were indicated were chosen, and two concentrations indicating no viable cells. These concentrations were applied in a new TTC assay and the validity of the results were tested with a CFU count and a CV assay.

4.2.2 Detecting metabolically active cells and the efficiency of biofilm removal

The results from the new TTC assay coincided with the results from the characterization of MIC-value. Optical density was measured at 595 nm to indicate possible growth, and these results supported the theory of no metabolically active cells in the wells that had no TPF. The wells treated with 1 % MX and 1 % QA had values indicating growth. This can be explained in a reaction that occurred between the disinfectant and the neutralizing broth, giving a blue color which stained the wells.

4.2.2.1 Assessment of the TTC-assay using CFU count

Based on the results from the TTC assay, it was not expected to detect viable cells at the concentrations where no red color had been observed. However, where metabolically active cells had been indicated, a reduction in bacterial number with increasing concentration of

disinfectant was expected. Results from the CFU count showed that there were viable cells after treatment with all concentrations, except for treatment with 1 % QA and MX. All disinfectants had an effect on killing bacteria. Log cfu/biofilm was reduced with increased concentration of disinfectant, with the highest effect seen with QA and MX. Treatment of 1% concentration gave a reduction of 8.9 and 9.4 log cfu/biofilm. The treatment with disinfectants QA and OX gave higher standard deviations compared with the others. This is likely due to a low effect of some of the concentrations, giving varying results between the replicates. Before applying the disinfectants to the microtiter wells, the wells were washed with MQ-water. Pipetting was used to empty the wells, and small remains of water could have been present and possibly lead to a small dilution of the disinfectant.

PE, OX, PF and SH did not kill all bacteria even at the highest concentration being 0.5 %. A reduction of 5.8, 6.4, 6.9 and 4.1 log cfu/biofilm was observed, respectively. Even though SH had the lowest effect at 0.5 %, it was more effective at the second lowest concentration, compared to the other disinfectants. Cruz and Fletcher investigated the effect of commercial sanitizers on a 48 h biofilm of *L. monocytogenes* grown in microtiter plate (2012). They scraped the bottom of the wells with cotton wool swabs, and enumerated cells by using a drop plate count. They found that several sanitizers, among them a QAC, could not achieve a 5-log reduction of bacteria with the recommended time (5 min) and concentration suggested by the supplier. This differed from the results of treatment with QAC in this study.

Many researchers have used enumeration of cells from a biofilm to measure the effect of antibiotics or disinfectants. Most often these involve detaching cells from the biofilm by swabbing (Joseph et al. 2001; Pan et al. 2006), however, vortexing coupons with bead glass is also a common method (Giaouris et al. 2005; Kim et al. 2007). Few studies can be found where the biofilm is dislodged from microtiter wells using a cell scraper. Some of the variations seen in the CFU data could be caused by differences in the amount of bacteria dislodged from the biofilm in each replicate. When counting the colonies on BA, it was sometimes observed what looked like aggregates of colonies. To obtain the most appropriate estimate, drop platings containing 5-25 colonies were used. The aggregates could be explained by that the cells still are attached to each other from the biofilm. After scraping the biofilm, the suspension made in LB was vortexed. This may not be sufficient to dislodge the cells from each other and thus leads to aggregates when being plated. Enumerating biofilm bacteria is a well-known problem due to the fact that biofilm bacteria are adhering to each other via an extracellular matrix.

4.2.2.2 Removal of biofilm

From the biofilm assay it was of interest to see if the decrease in TTC to TPF conversion at higher concentration of disinfectants and the lower CFU count was due to removal of biofilm, or killing the biofilm bacteria. The results showed that the amount of biofilm increased with increasing concentration of disinfectant. This could indicate that the disinfectants had an effect on killing the bacteria in the biofilm, but had no effect on removing the biofilm. Screening of the biofilm forming ability in chapter 3.2.1.2, showed that isolate 73 had biofilm values about 0.5. The values obtained in the control biofilm in the CV assay were lower (about 0.2) and inconsistent with the first experiment. Because of these results, it was of interest to investigate whether the isolate's biofilm forming ability had changed during the time of which the study was carried out (results not presented). This was not the case, thus the assay suffers from a technical problem that has not yet been solved. Despite the inconclusive results from the CV assay, it seems that the effect of the disinfectants was not due to removal of biofilm, but rather to killing the biofilm bacteria.

