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The effect of cold atmospheric plasma on biofilms developed by *L. monocytogenes* and *S.* Typhimurium

Laurens VERGAUWEN

Promotor: Prof. Dr. ir. Jan Van Impe

Co-promotoren: ing. Marlies Govaert

Dr. ir. Cindy Smet

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Foreword

First, I would like to thank Prof. Dr. ir. Jan Van Impe for giving me the chance to work on this project.

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For the moral support and relaxation during this project, I would like to thank my parents and my girlfriend.

Samenvatting

Het doel van dit onderzoek was het bestuderen van het effect van koud atmosferisch plasma op biofilms ontwikkeld door *S.* Typhimurium en *L. monocytogenes*. Om dit effect te onderzoeken, werden twee verschillende elektrodes getest, nl. de 'dielectric-barrier discharge' (DBD) en de 'surface dielectric-barrier discharge' elektrode (SBD), elk in combinatie met drie verschillende samenstellingen van het gebruikte voedingsgas, nl. puur heliumgas, heliumgas met 0,5% (v/v) zuurstofgas en heliumgas met 1,0% (v/v) zuurstofgas. Elk van deze combinaties werd getest voor inactivatie van biofilms ontwikkeld door *S.* Typhimurium en *L. monocytogenes* op een abiotisch oppervlak (in een petrischaal). Voor elke test werd het koud atmosferisch plasma (KAP) gegenereerd bij een inputvoltage van 13,88 V en een frequentie van 15 kHz. De biofilms werden behandeld tot 30 minuten lang, gedurende vastgelegde intervallen. Na de behandeling met KAP werden de cellen van de overblijvende biofilm teruggewonnen en werd een 'viable plate counting' (tellen van levensvatbare kolonies) uitgevoerd op zowel het algemene als het selectieve medium. Door op beide media uit te platen, was het mogelijk het percentage subletaal letsel (sublethal injury, SI) in functie van de KAP-behandelingstijd te bestuderen. De resultaten van de 'viable plate counting' werden gefit volgens het predictieve model dat door Geeraerd et al. (2000) ontwikkeld werd. De resulterende inactivatiecurves toonden geen schouderfase, maar de staartfase was altijd aanwezig. De optimale combinatie van elektrode en type voedingsgas was de DBD-elektrode in combinatie met puur heliumgas. Bij deze combinatie werden log-reducties van 2,4 ± 0,2 en 2,3 ± 0,2 (op algemeen medium) bekomen, en dit binnen 5 minuten KAP-behandelingstijd voor respectievelijk *S.* Typhimurium en *L. monocytogenes.* Bij het bepalen van het subletaal letsel werd een algemeen verloop van het percentage SI in functie van de behandelingstijd waargenomen, nl. een stijging in SI in het begin van de behandeling, gevolgd door een maximum, waarna de SI weer daalt naar een bepaalde waarde.

Trefwoorden: Biofilms, koud atmosferisch plasma, *Listeria monocytogenes*, *Salmonella* Typhimurium

Abstract

The goal of this research was to study the effect of cold atmospheric plasma on biofilms developed by *S.* Typhimurium and *L. monocytogenes*. To study this effect, two different electrodes were tested, i.e., the DBD and SBD electrode, each in combination with three different types of feed gases, i.e., pure helium gas, helium gas with an addition of 0.5% and helium gas with 1.0% (v/v) oxygen. Each of these combinations was tested for inactivation of both *S.* Typhimurium and *L.* monocytogenes model biofilms developed on an abiotic surface (petri dish). For each test, the cold atmospheric plasma (CAP) was generated at an input voltage of 13.88 V and a frequency of 15 kHz and biofilms were treated up to 30 minutes using fixed intervals. After the CAP treatment, cells were recovered from the remaining biofilm, and viable plate counting was performed on both general and selective media. By plating on both media, it was possible to study the percentage of sublethally injured cells as function of the CAP treatment time. The results of the viable plate counting were fitted with the predictive model developed by Geeraerd et al. (2000). The resulting inactivation curves showed no shoulder phase, but the tailing phase was always present. The optimal combination of electrode and type of feed gas was the DBD electrode in combination with pure helium gas. Using this combination, overall log reductions of 2.4 ± 0.2 and 2.3 ± 0.2 (on general media) were achieved within 5 minutes of CAP treatment for *S.* Typhimurium and *L. monocytogenes* biofilms respectively. For the sublethal injury (SI), a general trend of the percentage of SI as function of the treatment time was observed, i.e., there was an increase in sublethal damage at the beginning of the treatment, followed by a maximum, after which the sublethal damage decreases again to a residual value.

Keywords: Biofilms, Cold atmospheric plasma, *Listeria monocytogenes*, *Salmonella* Typhimurium

Wetenschappelijke samenvatting

Een biofilm is een samenleving van bacteriën die omgeven is door een extracellulaire matrix die bescherming biedt tegen verschillende omgevingsfactoren. Biofilms zijn een significant probleem in de voedingsindustrie waar ze kunnen voorkomen op omgevingsoppervlakken (vb. muren en vloeren), oppervlakken die direct in contact staan met voeding (vb. transportbanden) en op het oppervlak van de voedingsproducten zelf. Wanneer een biofilm aanwezig is, kan deze aanleiding geven tot contaminatie van voedingsproducten met bederfbacteriën en/of pathogene bacteriën. Wanneer het eindproduct met deze bacteriën besmet zou zijn, kan dit respectievelijk aanleiding geven tot economische verliezen en/of risicovol zijn voor de volksgezondheid.

Het verwijderen van deze biofilms is echter niet eenvoudig wegens het beschermend effect van de extracellulaire matrix. Conventionele methoden maken gebruik van oxiderende chemicaliën, zoals perazijnzuur of natriumhypochloriet, en zijn veelal onvoldoende efficiënt. Daarnaast brengt het gebruik van chemicaliën nog enkele andere nadelen met zich mee, zoals corrosie, toxiciteit, residuen die kunnen achterblijven, het gebruik van water, en het species-specifieke karakter. Het gebruik van actieve chloor (natriumhypochloriet) kan bijvoorbeeld corrosie teweegbrengen en is efficiënter tegen gramnegatieve dan tegen grampositieve bacteriën. Door deze nadelen worden er nieuwe technologieën onderzocht voor de verwijdering van biofilms, zoals het gebruik van koud atmosferisch plasma (KAP).

Koud atmosferisch plasma is een geïoniseerd gas, bestaande uit reactieve deeltjes zoals ionen, elektronen, reactieve zuurstof- en stikstofverbindingen en UV-fotonen. De ionisatie van het gas wordt in dit onderzoek tot stand gebracht met behulp van elektrische ontlading. Deze ontlading wordt gerealiseerd door een alternerende stroom met een hoge frequentie en een hoog voltage over een elektrode te sturen.

In deze thesis werden twee types elektroden getest, de 'dielectric-barrier discharge' (DBD) en de 'surface barrier discharge' (SBD) elektrode. Naast het effect van het type elektrode op de inactivatie van biofilms werd ook de gassamenstelling bestudeerd. Hierbij werden puur heliumgas en twee mengsels van heliumgas met zuurstof (He + 0,5/1,0% (v/v) O_2) getest. Het gas werd bij ieder experiment geïoniseerd bij een inputvoltage van 13,88 V en een frequentie van 15 kHz. Iedere combinatie van elektrode en zuurstofpercentage werd uitgetest op biofilms ontwikkeld door zowel *Listeria monocytogenes* als door *Salmonella* Typhimurium. Dit zijn twee belangrijke voedselpathogenen omwille van de hoge abundantie van *Salmonella* en de hoge letaliteit van *Listeria* (*monocytogenes*).

De invloed van de behandelingstijd werd nagegaan door 10 behandelingstijden te hanteren binnen een bereik van 0 tot 30 minuten. Deze behandelingstijden werden voor iedere combinatie van elektrode en zuurstofpercentage getest. Na de behandeling met KAP werden de cellen in de behandelde biofilm teruggewonnen (met behulp van een celschraper) en vervolgens verdund volgens een decimale verdunningsreeks. Iedere verdunning werd uitgeplaat op een selectief en een niet-selectief medium, zodat het percentage van subletaal verwonde cellen kon bepaald worden. De resultaten van de tellingen werden vervolgens gefit met het primair predictief schouder-staart type inactivatie model dat ontwikkeld werd door Geeraerd et al. (2000).

De inactivatiecurves die werden verkregen door de resultaten te fitten met het model van Geeraerd et al. (2000) vertoonden geen schouderfase, maar wel steeds een staartfase. De afwezigheid van de schouderfase impliceert dat vanaf het moment dat de behandeling gestart wordt, er inactivatie/beschadiging van cellen in de biofilm plaatsvindt. De aanwezigheid van de staart toont de aanwezigheid aan van een subpopulatie die meer resistent is tegen de KAP-behandeling.

De resultaten wezen eveneens uit dat het effect van het gebruik van verschillende elektroden op de efficiëntie van de KAP-behandeling afhankelijk is van de gebruikte gassamenstelling. Zo werd er gevonden dat wanneer er puur heliumgas gebruikt wordt, de DBD-elektrode efficiënter is (doordat er hogere reducties verkregen werden, of een eenzelfde reductie sneller behaald werd). Echter, wanneer er heliumgas werd gebruikt met 0,5% of 1,0% (v/v) zuurstof, dan werd er geen uniforme trend waargenomen. Voor de invloed van het type voedingsgas op de efficiëntie van de KAP-behandeling kon echter een semi-uniform verband vastgesteld worden. Over het algemeen bleek namelijk dat het gebruik van puur heliumgas tot hogere reducties leidt dan wanneer er 0,5% of 1,0% (v/v) zuurstof werd toegevoegd. Echter, bij het gebruik van deze laatste twee gassamenstellingen werden geen significante verschillen bekomen wanneer de SBD-elektrode werd gebruikt en in het geval van de DBD-elektrode, werden er tegenstrijdige resultaten bekomen. Er werden namelijk hogere reducties bekomen voor *S.* Typhimurium biofilms wanneer de DBD-elektrode werd gebruikt in combinatie met heliumgas met 0,5% (v/v) zuurstof, in vergelijking met wanneer heliumgas met 1,0% (v/v) zuurstof werd gebruikt, terwijl het omgekeerde waargenomen werd bij behandeling van *L. monocytogenes* biofilms onder dezelfde omstandigheden.