Many researchers have investigated the susceptibility of biofilm to disinfectants, yet little research is found on assessing biofilm removal in microtiter plates with the CV assay. Peeters et al. evaluated the efficiency of biofilm disinfection and removal using the resazurin assay and the CV assay on the bacteria *Burkholderia cenocepacia* (2008b). They found no relationship between the disinfectants potential to kill biofilm bacteria and removal of biomass. The experimental design and variables differed from this study, but still the indication that some disinfectants have a minimal effect on biofilm removal is interesting. Similar results were obtained by Lee et al. where biofilm removal was investigated on *Staphylococcus aureus* (*S. aureus*) and *L. monocytogenes* by applying peracetic acid (PAA) on biofilm in microtiter plates (2016). The cells of *S. aureus* were removed, but not the adhered cells of *L. monocytogenes.*

4.2.3 TTC assay as a method for detecting viable cells in biofilm after treatment with disinfectants

Moussa et al. investigated the use of TTC to detect antibacterial activity of the fungal chitosan on planktonic cells. They stated that the absorbance of formazan was directly proportional to amount of metabolically active cells. They observed a reduction in color intensity with prolonged treatment of chitosan. A correlation was found between the absorbance of

formazan and viable cells from colony count. Their conclusion was that TTC was a promising method for detecting antimicrobial activity. In the present study differences in color intensity were also observed, which coincided with the absorbance values. However, the TTC assay failed to give an indication of the viable cells detected by the CFU count. The amount of metabolically active cells in the biofilm may not be in sufficient number to give a reduction in TTC. This could indicate that the method is more appropriate for detecting antimicrobial effect on planktonic cells rather than in biofilm. In a review by Berridge et al. it was stated that tetrazolium salts may not be able to differentiate dormant cells from dead ones, and that reduction to formazans depends on the cells growth phase. Cells that are in a viable but not culturable state (VBNC) have been postulated as a mechanism for survival due to environmental stress, and dormant cells or VBNC are often enriched in biofilms (Besnard et al. 2002; Lindbäck et al. 2010).

Based on the results from this study, the TTC assay is not considered as a good method to estimate the bactericidal effect, hence effectiveness of disinfectants on biofilm on biofilm bacteria. The TTC assay did not detect viable cells at all the concentrations, at which it was detected by plate count.

4.2.3.1 Methodological consideration

Tapping the microtiter plates on paper could be a source of contamination, both from the paper and from well to well in the same plate. The tapping method should not be used for the CFU count as a small contamination could possibly lead to big errors. An alternate, more time and material consuming-approach is to empty the wells by pipetting. Both these methods were tested in the study with similar outcomes, indicating that the potential contamination risk of the tapping method did not impact on the results, presented in this thesis.

5. Conclusion

In the first part of this study, 84 isolates of *L. monocytogenes* sampled from fish processing facilities were characterized with regard to biofilm forming ability and PFGE. Biofilm formation occurred at all temperatures in the growth medium LB. There were no biofilm formation in LB w/o NaCl. The biofilm forming ability varied between isolates having the same PFGE-type, but also between different PFGE-types. A significant difference was seen for the biofilm forming ability between serogroup IIa and IVb, at all temperatures.

For the second part of the study, the TTC assay was evaluated as a fast and simple method to measure the effect disinfectants had on killing biofilm bacteria. The results from the TTC assay indicated that the disinfectants had a good killing effect, but these results were not supported by the CFU count. The results from the CV assay were inconclusive, but indicated that the biofilm was not removed by treatment with disinfectants. Of the six disinfectants used in this study, two disinfectants killed all cells using the highest concentration. To prevent contamination of food, it is important that the chemical agents used in the cleaning procedures are efficient. The results indicate that the concentration of these disinfectants should be increased, or that the treatment time should be prolonged compared to the suppliers recommendations.

Taken together, the TTC assay is not a good method to measure eradication of biofilm bacteria after treatment with disinfectants.

6. Further research

Validation of the TTC-assay gave inconclusive results for the CV assay. It was indicated that the biofilm was not removed after treatment of disinfectant. Removal of biofilm in the food industry is important to prevent reattachment of new cells and formation of new biofilm. For further research, it would be interesting to investigate the effect disinfectants have on dissolving and removing biofilm, and if this effect is influenced by the structure and components in the biofilm.

7. References

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Appendix 1

Composition for media used in the study produced at the Norwegian Veterinary Institute.

Heart infusion buljong (HIB)

Bacto Heart Infusion Broth	25.0 g
Destilllert vann	1.0 g

Dey-Engley neutralizing Broth

Casein Enzymic Hydrolysate	5.0 g/l
Yeast extract	2.5 g/l
Dextrose	10.0 g/l
Sodium Thiosulfate	6.0 g/l
Sodium Thiogllycollate	1.0 g/l
Sodium Bisulphite	2.5 g/l
Lecithin	7.0 g/l
Polysorbate	5.0 g/l
Bromo Cresol Purple	0.02 g/l

Crystal Violet 0.1 % solution

Crystal Violet, 92 %	1.09 g
Destillated water	1000 mL

Luria Bertoni broth (LB)

(bacto)Tryptone	5 g
Yeast extract	2.5 g
NaCl	2.5 g
Destillated water	500 ml

Adjusted to pH 7.5

Luria Bertoni broth w/o NaCl

(bacto)Tryptone	5 g
Yeast extract	2.5 g
Destillated water	500 ml

Trypton soya buljong (TSB)

Tryptic Soy Broth	30.0 g
Tryptone Soy Broth	30.0 g
CASO Broth	30.0 g
Destillated water	1.0 L

Appendix 2

Modified protocol for pulsed-field gel electrophoresis (PFGE).

Molekylær typing av *Listeria monocytogenes* stammer med PFGE

1 Anvendelsesområde

Molekylær subtyping av Listeria monocytogenes med Pulse-Field Gel Electrophoresis (PFGE). PFGE er en fingerprinting metode som brukes for å kunne sammenligne stammer innenfor samme art.
Denne metoden kan brukes til å sammenligne stammer innad ett eller mellom flere laboratorier.

2 Referanser

2.1 Molecular typing of Listeria monocytogenes strains isolated from food, feed and animals: state of play and standard operating procedures for pulse field gel electrophoresis (PFGE) typing, profile interpretation and curation. Roussel et. al. ANSES. EFSA supporting publication 2014:EN-702.

2.2 Molekylær typing av *Salmonella sp*. MExx_xxx

3 Definisjoner

4 Prinsipp

Bakterier blir støpt inn i gelplugger og lysert, deretter blir cellerestene vasket. Bakterienes kromosomale DNA blir så kuttet med restriksjonsenzymer som gjenkjenner spesifikke sekvenser på DNA. Dette genererer store DNA-fragmenter av ulik størrelse. DNA-fragmentene kan, på grunn av størrelsen, ikke separeres fra hverandre ved konvensjonell elektroforese. Ved å periodisk forandre retningen av det elektriske feltet over en agarosegel (pulsfelt gelelektroforese) kan man effektivt separere DNA-fragmentene etter størrelse. Hvor langt de ulike DNA fragmentene vandrer er avhengig av størrelsen. De korteste vandrer fortest og derfor lengst, de lengste bruker lengre tid på å forflytte seg og vandrer derfor kortere i løpet av kjøretiden. På den måten vil ulike stammer få ulike profiler "fingeravtrykk" avhengig av hvor restriksjonsenzymene har kuttet DNAet.

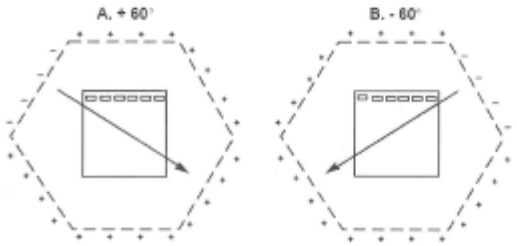

Figure 1.1. Voltage clamping by the CHEF-DR III system. A. Relative electrode potentials when the + 60° field vector is activated. **B.** Relative electrode potentials when the - 60° field vector is activated.

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5 Kontroll av laboratoriemiljøet

6 Advarsler/sikkerhetstiltak

6.1 *Listeria monocytogenes* er patogen og kan forårsake alvorlig sykdom hos foster, eldre og personer med nedsatt immunforsvar.

7 Kjemikalier, løsninger, medier og reagenser

Sammensetning, tillaging og kvalitetskontroll - se Mediekatalog for Veterinærinstituttet (KA M xxxx).