De meest efficiënte behandelingen werden bijgevolg verkregen wanneer de DBD-elektrode gebruikt werd met puur helium gas. Bij gebruik van deze combinatie werden voor de *S.* Typhimurium en *L. monocytogenes* biofilms $\log_{10}$-reducties behaald van respectievelijk 2,4 ± 0,2 en 2,3 ± 0,2, en dit voor beiden binnen een behandelingstijd van 5 minuten. Deze behaalde $\log_{10}$-reducties zijn echter niet hoog genoeg om voedselveiligheid te kunnen garanderen. Aan de positieve kant, de finale (relatief lage) reducties worden snel behaald. Op industriële schaal komt dit neer op korte periodes van 'equipment downtime'. Vooraleer deze technologie echter gerealiseerd kan worden op industriële schaal, moeten er hogere $\log_{10}$-reducties behaald worden. Om deze hogere reducties te bekomen, kunnen er bijvoorbeeld hogere voltage levels getest worden. Daarnaast zou het ook mogelijk zijn om eventuele voor- en/of nabehandeling te implementeren (bv. een voorbehandeling met een verdunde waterstofperoxide oplossing).

Dat een gelijkaardige $\log_{10}$-reductie bekomen werd voor de *S.* Typhimurium en *L. monocytogenes* biofilm, onder de bovenstaande condities, suggereert dat onder deze condities de KAP-behandeling even efficiënt is tegen grampositieve (*L. monocytogenes*) en gramnegatieve (*S.* Typhimurium) species. Om deze indicatie te bevestigen moeten er echter een grotere variatie aan grampositieve en gramnegatieve species getest worden.

Aan de hand van de resultaten van het subletaal letsel kon vastgesteld worden dat er reeds subletaal verwonde cellen aanwezig zijn in een biofilm alvorens de behandeling gestart werd. Gedurende de behandeling kon een algemene trend van het percentage subletaal letsel in functie van de behandelingstijd vastgesteld worden, namelijk een stijging gevold door een maximum, waarna terug een daling plaatsvond tot een residuele waarde bereikt werd. Deze trend suggereert een mechanisme van accumulatie aan subletale letsels, die vervolgens leidt tot celdood (Noriega et al., 2013). Tussen het gebruik van verschillende elektroden en het

gebruik van de DBD-elektrode in combinatie met de verschillende types geteste voedingsgassen, zijn de resultaten met betrekking tot de subletale letsels niet eenduidig. Echter, bij het gebruik van de SBD-elektrode in combinatie met de verschillende geteste voedingsgassen kon er wel een verband vastgesteld worden. Met deze elektrode werd namelijk het hoogste residuele percentage aan subletaal letsel bekomen bij het gebruik van helium met een additie van 0,5% (v/v) zuurstof, gevolgd door het residuele percentage bij gebruik van puur heliumgas, en de laagste residuele waarde werd bekomen wanneer helium gas met een additie van 1,0% (v/v) zuurstof gebruikt werd. Ten slotte dient vermeld te worden dat deze subletaal verwonde cellen niet over het hoofd gezien mogen worden aangezien deze cellen mogelijks kunnen herstellen van hun letsels (na de behandeling met KAP) en daarom nog steeds een gevaar vormen voor de volksgezondheid.

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k_{max}	The maximum specific death rate	[1/min]
$N(t)$	Cell density at time t	[CFU/mL]
N_0	Initial cell density	[CFU/mL]
N_{res}	Residual population	[CFU/mL]
t	Time	[min]
t_l	The length of the shoulder	[min]

List of abbreviations

BCCM	Belgium Co-ordinated Collections of Microorganisms
BHI	Brain Heart Infusion
CAC	The Codex Alimentarius Commission
CAP	Cold atmospheric plasma
CCP's	Critical control points
CFU	Colony forming units
DBD	Dielectric-barrier discharge
DC	Direct current
DNA	Deoxyribonucleic acid
e.g.	Exempli gratia
EFSA	European Food Safety Authority
EPS	Extracellular polymeric substances
FAO	Food and Agriculture Organization of the United Nations
FASFC	Federal Agency for Safety of the Food Chain
FAVV	Federaal Agentschap voor Veiligheid van de Voedselketen
FSA	Food Standard Agency
GHP	Good Hygiene Practices
HACCP	Hazard Analysis and Critical Control Points
He	Helium
HIV	*Human immunodeficiency virus*
i.e.	Id est
INFOSAN	International Food Safety Authorities Network
KAP	Koud atmosferisch plasma
L. monocytogenes	*Listeria monocytogenes*
LB	Luria-Bretani
LSD	Fisher's Least Significant Difference
min	Minute(s)

NTS	Non-typhoidal *Salmonella* serovars
O_2	Oxygen
PBS	Phosphate Buffered Saline
Pct.	Percentage
ppm	Parts per million
QACs	Quaternary ammonium compounds
RASFF	Rapid Alert System for Food and Feed
RMSE	Root Mean Square Error
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S. Typhimurium	*Salmonella* Typhimurium
SAP	Salmonella Action Plan
SBD	Surface dielectric-barrier discharge
SI	Sublethal injury
SPI	*Salmonella* pathogenicity island
spp.	Species
subsp.	Subspecies
T3SS	Type Three Secretion System
TSB	Tryptic Soy Broth
UV	Ultraviolet
v/v %	Volume/volume percent
w/v %	Weight/volume percent
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate

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1. INTRODUCTION

Biofilms are communities of bacterial cells embedded in an extracellular matrix, produced by the microorganisms themselves. The presence of these biofilms in the food industry are a significant problem regarding the food safety and quality. Biofilms can be present on environmental surfaces (such as floors and walls), food production surfaces (e.g., conveyor belts), and on the surface of food products. When biofilms develop on these surfaces, it is possible that these can get into contact (directly or indirectly by means of cross-contamination) with the food products, and possibly contaminate them with spoilage and/or pathogenic bacteria. When the end product is contaminated with these bacteria, it can respectively lead to economic losses and/or hold a risk for the public health.

Conventionally, biofilms are inactivated/removed by using chemical sanitizing agents such as oxidizing agents and quaternary ammonium compounds (QACs). The usage of these chemical agents has certain drawbacks, i.e., corrosion of the treated surfaces, toxicity, species-specificness, water consumption, and price. Due to these drawbacks, there is a need for novel approaches for the inactivation of biofilms. One promising novel approach is the usage of cold atmospheric plasma (CAP). Cold atmospheric plasma is an ionized gas composed out of different reactive species such as reactive oxygen and nitrogen species, electrons, and ions. The usage of CAP has been shown to be effective for the reduction of planktonic cells, as studies have reported to achieve reductions of 5 logs or greater (Niemira, 2012). However, the effectiveness of CAP for the inactivation of biofilms remains to be determined, which is the mean objective of this thesis.

The ionization of the gas can be obtained by supplying energy to the gas flow. This energy can be derived from electric discharge, laser light, or microwaves. In this thesis, however, the gas is ionized by means of an electric discharge, which is achieved by applying a high voltage (kV-range) alternating current with a high frequency (kHz-range) to an electrode. In this thesis two different types of electrodes will be tested, the Dielectric-Barrier Discharge (DBD) and Surface Barrier Discharge (SBD).

Besides the effect of the electrode type on the inactivation of biofilms, the gas composition will be tested as well, as studies have shown that an addition of a small percentage of oxygen to a helium gas leads to a higher reduction of planktonic cells (by one log or more) (Kim et al., 2011). To study this effect on biofilms, pure helium gas and helium with an addition of 0.5 and 1.0 % (v/v) oxygen will be tested. For each experiment, the gas or gas mixture will be ionised at an input voltage of 13.88 V (which is amplified by the plasma power supply into the kV-range) and a frequency of 15 kHz. Each combination of oxygen concentration and electrode type will be tested on biofilms developed by *Salmonella* Typhimurium and by *Listeria monocytogenes*, which are two important foodborne pathogens, i.e., *Salmonella* has a high occurrence and *Listeria (monocytogenes)* has a high lethality rate.

To study the effect of the treatment time, nine different treatment times within a range from 0 to 30 min. will be tested for each combination of oxygen concentration and electrode type. After the CAP treatment, microbiological analysis will be performed via viable plate counting on selective and general media. Plating on both these media makes it possible to calculate the sublethal injury. Finally, the results will be fitted with a predictive model, developed by Geeraerd et al. (2000).

2. FOOD SAFETY

Food safety holds a wide variety of subjects including the prevention of foodborne illnesses. The Food and Agriculture Organization of the United Nations defines food safety as:

> *"Food safety is about handling, storing and preparing food to prevent infection and help to make sure that our food keeps enough nutrients for us to have a healthy diet."* (Food and Agriculture Organization of the United Nations, 2001).

Not only is there a possibility of toxins and chemicals in the food chain, whether produced by microorganisms, microorganisms themselves can also cause significant damage. It is common knowledge that food safety is very important and must be very strictly controlled. Even a small contamination can have immense consequences, i.e., yearly around 420,000 people worldwide die because of illnesses caused by foodborne pathogens, of which 125,000 children under the age of five (World Health Organization, 2015).