	Navn (forkortelse)	Referanse
7.1	Blodagar (BA)	KA M 0022
7.2	Sterilt MQ-vann	(50.002.02)
7.3	TE-buffer (Tris 10 mM, EDTA 1 mM, pH 8(± 0,2))	(30.119.01)
7.4	20 % SDS	(30.110.03)
7.5	1M Tris, pH 8(± 0,2)	(30.119.00)
7.6	1 mg/ml BSA	(Fermentas #B14)
7.7	250 mM EDTA, pH 8(± 0,2)	(30.018.06)
7.8	10 % Sarcosine (= Sarcosyl (N-Lauroylsarcosine, Sodium salt)	
7.9	SeaKem Gold agarose (SKG agarose)	(AG4163)
7.10	20 mg/ml Proteinase K (løst i MQ, kan kun fryses en gang)	(Sigma P2308)
7.11	10 mg/ml Lysozyme (løst i MQ, kan kun fryses en gang)	(Sigma L6876)
7.12	H-buffer	(11417991001, Roche(Sigma))
7.13	*XbaI*-restriksjonsenzym	(10674265001, Roche(Sigma))
7.14	NE4 Buffer	(B7004S, NEB (Bionordica))
7.15	*AscI*- restriksjonsenzym	(R0558S, NEB (Bionordica))
7.16	Buffer A	(11417959001, Roche(Sigma))
7.17	*ApaI*- restriksjonsenzym	(10899208001, Roche(Sigma))
7.18	10X TBE,	(1666703001, Roche)
7.19	Gelred	(730-2958, VWR)

8 Instrumenter og utstyr

8.1 Spektrofotometer: GeneQuant pro
8.2 1 ml engangs kyvetter til spektrofotometer.
8.3 Varmeblokk med risting til 50 ml falconrør (vi har plass til 8 om gangen).
8.4 Varmeblokk til eppendorfrør.
8.5 Vannbad: 50 °C og 55-60 °C
8.6 Grønne silkorker.
8.7 PFGE-maskin: CHEF-DR III

9 Arbeidsbeskrivelse

9.1 Så ut stammene som skal analyseres og referansestamme Listeria monocytogenes H2446 (VI 51269) på BA. Innkuber i 37 °C i 18-48 t.
9.2 Overfør til ny BA. Innkuber i 37 °C i 14-18 t.

9.3 Pluggstøping:

9.3.1 Lag SSP-gel: Se vedlegg 1. La stå i 50 °C vannbad.
9.3.2 Pluggformer tapes. Sett på en så lang tape at den kan merkes med stammenummer.
9.3.3 Ta noen kolonier fra BA-skålen over i reagensrør med 2 ml TE-buffer. Bland godt.
9.3.4 Overfør til kyvetter og mål konsentrasjonen på spektrofotometer. Optisk tetthet (OD) ved 600 nm skal være mellom 1,6 og 1,8. Juster med bakterie-kultur eller TE-buffer.
9.3.5 240 µl justert bakterieløsning overføres til 1,5 ml eppendorfrør.
9.3.6 Tilsett 60 µl 10 mg/ml lysozym. Bland forsiktig opp og ned for å ikke skjære cellene istykker. Innkuber i varmeblokk ved 37 °C i 10 min.
9.3.7 Tilsett 300 µl SSP-gel til bakterieløsningen, bruk 1 ml spisser med stort hull. Bland godt, men forsiktig slik at ikke DNAet skjæres i biter.
9.3.8 Umiddelbart etter overføres blandingen til pluggformene. Lag 3-5 plugger per stamme. Pass på at det ikke dannes luftbobler.
9.3.9 La gel-pluggene stivne i minst 10-15 minutter ved romtemperatur, eller 5-10 minutter i kjøleskap.

9.4 Cellelysis i pluggene

9.4.1 Lag lysisbuffer: Se vedlegg 2. Fordel på 50 ml falconrør, 4 ml/rør, 1 rør per stamme.
9.4.2 Fjern tapen fra pluggformene. Bruk en 1 µl hvit podeøse til å dytte pluggene ut av formene og ned i lysisbufferen. Pass på at pluggene er i væsken.
9.4.3 Skru på de grønne silkorker på falconrørene og de blå korkene på toppen.
9.4.4 Inkuber ved 37 °C i varmeblokk med forsiktig risting, (110-115 rpm) vi bruker 300 rpm, i 2 timer.

9.5 Vasking pluggene etter cellelysis.

9.5.1 Forvarm MQ-vann og TE-buffer i 50 °C vannbad.
9.5.2 Skru av de blå korkene.
9.5.3 Hell av lysisbufferen gjennom silkorken. Tørk av den siste dråpen på papir.