In this thesis, there will be a focus on inactivation/reduction of two pathogenic species, i.e., *Salmonella* Typhimurium and *Listeria monocytogenes*. Therefore, the following subjects will also focus on these microbial species.

2.1. Regulation of food safety

Needless to say, food safety regulation is very important. Several organizations control food safety, globally as well as nationally.

2.1.1. Global regulations

Globally, the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) are the most important institutions to ensure international health and eliminate hunger and malnutrition in the United Nations, which includes 194 Member States (Food and Agriculture Organization of the United Nations, 2017b).

WHO and FAO established the International Food Safety Authorities Network (INFOSAN) to assist in managing food safety risks and making sure the spread of contaminated food to other countries is stopped during food safety emergencies. Also optimizing for future interventions is one of INFOSAN's responsibilities. For laboratory-based supervision, WHO brought the Global Foodborne Infections Network into existence. In 2010, WHO composed a strategy which includes three steps: decreasing foodborne health risks in the entire food chain, improve collaboration between countries and provide leadership to intensify risk-based, integrated national systems for food safety (Fukuda, 2015; World Health Organization, 2016).

To assure consumer health and insure fair food trading, WHO and FAO established The Codex Alimentarius Commission (CAC). The CAC maintain the Codex Alimentarius, a collection of standards, codes and guidelines of practice concerning food safety and fair trading. The Codex focuses on many things, such as food hygiene, use of pesticides and veterinary drugs, limits for additives, and chemical and microbiological contamination (Food and Agriculture Organization of the United Nations, 2017a).

2.1.2. European regulations

In 2002, the European Food Safety Authority (EFSA) was founded by the European Commission under the General Food Law. This was necessary after several food crises in de late 1990's. EFSA does not have any direct executive power, but advises the European Commission on risk assessments and is responsible for scientific advice and communication (European Food Safety Authority, 2017a).

Besides EFSA there is also RASFF, the Rapid Alert System for Food and Feed. The RASFF was created by the European Commission in 1979 and is responsible for fast exchange of information between its members, including EFSA and other European food safety authorities. In general, the function of the RASFF is to protect people from food that does not meet the European food safety standards, which includes banned substances, high-risk substances, veterinary medicines, or carcinogenic food colouring (European Commission, 2016; European Union, 2014).

2.1.3. Belgian regulations

Food safety in Belgium is under the control of the FAVV, Federaal Agentschap voor Veiligheid van de Voedselketen, which translates to Federal Agency for Safety of the Food Chain (FASFC). FASFC was founded in 2000 and has the authority over food safety, with emphasis on hygiene in the food chain (Federal Agency for Safety of the Food Chain, 2016b; FOD Volksgezondheid, 2016b).

One of the basic principles of the FASFC is "auto control" or self-checking. The essence of self-checking is that all products under control of an operator in all stages of production, processing and distribution, meet the legal demands concerning (i) food safety, (ii) the quality of the products, and (iii) traceability of substances. The system of self-checking includes Good Hygiene Practices (GHP), which is based on Hazard Analysis and Critical Control Points (HACCP) (Federal Agency for Safety of the Food Chain, 2017b).

HACCP has seven principles: (i) conducting a hazard analysis, (ii) identifying critical control points (CCP's), (iii) establish critical limits for each CCP, (iv) establish CCP monitoring requirements, (v) establish corrective actions, (vi) establish procedures to guarantee HACCP works as it was meant, and (vii) establish record keeping procedures (FOD Volksgezondheid, 2016a).

GHP relate to personal hygiene as well as hygiene of facilities, furnishing, and equipment. It focuses on cleaning and disinfecting, good water quality, correct disposal of waste and avoiding pests like insects and rodents. Every person who works in the food sector has to be aware of GHP and, if necessary, get a basic education on GHP (Federal Agency for Safety of the Food Chain, 2017a).

Besides self-checking, FASFC has authority over foodstuffs (additives, aroma's, allergens, contaminants, genetically modified organisms, insects in the food chain, mineral oils, nutritional supplements, novel foods, etc.) and everything related to it (labelling of foodstuffs, food waste, food trading, shelf life data, hygiene, radioactivity, water quality, etc.) (Federal Agency for Safety of the Food Chain, 2017c).

Finally, an important benefit of the FASFC is the compulsory notification. Every operator is obligated to inform the FASFC if there is any reason to believe that one of the products that were

imported, produced, reared, grown, processed, manufactured or distributed by the operator is harmful to the health of humans, animals, or plants. If necessary, products can be recalled (Federal Agency for Safety of the Food Chain, 2016a).

2.2. Types of contamination and corresponding risks

To ensure food safety, it is not only important to prevent contamination but also to reduce the risks of contamination. Therefore, knowledge of different contamination types is crucial. One can distinguish three types of contamination, i.e., chemical, biological and physical contamination (Australian Institute of Food Safety, 2016).

Chemical contamination is caused by chemicals or toxins which are not produced by living organisms. For example, the improper use of pesticides and cleaning agents. Another way of chemicals entering the food chain is by pollution. For instance, the pollution of heavy metals in lakes or rivers used for fishing, which causes poisoned fish to enter the food chain. These heavy metals can for example cause neurological and kidney damage in humans and other animals (Australian Institute of Food Safety, 2016).

Biological contamination is contamination by living organisms or substances that are produced by organisms. This includes bacteria, moulds, viruses, and parasites. When the organisms are pathogenic, contamination can lead to foodborne intoxication or infection. Bacterial contamination is the biggest cause of food poisoning worldwide (Australian Institute of Food Safety, 2016).

Finally, there is physical contamination. This takes place when an external object enters the food production process, which can lead to two significant problems. Firstly, when the object is hard and/or sharp (e.g., nails, wood, glass, etc.), this may lead to suffocation, broken teeth, or internal bleedings when the final food product is consumed. Secondly, these objects may carry pathogens, which contaminate the food and may lead to foodborne illnesses. This type of contamination is referred to as cross-contamination, which is defined as: the unintentionally transfer of microorganisms from one substance/object to another (Australian Institute of Food Safety, 2016).

The risks of food contamination vary widely. It is possible that there is no effect at all, however, disease with hospitalization and even death are two other dangerous possible outcomes. As mentioned before, estimates of 420,000 people die and 10 % of the world population gets sick every year because of infected food (World Health Organization, 2015).

2.3. Consumption of foodborne pathogens

When food is contaminated with pathogenic microorganism, these microorganisms are described as foodborne pathogens. The contamination of food can occur at any stage of the food production chain (production, processing, distribution, and preparation). The most common food pathogens include bacteria, viruses, and parasites. Prions can be present as well, but these are rather exceptional. Foodborne pathogens can lead to foodborne illnesses, which in most cases exhibit gastrointestinal symptoms and fever, yet other symptoms may occur (Centers for Disease Control and Prevention, 2017c).

Table 1 shows the number of confirmed illnesses and deaths caused by the top five most frequent foodborne diseases within the European Union in the year 2016. These data were published in

the EFSA journal of 2017 (European Food Safety Authority & European Centre for Disease Prevention and Control, 2017).

Table 1: Top five most frequent diseases caused by foodborne bacterial pathogens within the European Union (European Food Safety Authority & European Centre for Disease Prevention and Control, 2017)

Disease	Number of confirmed illnesses	Number of reported deaths
Campylobacteriosis	246,307	62
Salmonellosis	94,530	128
Yersiniosis	6,861	5
STEC* infections	6,378	10
Listeriosis	2,536	247

* Shiga toxin-producing *Escherichia coli*

Out of the top five most frequent foodborne diseases shown in Table 1, listereriosis followed by salmonellosis account for the highest number of deaths. These diseases are respectively caused by *Listeria monocytogenes and* non-typhoidal *Salmonella* serovars (NTS). Listeriosis caused more deaths than salmonellosis, but the disease is less frequent than salmonellosis. This insists that the disease caused by *Listeria* has a higer mortality rate than the disease caused by *Salmonella*. Because of the frequent occurrance of *Salmonella* and the high mortallity rate of *Listeria monocytogenes*, this thesis focusses on the inactivation/reduction of these two bacterial species. Therefore, these bacterial species will be further discussed in the next paragraph.

2.3.1. Salmonella Typhimurium

Salmonella is among the most frequent occurring foodborne pathogens and can cause salmonellosis. The EFSA reports that over 100,000 cases are reported in the European Union each year. The serotype Typhimurium is one of the most common serotypes in most parts of the world (European Food Safety Authority, 2017b; World Health Organization, 2017).

2.3.1.1. Classification

The genus *Salmonella* is classified within the family of the *Enterobacteriaceae* and contains two species, i.e., *S. enterica* and *S. bongori*. These two species are respectively found in warm-blooded and cold-blooded animals. *S. enterica* is divided into six subspecies, of which the most important one is the subspecies *enterica*. This subspecies accounts for most cases of salmonellosis and includes more than 2500 serotypes, including the serotype Typhimurium. Thus, the full scientific name of *Salmonella* Typhimurium is *Salmonella enterica* subsp. *enterica* serotype Typhimurium (Gurtler, Doyle, & Kornacki, 2017; Schultz, 2008).