9.5.4 Vasking med MQ-vann:
9.5.4.1 Tilsett 15-20 ml MQ-vann (50 °). Pass på at pluggene er i væsken.
9.5.4.2 Inkuber ved 50 °C i varmeblokk med forsiktig risting, (110-115 rpm) vi bruker 300 rpm, i 10 minutter.
9.5.4.3 Hell av vannet gjennom silkorken. Tørk av den siste dråpen på papir.
9.5.4.4 Gjenta vasketrinnet.

9.5.5 Vasking med TE-buffer:
9.5.5.1 Tilsett 10-15 ml TE-buffer (50 °). Pass på at pluggene er i væsken.
9.5.5.2 Inkuber ved 50 °C i varmeblokk med forsiktig risting, (110-115 rpm) vi bruker 300 rpm, i 15 minutter.
9.5.5.3 Hell av TE-bufferen gjennom silkorken. Tørk av den siste dråpen på papir.
9.5.5.4 Dette vasketrinnet gjentas tre ganger.
9.5.6 Overfør pluggene til eppendorfrør med 1 ml TE-buffer. Pass på at pluggene er helt nede i væsken.
9.5.7 Oppbevares ved 5±3 °C.

9.6 Kutting med restriksjonsenzym av DNA i pluggene.

Viktig! Sammen med restriksjonsenzymkutting av stammene som skal testes, skal det også foretas restriksjonsenzymkutting av referansestammen som er støpt sammen med stammene, og på en gammel plugg med referansestamme som har gitt godkjent resultat før. Dessuten skal plugger med *Salmonella braenderup* H9812 (VI51270), 1 per hver 5 stamme + 1 kuttes med *Xba*I, dette er størrelsesmarkøren.

9.6.1 *S. braenderup* H9812-stamme: kutting med restriksjonsenzym *Xba*I.

Restriksjons-sete:

T▼CTAG A
A GATC▲T

9.6.1.1 Kutt 2 mm av en plugg med en skalpell på et objektglass.
9.6.1.2 Plasser biten i eppendorfrør med 100 µl miks A (løsning uten enzym *Xba*I, se vedlegg 3). Pass på at biten er helt nedi væsken.
9.6.1.3 Innkuber ved 37 °C varmeblokk i 10 min.
9.6.1.4 Fjern all væske med en pipette, vær forsiktig slik at ikke biten skades.
9.6.1.5 Tilsett 100 µl med miks B (løsning med *Xba*I, se vedlegg 3). Pass på at biten er helt nedi væsken.
9.6.1.6 Innkuber ved 37 °C varmeblokk i 4 timer.
9.6.1.7 Valgfritt: Fjern restriksjonenzymløsningen, og erstatt med 200 µl 0,5 X TBE-buffer.

9.6.2 Test-stammene og *L. monocytogenes* H9812-referansestamme: kutting med restriksjonsenzym *Asc*I.

Restriksjons-sete:

GG▼CGCGCC
CC GCGC▲GG

9.6.2.1 Kutt 2 mm av en plugg med en skalpell på et objektglass.
9.6.2.2 Plasser biten i eppendorfrør med 100 µl miks A (løsning uten enzym *Asc*I, se vedlegg 4). Pass på at biten er helt nedi væsken.
9.6.2.3 Innkuber ved 37 °C varmeblokk i 10 min.
9.6.2.4 Fjern all væske med en pipette, vær forsiktig slik at ikke biten skades.
9.6.2.5 Tilsett 100 µl med miks B (løsning med *Asc*I, se vedlegg 4). Pass på at biten er helt nedi væsken.
9.6.2.6 Innkuber ved 37 °C varmeblokk i 4 timer.
9.6.2.7 Valgfritt: Fjern restriksjonenzymløsningen, og erstatt med 200 µl 0,5 X TBE-buffer.

9.6.3 Test-stammene: Kutting med restriksjonsenzym *Apa*I.

Restriksjons-sete:

G GGCC▼C
C▲CCGG G

9.6.3.1 Kutt 2 mm av en plugg med en skalpell på et objektglass.
9.6.3.2 Plasser biten i eppendorfrør med 100 µl miks A (løsning uten enzym *Apa*I, se vedlegg 5). Pass på at biten er helt nedi væsken.
9.6.3.3 Innkuber ved 30 °C varmeblokk i 10 min.