2.3.1.2. Characteristics

Salmonella Typhimurium is a gram-negative, facultative anaerobic, and rod-shaped bacterium. The bacterium is motile due to its peritrichous flagella (Figure 1). It's optimum growth temperature is 37°C, however growth at temperatures between 5.9°C and 46°C have been reported for specific food matrices. The optimum growth pH can be found between 6.5 and 7.5, however, growth is

possible in a pH range of 4 – 9. The minimum pH at which *S.* Typhimurium can grow is dependent on the type of acid, temperature, and presence of salt. The lowest reported water activity needed for growth is 0.93. In food with a low water activity, *Salmonella* spp. show an increase in heat tolerance. In products with a low pH, the heat tolerance is reduced. When food products are frozen, there is an initial decrease in the number of viable cells because of freezing damage. However, *Salmonella* spp. can survive at low temperatures for an extended period of time. Strawn an dayluk (2010) showed that *Salmonella* was able to survive on papayas and mangoes for more than 180 days at a temperature of -20°C (Food Standards Australia New Zealand, 2017; Keerthirathne et al., 2016; Strawn & Danyluk, 2010).

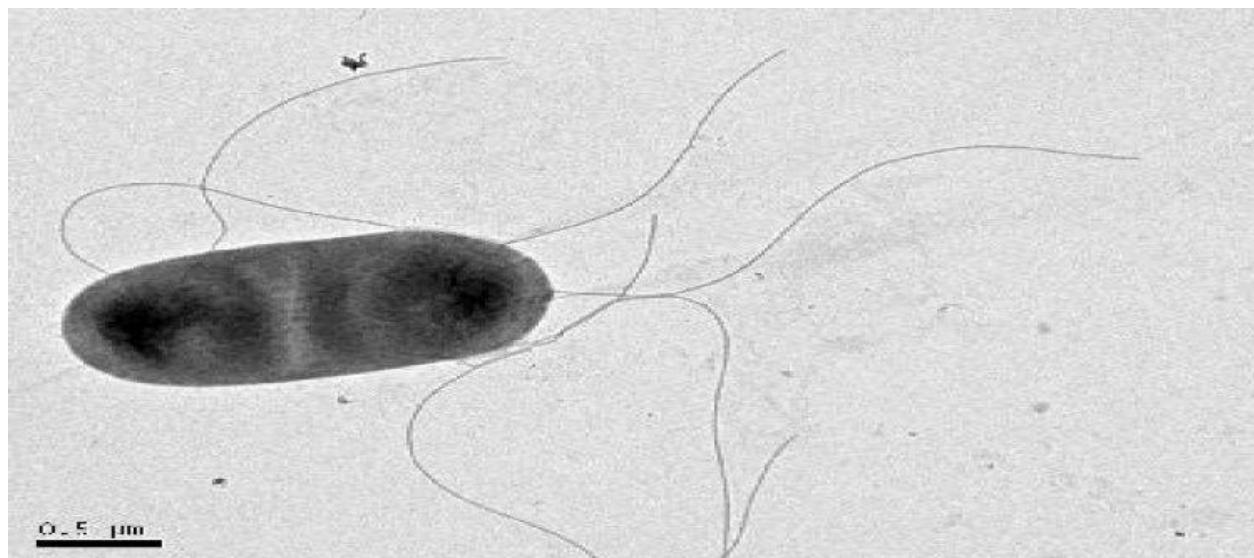


Figure 1: Microscopic picture of Salmonella (Sheba - Academic Medical Center Hospital, 2018)

2.3.1.3. Salmonellosis

Salmonella serovars are generally divided into two categories, typhoidal and non-typhoidal serovars (NTS). The non-typhoidal serovars generally have a broad host specificity. Serovar Typhimurium and Enteritidis are examples of NTS. Typhoidal serovars, such as Typhi and Paratyphi are highly adapted to the human host and can cause enteric fever, which is an invasive and life-threatening systemic disease. Enteric fever confines to developing countries that lack clean water and sufficient sanitation, which stimulates faecal-oral transmission. NTS salmonellosis is not confined to developing countries, but occurs worldwide. NTS infections lead to self-limiting (in immunocompetent patients) acute gastroenteritis. Symptoms such as diarrhoea, nausea, fever, vomiting, and abdominal pain can appear 6-12 hours after ingestion of the pathogen and resolve typically between 3 to 7 days. In 5% of the NTS-infections, the bacteria cause an invasive extra-intestinal disease. This can lead to bacteraemia and systemic infections. It appears that some serovars have a higher chance to cause these extra-intestinal infections, which means that there is a genetic basis for the invasive behaviour. However, to date, little is known about the difference between NTS with a higher chance of extra-intestinal infection and general NTS. Serovar Typhimurium is an example of a serovar which has a higher potential to cause an extra-intestinal infection (Darwin & Miller, 1999; Gal-Mor, Boyle, & Grassl, 2014).

To initiate an infection, a dose between 10^5 and 10^{10} cells is needed. This dosage depends on the serovar and the type of food matrix. Food that passes rapidly through the stomach and/or that neutralizes the acidic pH of the stomach, requires a lower dosage. When *S.* Typhimurium reaches the small intestine, it adheres to the epithelia cells by its fimbriae. After adhesion, it injects effector proteins (e.g., SpA, SopE) into the host cell by means of a Type Three Secretion System 1 (T3SS), which is a needle-like structure. This structure is expressed by the *Salmonella* pathogenicity island 1 (SPI-1). When the effector proteins are injected in the host cell, rearrangement of actin filaments is induced. These rearrangements lead to the formation of plasmatic membrane extensions (ruffles), which engulf the bacteria. A second T3SS (encoded by SPI-2) plays a role in the intercellular survival of the bacteria. During this process, the infected cells produce pro-inflammatory cytokines, which attract white blood cells. In normal circumstances, as mentioned above, the disease is self-limiting. When the patient is not immunocompetent (e.g., babies, elderly, and, HIV-positive people), antibiotic therapy is necessary. However, multidrug-resistance is an increasing problem within the *S. enterica* serovars. For example, some strains of *S.* Typhimurium are resistant to ampicillin, chloramphenicol, tetracycline, streptomycin, and sulphonamides (Bueno et al., 2012; Darwin & Miller, 1999; Gal-Mor et al., 2014).

2.3.1.4. Sources of infection

The most common source of infection is the consumption of raw food that is derived from animals infected with *Salmonella* or that has been contaminated with faeces from an infected animal. For example, when tools get contaminated during meat-processing by puncturing the intestines of an infected animal, cross-contamination to different animals can occur if the tools are not disinfected in between. Other food products that are possible sources of infection include: raw milk, poultry, undercooked eggs, contaminated water, and other products containing potentially contaminated ingredients. Next to food products, pets and humans (by faecal-oral transmission) are possible sources of infections (Mahmoud, 2012).

2.3.1.5. Regulations

In Europe, the commission regulation (EC) No 2073/2005 defines the following (European Commission, 2018):

- For minced meat and meat preparations intended for raw consumption, five samples must be taken. These samples must show an absence of *Salmonella* in 25 grams food product.
- For minced poultry meat and meat preparations intended to be cooked before consumption, five samples must be taken. These samples must show an absence of *Salmonella* in 25 grams food product.
- For minced non-poultry meat and meat preparations intended to be cooked before consumption, five samples must be taken. These samples must show an absence of *Salmonella* in 10 grams food product. The same applies for mechanically separated meat.

In Belgium there is also the Salmonella Action Plan (SAP) which describes the National *Salmonella* Containing Program. This program must be implemented by factories that contain more than 200 chickens or turkeys. The program consist out of three components, (i) sample taking, (ii) prevention (e.g. vaccination), and (iii) guidelines in case samples are positive (Federal Agency for Safety of the Food Chain, 2018).

2.3.2. Listeria monocytogenes

Among the foodborne pathogens, *Listeria* is one of the most dangerous pathogens. According to the Food Standard Agency (FSA), the FAVV equivalent in the United Kingdom, 126 out of 358 cases of *Listeria* infection in the United Kingdom in 2008 were deadly. In comparison with their reports for *Campylobacter* infections (321,179 cases of which 76 were deadly), the *Listeria* mortality rate of 35% is quite high. Globally, the mortality rate of *Listeria* infection, or listeriosis, is estimated to be 20-30% (The BioCote Team, 2012).

2.3.2.1. Classification

Listeria monocytogenes is a gram-positive pathogenic bacterium from the *Listeriaceae* family. It was discovered in 1926 and was named *Bacterium monocytogenes* after observing an increase in monocytes after infection. In 1940 it was renamed *Listeria monocytogenes* after Joseph Lister, a pioneer in antiseptic surgery (Rosaler, 2004; The BioCote Team, 2012).

2.3.2.2. Characteristics

L. monocytogenes is rod-shaped (Figure 2), facultative anaerobic, and motile due to peritrichous flagella. This pathogen can very well adapt to severe conditions in the human body and can offset changes in acidity, variations in the partial pressure of oxygen (oxygen tension), bile, and antimicrobial peptides. It is a psychotropic pathogen which grow in temperatures as low as -1.5°C and which can easily survive in frozen food. Growth is possible up to 45°C and survival is even possible up to 70°C (the temperature where *L. monocytogenes* is too damaged to survive). The pH range for *L. monocytogenes* growth can be found between 4.3 and 9.4, but also depends on other conditions such as temperature. The pathogen needs a water activity of at least 0.92 and can survive in salt concentrations of 20-30%. Additionally, it can even survive some food drying processes (Lawley, 2013; The BioCote Team, 2012).

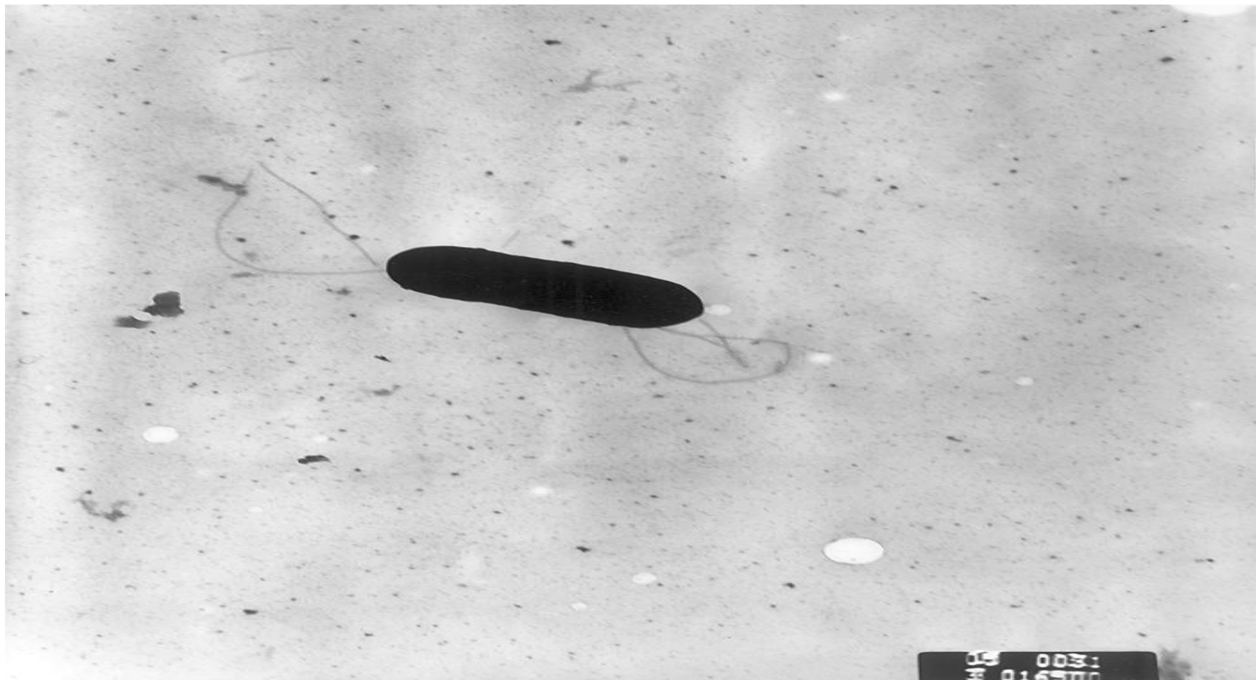


Figure 2: Microscopic picture of Listeria monocytogenes (Centers for Disease Control and Prevention, 2002)

2.3.2.3. Listeriosis

Listeriosis is a serious disease: every year, around 2,536 people get ill, of which 247 people do not survive. People at risk are pregnant women, people of 65 years or older, and people with a weakened immune system (European Food Safety Authority & European Centre for Disease Prevention and Control, 2017)

When *Listeria* (*monocytogenes*) is ingested due to consumption of contaminated food, the bacteria will infect the epithelial cells of the small intestine. This happens by means of membrane bound internalin. When interaction takes place between these bacterial surface molecules and cellular receptors (of epithelial cells), the host cell is triggered to take up the bacteria. *Listeria* (*monocytogenes)* can survive in macrophages and is able to cross the blood-brain, fetoplacental, and intestinal barriers. This means that when the person is not immunocompetent and/or is pregnant, severe symptoms could develop (Pizarro-Cerdá, Kühbacher, & Cossart, 2012).

The incubation period of listeriosis ranges from 1 to 90 days, but the average period is 30 days. Typical symptoms in immunocompetent patients are flu-like symptoms, such as fever and headaches, along with nausea, vomiting, and diarrhoea. Also, a stiff neck and muscle aches are common symptoms. Patients with a weakened immune system have a higher chance of septicaemia (when the infection spreads from the intestine to the blood), which can lead to meningitis and convulsions. When a pregnant woman is infected, it can affect the foetus and lead to the birth of an infected child, miscarriage, and stillbirth (Centers for Disease Control and Prevention, 2017d; Lawley, 2013)

2.3.2.4. Sources of infection

L. monocytogenes is a bacterium that is common in soil, where it can infect plants, fruits and vegetables. In marine environments, this pathogen is often found and is therefore often associated with seafood. Various animals are carriers of *L. monocytogenes*, including cattle, sheep, and goats (The BioCote Team, 2012).

Lots of outbreaks of *L. monocytogenes* are caused by infected dairy, such as cheese, ice cream, and milk. Especially raw cheeses (not pasteurized) form a great risk of infection. In addition, there have been several outbreaks because of packaged or frozen vegetables and, to a lesser extent, fruits (Centers for Disease Control and Prevention, 2017a).

The best way to prevent infection is to avoid dairy products that are not pasteurized, such as raw cheese and some soft cheeses. It is recommended to properly cook vegetables, such as sprouts. Fruits, such as melons, must be refrigerated well and must be eaten immediately after cutting. “Cold meat”, like hot dogs or pâté, and seafood should be refrigerated properly and heated to at least 70°C for several minutes when cooked (Centers for Disease Control and Prevention, 2017b).

2.3.2.5. Regulations

Rules and regulations are different in every country. In Europe, the commission regulation (EC) No 2073/2005 defines the following (European Commission, 2018):

- For ready-to-eat foods intended for infants, an absence of *Listeria* in 25 g of the food product is required and 10 samples must be taken.

- For ready-to-eat foods which are not intended for infants and that may support the growth of *L. monocytogenes*, the limit is 100 cfu (colony forming units) of *Listeria* in one gram of food product and 5 samples must be taken. This limit may not be exceeded during the shelf life. If the manufacturer cannot assure that the limit will not be exceeded, an absence of *Listeria* in 25 g in 5 samples must be implemented.
- For ready-to-eat foods which are not intended for infants and that cannot support the growth of *L. monocytogenes*, the limit is 100 cfu of *Listeria* in one gram of food product and 5 samples must be taken.

3. BIOFILMS

3.1. Definition and presence in the food industry

Biofilms are defined as a structured microbial community that is associated with a surface and embedded in an extracellular polymeric substances (EPS), produced by the microorganisms themselves. These biofilms can form on abiotic as well as on biotic surfaces and can consist out of different species. Biofilms are a significant problem in the food processing industry. They are found on food production surfaces that are hard to reach during cleaning, such as gaskets, pasteurizers, and equipment containing dead spaces or narrow openings. The formation of biofilms on these surfaces is the main cause of contamination of the final product, which leads to economic losses due to food spoilage and a risk for the public health due to the possible presence of foodborne pathogens. Biofilms are also found on environmental surfaces such as floors or walls, which can lead to cross-contamination. In some cases the biofilms are also found on the food products themselves (e.g., sprouts or spinach) (Deibel & Schoeni, 2003; Téllez, 2010). Other problems caused by biofilm formation include (i) a decrease in heat transfer when biofilms are formed in heat exchangers, (ii) the corrosion of metal surfaces due to the production of acids by the microorganisms, and (iii) blockage of the pores of membrane systems, which leads to capacity losses (Garrett, Bhakoo, & Zhang, 2008; Téllez, 2010). In the following paragraphs, the different steps of the bacterial biofilm formation procedure will be discussed.

3.2. Formation of a biofilm

The formation of a biofilm is a multi-step process in which the bacterial cells transition from a planktonic to a sessile (immobile) mode of growth. It is important to note that the gene expression in planktonic cells differs from that in sessile cells. This implies that during the transition, some genes become up- or downregulated. The different steps in the biofilm formation include: (i) the formation of a conditioning layer, (ii) the reversible and irreversible adhesion to the (conditioned) surface, (iii) maturation of the biofilm and (vi) biofilm dispersal (Deibel & Schoeni, 2003). These steps (except for the formation of the conditioning layer) are illustrated in Figure 3 (Flemming, Wingender, & Szewzyk, 2011).

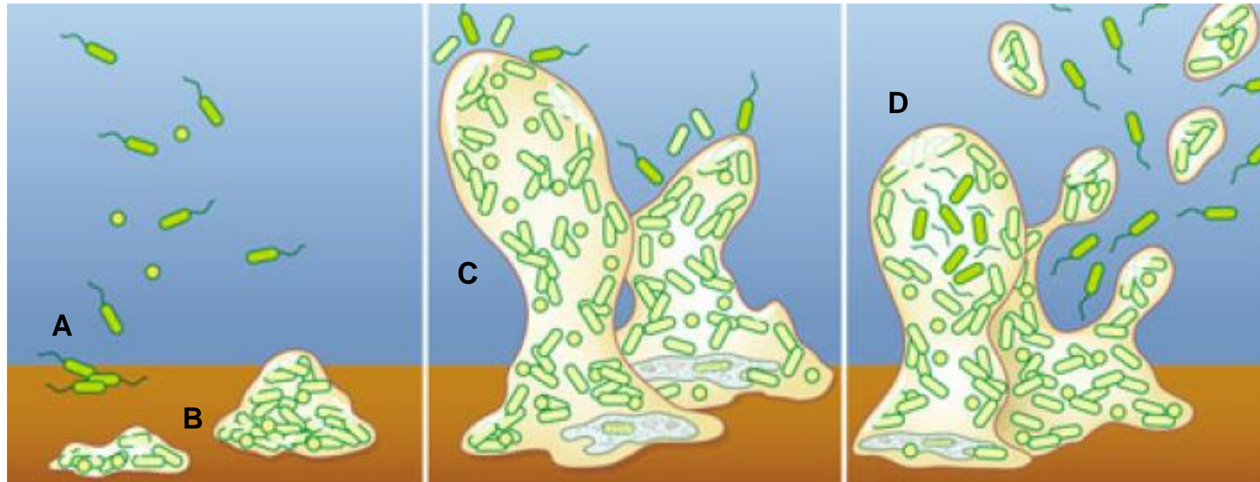


Figure 3: The different stages of the biofilm formation, with (A) the reversible adhesion, (B) the irreversible adhesion, (C) the maturing and (D) the dispersal. The surface might also contain a conditioning layer, which is not illustrated in the figure (Flemming et al., 2011).