9.6.3.4 Fjern all væske med en pipette, vær forsiktig slik at ikke biten skades.
9.6.3.5 Tilsett 100 µl med miks B (løsning med *Apa*I, se vedlegg 5). Pass på at biten er helt nedi væsken.
9.6.3.6 Innkuber ved 30 °C varmeblokk i 4 timer.
9.6.3.7 Valgfritt: Fjern restriksjonenzymløsningen, og erstatt med 200 µl 0,5 X TBE-buffer.

9.7 Forberedelse av gelkaret og støping av agarose gel:
9.7.1 Lag 2 L 0,5 x TBE-buffer.
9.7.2 Lag 150 ml 1 % SKG agarose i 0,5 x TBE-buffer (30-brønners gel) eller 100 ml 1 % SKG agarose i 0,5 x TBE-buffer (15-brønners gel). La stå i 55-60 °C vannbad 15 min før støping av gelen.
9.7.3 Lag 10 ml 0,8 % SKG agarose i 0,5 x TBE-buffer, til forsegling av brønnene. La stå i 55-60 °C vannbad.
9.7.4 Sjekk at elektroforesekaret er i vater.
9.7.5 Ha bufferen som er igjen etter å ha lagd agarose-gel i elektoforesekaret.
9.7.6 Slå på PFGE-maskina og deretter på pumpa. Vent til bufferen sirkulerer gjennom hele systemet. Sjekk at det ikke er noen bobler. Slå tilslutt på kjøleren (14 °C).
9.7.7 Sett sammen gelformen (stor eller liten), sjekk at den er i vater. Sett kam i formen (15 brønner til liten gel og 30 brønner til stor gel).
9.7.8 Hell 1 % agarosegelen forsiktig i gelformen. Eventuelle luftbobler må fjernes. La stivne i minst 30 min.
9.7.9 Fjern kammen forsiktig. Fyll brønnene med 0,5 x TBE buffer.
9.7.10 Plasser pluggene forsiktig ned i brønnene, bruke 2 spatler eller hvite podeøser. Unngå at det blir luftbobler i brønnene.
9.7.10.1 Plasser plugger av størrelsesmarkøren *S. braenderup* H9812 (VI51270) i hver 5-6 brønn, dessuten i første og siste brønn.
9.7.11 Forsegl brønnene med 0,8 % SKG agarose.
9.7.12 Fjern gelen fra formen, men behold den svarte platen. Tørk overskudds agarose av undersiden og kantene på den svarte platen.
9.7.13 Plasser gelen og den svarte platen ned i platformen i elektroforeskammeret. Lukk lokket.

9.8 Elektroforesen.
9.8.1 Volts/cm = 6
9.8.2 Included angle = 120
9.8.3 Temperature = 14 °C
9.8.4 Block 1: Initial switch time = 4 sec
Final switch time = 40 sec
9.8.5 Pumpen skal stå på 70 L/min
9.8.6 For 14 x13 cm (15 brønner) gel, vandringstid = 18-19 t.
For 21 x14 cm (30 brønner) gel, vandringstid = 20-21 t.
9.8.7 Trykk run for å starte kjøringen. Strømstyrken skal være ca 120 mA ved start, og ikke overstige 160 mA ved slutt.

9.9 Farging og dokumentering av gelen
9.9.1 Tilsett 120 µl GelRed™ til 400 ml MQ-vann.
9.9.2 Helles i en bakke, plasser gelen forsiktig ned.

9.9.3 Gelen farges i 20-30 min på et vippebrett.
9.9.4 Gelen visualiseres i UV-lys kammer (GelDoc), bilde tas og lagres for videre bearbeiding. Lagre som TIFF for å kunne jobbe med det i BioNumerics.
9.9.5 Hvis det er for mye bakgrunn på bilde, avfarges gelen i MQ-vann i 15-30 min.

10 Resultatberegning og – rapportering

10.1 Hvis alle test-stammene som skal sammenlignes kjørt på samme gel, kan disse sammenlignes og resultat angis ut i fra dette som PFGE-profiler. Hver profil gis et nummer. Henvisning til bilde av gelen er en del av profilnavnet og deretter fortløpende nummer. F.eks: 20160819-1, 20160819-2 osv, der 20160819 er datoen bilde av gelen er tatt og -1, -2 osv er de ulike profilene på den enkelte gelen. Profil ååååmmdd-1 er den første profilen som dukker opp fra venstre etter størrelsesmarkører og referansestamme (når brønnene er øverst).
10.2 Hvis profiler fra ulike geler skal sammenlignes, eller stammeprofilene skal sendes inn til EURL sin Listeria monocytegenes PFGE database, må videre bearbeiding utføres i BioNumerics.