3.2.1. Conditioning layer

The formation of a conditioning layer is often seen as the first step of the biofilm formation and forms the foundation on which a biofilm can grow. During this step, organic and/or inorganic substances from an aqueous medium are adsorbed to a surface. This changes the physicochemical properties of the surface (e.g., electrical charges, hydrophobicity) and affects the adhesion of bacteria. In the food industry, the conditioning layer is mainly formed by proteins (Deibel & Schoeni, 2003; Kumar & Anand, 1998). These may inhibit or facilitate the adhesion of certain bacteria. For example, albumin, casein and β-lactoglobulin inhibit the attachment of *L. monocytogenes* and *S.* Typhimurium, while whey proteins show an increase in adhesion of milk-associated bacteria. Processing parameters that facilitate the formation of conditioning layers include a high or low pH and high temperatures. This is due to the denaturation of proteins in these circumstances. When proteins denature, more hydrophobic groups are exposed, which are able to interact with hydrophobic surfaces (e.g. stainless steel) (Kumar & Anand, 1998).

3.2.2. Adhesion

The adhesion of bacterial cells to a surface can be divided in three phases. In the first phase, planktonic cells are transported form the bulk of the aqueous medium to the boundary layer. The transport can either be passive or active. Passive transport includes transport by means of gravity and/or the flow of the aqueous medium. Active transport can be achieved by motile bacteria by means of their flagella. When bacterial cells traverse the boundary layer, the second and the third phase of the adhesion can take place. During these two phases, interactions between the cells and the surface occur. Initially, these interactions between the cells and the substratum (surface) are weak and therefore reversible. For this reason, this second phase of the adhesion is called the *reversible adhesion phase*. The bacteria will detach from the surface when the repulsive forces are greater than the attractive forces, which is more likely to happen when the surface is not conditioned. Long range interaction forces such as van der Waals, hydrophobic, and electrostatic (double layer) forces affect the adhesion process during this stage. Due to the weak interactions, the bacterial cells are sensitive to the fluid shear forces, which may remove the cells from the

surface (e.g., by rinsing). Also, Brownian motion of the cells is still observed during this phase. The third phase is called the *irreversible adhesion or anchoring phase*. The transition to the irreversible adhesion phase is accomplished by reorientation of the cell and production of specific adhesins, which interact with the (conditioned) surface. These specific adhesins include flagella, fimbriae, pili, and EPS. The presence of adhesins makes the direct contact between the cells and the surface possible, which was prevented by repulsive forces in the previous face. The adhesins can overcome these repulsive forces and make short range interactions possible, such as hydrogen, ionic, and covalent bonds. To remove these anchored cells, much higher shear forces are necessary (e.g., scrubbing) (Garrett et al., 2008; Kumar & Anand, 1998).

The adhesion process is affected by several factors. During the first phase of the adhesion, the velocity of the liquid phase plays a significant role. The higher the velocity, the smaller the boundary layer becomes, which means that the cells can more easily traverse this layer. However, the velocity may not exceed critical levels, otherwise the bacteria might get swiped away by the liquid flow during the reversible adhesion phase. Another important factor that effects the adhesion of cells is the roughness of the surface, i.e., a higher roughness implies an increased surface area and less shear forces, which benefits the adhesion of cells. Other factors include the presence of a conditioning layer, physicochemical properties of the surface, nutrient availability, temperature, and pressure. The adhesion is also dependent on the properties of the cell surface. Cell surface structures with apolar sites, such as fimbriae, facilitate the attachment to hydrophobic surfaces. In contrary to the adhesion to hydrophilic surfaces, where lipopolysaccharides and EPS have a more important role (Donlan, 2002; Garrett et al., 2008).

3.2.3. Biofilm maturation

After the irreversible adhesion, the bacterial cells will grow and divide by using nutrients derived from the conditioning layer as well as from the surrounding fluid. This leads to the development of microcolonies, which form a layer of cells covering a surface area. During this stage, the cells continue the production of extracellular polymeric substances (EPS), which form an extracellular matrix. The EPS components include: polysaccharides, proteins, nucleic acid, lipids, and phospholipids. The extracellular matrix serves as a barrier which protects the cells from fluctuations in the environment and helps the anchoring of the cells to the surface. An example of the protective features of the extracellular matrix, is the protection against antibiotics and chemical agents. This is due to diffusion limitation and/or chemical interaction with the EPS components. In a second stage, multilayers of bacterial cells are formed. This gives rise to a three-dimensional network of cells imbedded in the extracellular matrix, in which the cells are not uniformly distributed. It is possible that other species from the surrounding medium attach to the maturing biofilm, which gives rise to a multi-species biofilm. In most cases, the biofilm grows into a mushroom like structure. The formation of this structure is believed to allow the transportation of nutrients deep into the biofilm (Garrett et al., 2008; Kumar & Anand, 1998; Simões, Simões, & Vieira, 2010).

3.2.4. Biofilm dispersal

Biofilm dispersal mechanisms can be divided into two categories, i.e., active and passive. Active dispersal means that the mechanisms are initiated by the bacteria themselves (Kaplan, 2010). This is often seen as the last step in the biofilm development, in which the bacteria leave the

mature biofilm due to unfavourable local conditions (Flemming et al., 2011). Bacteria can sense these unfavourable conditions, for example by means of quorum-sensing, which is a form of cell-to-cell communication that is based on auto-induction. The cell to cell communication is mediated by the production of diffusible organic signal molecules, which accumulate in the local environment. If the population grows, the concentration of these signal molecules increases. When this concentration reaches a critical threshold, the expression of certain genes is induced (Simões et al., 2010). This leads to the production of hydrolytic enzymes, which degrade the EPS, after which the bacterial cells can escape the biofilm and colonize new areas. The active dispersal of a biofilm does typically not involve the entire biofilm. Instead, only certain microcolonies will undergo a dispersion event, leaving void spaces behind in the biofilm. The remainder of the biofilm may still undergo growth, thus growth and dispersal may occur simultaneously but at a different location within the biofilm (Flemming et al., 2011).

Passive biofilm dispersal refers to cell detachment that is caused by external forces/factors (Kaplan, 2010). The mechanisms of passive dispersal can be categorized into four categories. These mechanisms are shown in Figure 4 and include, (i) abrasion, (ii) predator grazing, (ii) sloughing, and (iv) erosion. Abrasion refers to the release of bacterial cells due to particles from the fluid environment that collide with the biofilm. Predator grazing is caused by the feeding activity of eukaryotic organisms. Sloughing and erosion are both due to fluid shear forces, however, they differ in the number of cells that are released in a certain time period. Erosion is the continuous release of a small amount of cells, whereas sloughing is the sudden release of large intact pieces of biofilm (Flemming et al., 2011).

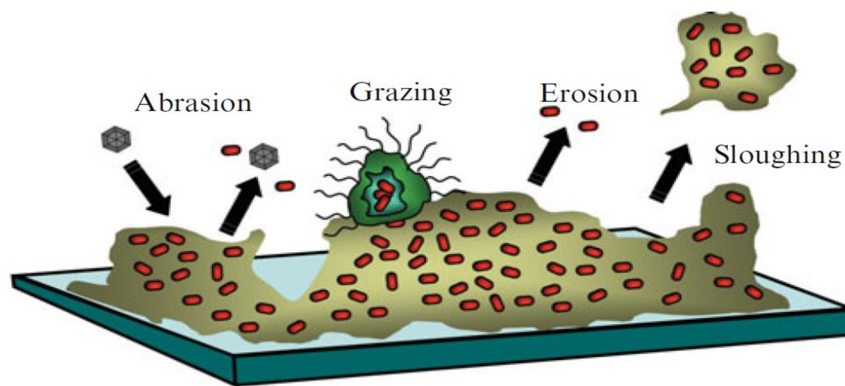


Figure 4: The four passive mechanisms by which bacteria may detach from the biofilm: (i) abrasion, (ii) predator grazing, (ii) sloughing, and (iv) erosion (Flemming et al., 2011)

3.3. Removal of biofilms

In the first place, the formation of biofilms should be prevented by regularly sanitizing. Since the adhesion phase of the biofilm formation process takes only a few hours, it is difficult to sanitize frequently enough to inhibit this adhesion phase, as this would cause too much equipment downtime. When biofilms do establish, they are rather hard to remove due to the protective effect of the extracellular matrix against antimicrobial compounds, such as chemical sanitizers. For example, an active chorine concentration of 10 ppm shows to be effective against planktonic cells, whereas a concentration of 1000 ppm is needed for a significant reduction of cells in a biofilm. Thus, for efficient removal of the biofilms, the extracellular matrix needs to be broken down before

adding chemical sanitizers (Meyer, 2003). The extracellular matrix may be removed mechanically (e.g., by scrubbing), chemically, or biochemically. The chemical approach involves strong oxidizing agents such as peracetic acid or hydrogen peroxide, which also kills the microorganisms. A mixture of peracetic acid and hydrogen peroxide was proven to be affective against biofilms formed by *Salmonella* and *Listeria* (Deibel & Schoeni, 2003). The biochemical approach is based on the enzymatic breakdown of the EPS by hydrolytic enzymes. After the enzyme treatment, sanitizers can be applied (Meyer, 2003).

The conventional use of chemical sanitizing agents has certain drawbacks i.e., corrosion of the treated surfaces, toxicity, species-specificness, water consumption, price, etc. Active chlorine is for example corrosive, relatively expensive, more effective to gram-negative bacteria compared to gram-positive bacteria, and can be used up to a concentration of 200 ppm. Higher concentrations demand a rinsing step to remove traces, which implies an increase in water consumption (Cramer, 2007). One other problem is the increasing chemical resistance of bacterial cells. For example, studies have indicated that the use of quaternary ammonium compounds (QACs) can select for genetic variants of *L. monocytogenes*, which are resistant to these QACs (Brandan A. Niemira, 2017). Due to the drawbacks of the use of chemical agents, there is need for novel approaches to eliminate biofilms. One of these novel approaches is the use of cold atmospheric plasma, which will be further discussed in the next chapter.

4. COLD ATMOSPHERIC PLASMA

Plasma is known as the fourth state of matter, which is a partially or fully ionized gas. The ionized gas is composed out of different reactive species, such as reactive oxygen and nitrogen species. The ionization is obtained by adding energy to a pure gas or mixture of gasses. Historically, plasma could only be generated at high temperatures or in partially vacuum. Present-day, it is also possible to generate plasma at room temperature and atmospheric pressure. This type of plasma is known as non-thermal atmospheric pressure plasma or cold atmospheric plasma (CAP). It is also referred to as non-equilibrium atmospheric plasma, since the electrons have a higher average kinetic energy than the heavier particles. These high energy electrons are therefore likely to transfer their energy to the heavier particles by means of collisions, upon which the larger particles get excited into a more reactive state (Chen et al., 2016; Niemira, 2012).

It has been shown that CAP has non-specific antibacterial effects. This makes it a promising sanitizing tool in the food industry, where it can be used as a non-thermal process to inactivate microorganisms on food contact surfaces as well as on food product surfaces (e.g., nuts and egg shells) (Niemira, 2017; Niemira, 2012). Treatment with CAP has already been shown to be effective against planktonic cells, for example a reduction of 5 logs (or greater) can be obtained for foodborne pathogens, such as *Salmonella* and *L. monocytogenes* (Niemira, 2012). However the effectiveness of CAP treatment against biofilms remains to be investigated (Helgadóttir et al., 2017).

4.1. Generation of cold atmospheric plasma

As previously mentioned, plasma is obtained by supplying energy to a pure gas or a mixture of gasses. In case of CAP, the energy to ionize the gas is derived from electricity, microwaves, or laser light. In most cases, CAP is generated by means of an electric discharge. There are different methods that are based on the generation of CAP by means of an electrical discharge, such as Dielectric-Barrier Discharge (DBD) and Surface Barrier Discharge (SBD). The DBD method is shown in Figure 5(a) and consist out of two electrodes which are separated from each other and both covered with a dielectric material such as glass or quartz. The dielectric material has two functions, i.e., it distributes the applied current over the whole surface area of the electrodes, and it prevents the formation of a continuous electric current between the two separated electrodes (referred to as an electric arc). To ionize the gas at atmospheric pressure, a high voltage (kV-range) alternating current is applied between the two electrodes, often at frequencies in the kHz-range (Hoffmann, Berganza, & Zhang, 2013). The surface that needs to be treated is located in between the two electrodes. This implies that the surface is positioned within the CAP generated field, which is the major advantage of this method. The SBD method is similar to the DBD method, but the configuration of the electrodes differs. The two electrodes are now only separated by a dielectric medium. The top electrode does not cover the entire surface of the dielectric, as can be observed Figure 5(b). Due to this configuration, the plasma can be formed on the surface area of the dielectric that is not covered by the top electrode. The plasma needs to be transported from the surface of the dielectric to the surface that needs to be treated, which is possible due to the flow of the feed gas. The major advantages of this method include a simpler design of the device and a higher flexibility regarding to the shape of the surface that needs to be treated, as it does not necessary need to be placed in between the two electrodes. However, the downside of this

method is that during the transport from the dielectric to the surface that needs to be treated, electrons may recombine with other plasma products, resulting in the formation of less reactive species (Niemira, 2012; Pavón, 2008). To date, different configurations of the electrodes have been developed by several companies, such as *SurfPlasma*. This company has recently created an innovative design for the SBD electrode, which they embedded in a pipeline. Due to this configuration, it is possible to create plasma on the inner surface of the pipeline (SurfPlasma, 2016).

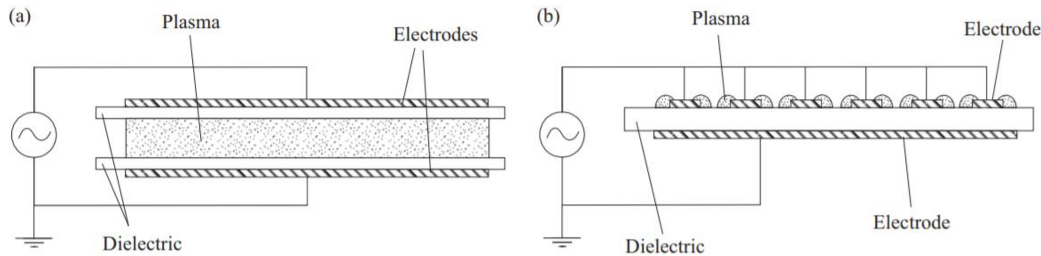


Figure 5: Illustration of two frequently used CAP set-ups: (a) DBD and (b) SBD electrode (Pavón, 2008)

4.2. Mechanism of inactivation

To date, the exact mechanism of inactivation by means of cold atmospheric plasma is not well known. However, several reactive species are believed to play a significant role, such as, reactive oxygen species (ROS, e.g., singlet oxygen, ozone), reactive nitrogen species (RNS, e.g., nitric oxide), electrons, positive and negative ions, and UV-photons. ROS and RNS can cause oxidative damage to a broad range of biomolecules, such as proteins, DNA, and lipids. The bombardment of ions and electrons on the cell wall can cause perforations in the cell membrane by breaking covalent bonds. These perforations result in a higher penetration of reactive plasma species into the cell. The UV photons result in DNA-damage, due to the formation of cyclobutane pyrimidine dimers, which inhibit DNA-polymerase activity, and thus the replication. All these different reaction pathways lead to sublethal or lethal cell damage. Sublethally injured cells still pose a risk for the food safety and/or economic losses, as these cells might recover. To recover from the sublethal damage, these cells need a suitable environment (nutrient rich), which is likely the case in the food processing industry (Bourke et al., 2017; Jawaid et al., 2016).

4.3. Influencing parameters

The composition (and hence the efficiency) of the cold atmospheric plasma is affected by several parameters, such as feed gas composition, humidity, temperature, voltage, and frequency. The most used feed gasses include helium and argon since these noble gasses produce a stable plasma. Plasma created with helium shows a higher concentration of ROS, compared to when argon is used to generate plasma. It is also an option to use gas mixtures, for example 1% oxygen and 99% argon to increase the concentration of ROS (Bourke et al., 2017; Jawaid et al., 2016). Studies have shown that when gas mixtures of helium (or argon) and oxygen were used to treat planktonic cells, higher reductions were achieved compared to the usage of pure helium gas. Kim et al. (2011) showed for example that when a gas mixture of helium and 0.1% oxygen was used, a higher reduction (by one log) of planktonic *Listeria* and *Salmonella* cells was achieved compared

to the use of pure helium gas. Whether the same applies for the inactivation of biofilms is yet to be determined. Han et al. (2016) studied the effect of the voltage level on the inactivation of *Listeria monocytogenes* biofilms. Three voltage levels were testes, i.e., 60, 70, and 80 kV. The highest voltage showed the highest inactivation after a treatment time of 60 s. Han et al. (2016) also reported that when a higher voltage is applied, the concentration of reactive oxygen species in the plasma increases. This could be a possible explanation for the increased inactivation at higher voltage levels.

4.4. Advantages and disadvantages of cold atmospheric plasma

One of the major advantages of this novel approach for decontamination is the fact that CAP treatment is a waterless process, which gives this technology the image of being a green technology. Other advantages are: a low running cost, effective against gram-positive as well as gram-negative species, no chemical residues remain on the treated surface, and only a short treatment time is required. It should also be mentioned that the multidrug and chemical resistance of bacteria has increased over the last years. Cold atmospheric plasma treatment has the advantage that its inactivation mechanism relies on the synchronic effect of multiple reactive species, which makes it harder for the bacteria to develop resistance (Banu et al., 2012; Niemira, 2017).

The price of helium/argon is the biggest cost with respect to the running cost. However, it is also possible to create plasma with atmospheric air as feeding gas, which would reduce the running cost significantly. However the effectiveness of plasma created by atmospheric air has yet to be determined (Khan et al., 2017).

When CAP is used to treat food product surfaces, the food quality can be affected. It has been shown that the reactive species of the plasma also lead to oxidation of lipids, proteins, and carbohydrates of the food matrix. This may lead to a more rapid development of off flavours and unwanted colour changes, which decreases the shelf life. Consequently, further optimization studies and the understanding of the mechanism of CAP are crucial to incorporate this technology on industrial scale (Pankaj, Wan, & Keener, 2018).

5. PREDICTIVE FOOD MICROBIOLOGY

Predictive microbiology is an emerging discipline within food microbiology. The discipline uses mathematical functions to predict the behaviour (e.g., growth, inactivation, or toxin production) of microorganisms to specified environmental conditions (e.g., oxygen level, pH, and temperature) and relies on the fact that these behaviours are reproducible. This implies that based on observations from the past, a prediction can be made by making use of such mathematical functions (models) to predict behaviour in other similar environments. During the last years, these predictive models have evolved to become more exact due to the increasing computational resources and statistical packages. This is the main reason why there is a recent increase in the interest of these models. The predictive models have two main goals i.e., guarantee food safety and assure food quality. The final models could be implemented in HACCP procedures to identify critical control points. It should be mentioned, however, that these models only serve as a guide to predict responses of microorganisms to certain environmental factors. They do not replace laboratory analysis (Pérez-Rodríguez & Valero, 2013).