11 Etterlagring av prøvemateriale

11.1 Stammene fryses ned i VI-stammearkiv i to paralleller.

12 Avfallshåndtering

13 Vedlegg

13.1 Vedlegg 1: Tillaging av SSP gel
13.2 Vedlegg 2: Tillaging av Lysis buffer
13.3 Vedlegg 3: Tillaging av restriksjons-løsning: *XbaI*
13.4 Vedlegg 4: Tillaging av restriksjons-løsning: *AscI*
13.5 Vedlegg 5: Tillaging av restriksjons-løsning: *ApaI*
13.6 Vedlegg 6: Flytskjema av prosedyren
13.7 Vedlegg 7: Oversikt over stammer og resultater OD_{600}
13.8 Vedlegg 8: Oppsett til 30-brønnergel.

14 Dokumentlogg

15 Utgave nr	*16 Dato*	*17 Punkt*	*18 Forandringer fra forrige utgave*
19	20	21	22
23	24	25	26

Vedlegg 1: SSP-gel

Alle løsninger lages til en stamme mer enn man trenger.
La stå i 50 °C vannbad/varmeblokk.

Antall stammer	20 % SDS (µl) Forvarmet til 50 °C	1,2 % SeaKem Gold agarose (µl)*	20 mg/ml Proteinase K (µl)
1	15	282	3
2	30	564	6
3	45	846	9
4	60	1128	12
5	75	1410	15
6	90	1692	18
7	105	1974	21
8	120	2256	24
9	135	2538	27
10	150	2820	30
11	165	3102	33
12	180	3384	36
13	195	3666	39
14	210	3948	42
15	225	4230	45
16	240	4512	48
17	255	4794	51
18	270	5076	54
19	285	5358	57
20	300	5640	60
21	315	5922	63
22	330	6204	66
23	345	6486	69
24	360	6768	72
25	375	7050	75
26	390	7332	78
27	405	7614	81
28	420	7896	84
29	435	8178	87
30	450	8460	90

*1,2 % SeaKem Gold agarose: Løs f.eks 0,3 g SeaKem Gold agarose i 25 ml MQ-vann i et falconrør ved å varme i mikrobølgeovn. Først 30 sek, deretter 10 og 10 sek til det er løst opp.

Vedlegg 2: Lysisbuffer

Alle løsninger lages til en stamme mer enn man trenger.

Antall stammer	1M Tris, pH 8 (ml)	250 mM EDTA, pH 8 (ml)	10 % L-lauryl sarcosine (ml)	MQ-vann (ml)	20 mg/ml Proteinase K (µl)
1	0,2	0,8	0,4	2,57	30
2	0,4	1,6	0,8	5,14	60
3	0,6	2,4	1,2	7,71	90
4	0,8	3,2	1,6	10,28	120
5	1,0	4,0	2,0	12,85	150
6	1,2	4,8	2,4	15,42	180
7	1,4	5,6	2,8	17,99	210
8	1,6	6,4	3,2	20,56	240
9	1,8	7,2	3,6	23,13	270
10	2,0	8,0	4,0	25,70	300
11	2,2	8,8	4,4	28,27	330
12	2,4	9,6	4,8	30,84	360
13	2,6	10,4	5,2	33,41	390
14	2,8	11,2	5,6	35,98	420
15	3,0	12,0	6,0	38,55	450
16	3,2	12,8	6,4	41,12	480
17	3,4	13,6	6,8	43,69	510
18	3,6	14,4	7,2	46,26	540
19	3,8	15,2	7,6	48,83	570
20	4,0	16,0	8,0	51,40	600
21	4,2	16,8	8,4	53,97	630
22	4,4	17,6	8,8	56,54	660
23	4,6	18,4	9,2	59,11	690
24	4,8	19,2	9,6	61,68	720
25	5,0	20,0	10,0	64,25	750
26	5,2	20,8	10,4	66,82	780
27	5,4	21,6	10,8	69,39	810
28	5,6	22,4	11,2	71,96	840
29	5,8	23,2	11,6	74,53	870
30	6,0	24,0	12,0	77,10	900

Vedlegg 3: *Xba*I

Alle løsninger lages til en stamme mer enn man trenger.

Antall stammer	MQ-vann (µl)	10 x buffer H (µl)	1 mg/ml BSA (µl)	Enzym *Xba*I - 10 U/µl (µl)*
1	82,5	10	6,5	1
2	165,0	20	13,0	2
3	247,5	30	19,5	3
4	330,0	40	26,0	4
5	412,5	50	32,5	5
6	495,0	60	39,0	6
7	577,5	70	45,5	7
8	660,0	80	52,0	8
9	742,5	90	58,5	9
10	825,0	100	65,0	10
11	907,5	110	71,5	11
12	990,0	120	78,0	12
13	1072,5	130	84,5	13
14	1155,0	140	91,0	14
15	1237,5	150	97,5	15
16	1320,0	160	104,0	16
17	1402,5	170	110,5	17
18	1485,0	180	117,0	18
19	1567,5	190	123,5	19
20	1650,0	200	130,0	20
21	1732,5	210	136,5	21
22	1815,0	220	143,0	22
23	1897,5	230	149,5	23
24	1980,0	240	156,0	24
25	2062,5	250	162,5	25
26	2145,0	260	169,0	26
27	2227,5	270	175,5	27
28	2310,0	280	182,0	28
29	2392,5	290	188,5	29
30	2475,0	300	195,0	30

*Miks A lages uten enzym, miks B lages med enzym, ellers like.

Vedlegg 4: *Asc*I

Alle løsninger lages til en stamme mer enn man trenger.

Antall stammer	MQ-vann (µl)	10 x NE4 buffer (µl)	1 mg/ml BSA (µl)	Enzym *Asc*I - 10 U/µl (µl)*
1	83	10	6,5	0,5
2	166	20	13,0	1,0
3	249	30	19,5	1,5
4	332	40	26,0	2,0
5	415	50	32,5	2,5
6	498	60	39,0	3,0
7	581	70	45,5	3,5
8	664	80	52,0	4,0
9	747	90	58,5	4,5
10	830	100	65,0	5,0
11	913	110	71,5	5,5
12	996	120	78,0	6,0
13	1079	130	84,5	6,5
14	1162	140	91,0	7,0
15	1245	150	97,5	7,5
16	1328	160	104,0	8,0
17	1411	170	110,5	8,5
18	1494	180	117,0	9,0
19	1577	190	123,5	9,5
20	1660	200	130,0	10,0
21	1743	210	136,5	10,5
22	1826	220	143,0	11,0
23	1909	230	149,5	11,5
24	1992	240	156,0	12,0
25	2075	250	162,5	12,5
26	2158	260	169,0	13,0
27	2241	270	175,5	13,5
28	2324	280	182,0	14,0
29	2407	290	188,5	14,5
30	2490	300	195,0	15,0

*Miks A lages uten enzym, miks B lages med enzym, ellers like.

Vedlegg 5: *Apa*I

Alle løsninger lages til en stamme mer enn man trenger.

Antall stammer	MQ-vann (µl)	10 x buffer A (µl)	1 mg/ml BSA (µl)	Enzym *Apa*I - 10 U/µl (µl)*
1	82,5	10	6,5	1
2	165,0	20	13,0	2
3	247,5	30	19,5	3
4	330,0	40	26,0	4
5	412,5	50	32,5	5
6	495,0	60	39,0	6
7	577,5	70	45,5	7
8	660,0	80	52,0	8
9	742,5	90	58,5	9
10	825,0	100	65,0	10
11	907,5	110	71,5	11
12	990,0	120	78,0	12
13	1072,5	130	84,5	13
14	1155,0	140	91,0	14
15	1237,5	150	97,5	15
16	1320,0	160	104,0	16
17	1402,5	170	110,5	17
18	1485,0	180	117,0	18
19	1567,5	190	123,5	19
20	1650,0	200	130,0	20
21	1732,5	210	136,5	21
22	1815,0	220	143,0	22
23	1897,5	230	149,5	23
24	1980,0	240	156,0	24
25	2062,5	250	162,5	25
26	2145,0	260	169,0	26
27	2227,5	270	175,5	27
28	2310,0	280	182,0	28
29	2392,5	290	188,5	29
30	2475,0	300	195,0	30

*Miks A lages uten enzym, miks B lages med enzym, ellers like.

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