5.1. Types of models

Predictive models are generally divided into two categories, i.e., probabilistic and kinetic models. Probabilistic models are often referred to as growth/no growth models and describe whether microorganisms can grow under certain conditions. These types of models are based on the hurdle theory, which is a food preservation technique. It makes use of a combination of mostly mild treatments, such as the addition of preservatives, addition of organic acids, reduction of water activity, removal of oxygen, chilling and heating. Each of these treatments act as an obstacle (hurdle) for bacterial growth. The kinetic models are classified based on their complexity. One can distinguish three levels of complexity, i.e., (i) primary, (ii) secondary, and (iii) tertiary. Primary models describe the change in population density against time. The aim of these primary models is to describe these changes in population with as few parameters (e.g., inactivation rate, 'shoulder', and 'tail') as possible, while still being accurate. Secondary models describe how parameters of primary models are affected by environmental conditions. Tertiary models are computer applications in which primary and secondary models are integrated. These applications can be used by non-modelers and enable them to make predictions on microbial behaviour under different environmental conditions (Fakruddin, Mazumder, & Mannan, 2011; Pérez-Rodríguez & Valero, 2013).

Figure 6 shows the most commonly used models that describe the inactivation of foodborne pathogens. In this thesis, a primary shoulder/tail model developed by Geeraerd et al. (2000) will be used to predict the level of inactivation by cold plasma treatment. Such a model consists out of three phases, i.e., (i) shoulder, (ii) logarithmic order of destruction, and (iii) tailing. The shoulder means that there is initially no reduction at the beginning of the treatment. During the logarithmic destruction phase, the microorganisms are inactivated. After this phase, the curve may flatten out again, since some microorganisms are resistant to the applied dose. This last phase is called the tailing phase (Pérez-Rodríguez & Valero, 2013).

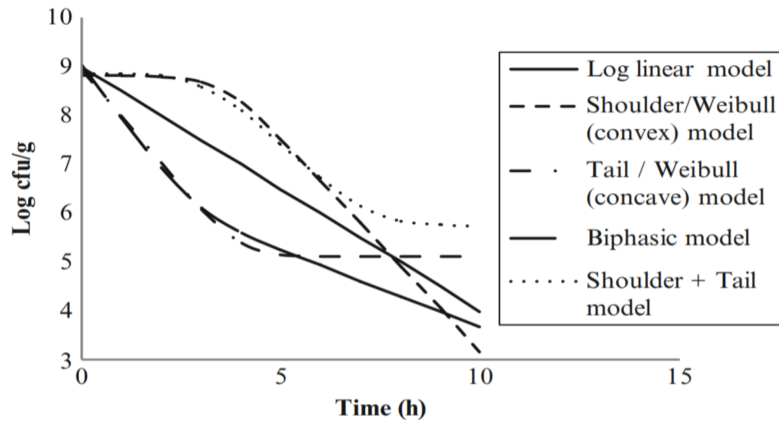


Figure 6: Commonly used models for the inactivation of foodborne pathogens (Pérez-Rodríguez & Valero, 2013)

The model developed by Geeraerd et al. (2000) is given by the following mathematical function:

$$N_t = [(N_0 - N_{res}) \cdot e^{(-k_{max} \cdot t)} \cdot \frac{e^{(-k_{max} \cdot t_l)}}{(1 + e^{((-k_{max} \cdot t_l) - 1)} \cdot e^{(-k_{max} \cdot t)})} + N_{res}]$$

with *N(t)* [CFU/mL], the number of bacterial cells that survived after a treatment with a duration *t* [min], N_0 [CFU/mL] is the initial amount of bacterial cells, N_{res} [CFU/mL] stands for the residual population, k_{max} [1/min] defines the maximum specific death rate, and t_l [min] is the duration of the initial lag phase (Pérez-Rodríguez & Valero, 2013).

6. MATERIALS AND METHODS

Every procedure regarding the inoculation of media and the recovery of cells from the biofilms should be performed under sterile conditions. This can be obtained by making use of a laminar flow cabinet or by working in the proximity of a Bunsen burner. For production of media and saline solutions, demineralized water was used.

6.1. Experimental design

In this thesis, two parameters that (possibly) influence the efficiency of the cold atmospheric plasma treatment are studied, i.e., the addition of oxygen to the helium feed gas and the design of the electrodes. In Section 4.3, it was mentioned that the addition of oxygen to a helium gas feed leads to an increase in the inactivation of planktonic cells during the CAP treatment (Kim et al., 2011). To study this effect on biofilms, three oxygen concentrations were used, 0.0%, 0.5%, and 1.0% (v/v). These oxygen concentrations were tested with two different electrodes, i.e., the dielectric-barrier discharge (DBD) and the surface-barrier discharge (SBD) electrode (see Section 4.1). Both electrodes created plasma at an input voltage of 13.88 V and frequency of 15 kHz. To study the effect of the treatment time, nine different treatment times within a range of 0 - 30 min. were applied for each combination of oxygen concentration and electrode type. After the CAP treatment, cells were recovered from the remaining biofilm for microbiological analysis. The obtained results were then fitted with the model of Geeraerd et al. (2000). For each possible combination of oxygen concentration, electrode type, and treatment time, at least two biological replicates were performed.

6.2. Microorganisms, preculture, and biofilm conditions

Two bacterial strains were used for the experiments, i.e., *Salmonella enterica* serovar Typhimurium LMG 14933 and *Listeria monocytogenes* LMG 23775. Both strains were acquired from the Belgium Co-ordinated Collections of Microorganisms (BCCM, Ghent, Belgium). The stock-cultures of these strains were stored at -80°C in Tryptic Soy Broth (TSB, Becton Dickinson, New Jersey, USA) supplemented with 20% (v/v) glycerol (VWR international, Pennsylvania, US). For every experiment, a new purity plate was prepared by inoculating a loopful of the stock-culture (*Salmonella* or *Listeria*) on Luria-Bretani (LB, Becton Dickinson, New Jersey, US) supplemented with 14 g/L technical agar (VWR international, Pennsylvania, US). Plates were incubated for 24 h at 30°C for *Listeria* and at 37°C for *Salmonella*. However, in some cases, it was needed to incubate the plates during the weekend at 20°C, which results in the same density of cells within one colony. After the incubation period, one colony from the purity plate is picked and transferred into a flask containing 20 mL LB medium, which is incubated for 24 h at 30°C or 37°C for *Listeria* and *Salmonella*, respectively. After 24 h of incubation, the precultures contain about 10^9 CFU/mL. These precultures are used to prepare the inoculum, which needs to contain about 10^7 CFU/mL. Consequently, the preculture needs to be 100x diluted. This is done by transferring 100 µl of homogenized preculture (by gently shaking) into a falcon tube (15 mL) containing 10 mL of dilution medium optimal for biofilm development. For *Listeria,* this medium is a Brain Heart Infusion broth (BHI, VWR international, Pennsylvania, US) and for *Salmonella,* it is a 20-fold diluted TSB. After vortexing the falcon tube, 1.2 mL of the inoculum is transferred into a sterile small polystyrene petri dish (diameter 5.5 cm). The inoculated petri dishes are then gently shaken to cover the entire

surface area and incubated for 24 h at 30°C (for *Listeria*) or 25°C (for *Salmonella)*. After the incubation period of 24 h, a biofilm is formed on the surface of the small petri dishes. The petri dishes are then emptied and rinsed 3 times with sterile Phosphate Buffered Saline (PBS) solution. After the rinsing process, the petri dishes are placed in the laminar flow cabinet to dry. When the petri dishes are dry, they are ready to be treated with cold atmospheric plasma.

6.3. Cold atmospheric plasma set up and treatment procedure

6.3.1. Plasma set up

Figure 7 shows the cold atmospheric plasma set-up. The set-up consists out of six essential components, i.e., (i) a DC power supply (PSP-603, GW Instek), (ii) an oscilloscope (DSO1052B, Agilent technologies), (iii) a function generator (TG1000, AIM-TTI), (iv) a plasma power source, (v) a gas flow meters, and (vi) a plasma reactor with either an SBD or DBD electrode. The DBD reactor (dimensions: 22.5 cm x 13.5 cm x 10 cm) consists out of two electrodes (diameter 5.5 cm), covered with a dielectric layer (diameter 7.5 cm). The gap between the two electrodes is 0.8 cm. The SBD reactor (dimensions: 10 cm x 10 cm x 4 cm) consists out of two electrodes (diameter 5.0 cm) separated by a dielectric layer (diameter 6.0 cm). Both reaction chambers are not fully airtight, as there is a small hole present. This hole is necessary to avoid overpressure, as the flow of the feed gas is continuous. Three feed gas compositions were used to create the plasma, i.e. (i) pure helium gas (purity ≥ 99.996%), (ii) helium gas with an addition of 0.5% (v/v), and (iii) a mixture of helium gas with 1.0% (v/v) oxygen (purity ≥ 99.995%). The helium flow was kept constant at 4 L/min.

To create the helium-oxygen mixtures of 0.5% and 1.0% (v/v), an oxygen gas flow of 20 mL/min and 40 mL/min were added to the 4 L/min of helium, respectively. These two flows were mixed before entering the plasma reactor. Finally, the plasma was generated at an input voltage of 13.88 V (DC) which is transformed by the plasma power source into a high voltage alternating current, with a frequency of 15 KHz.

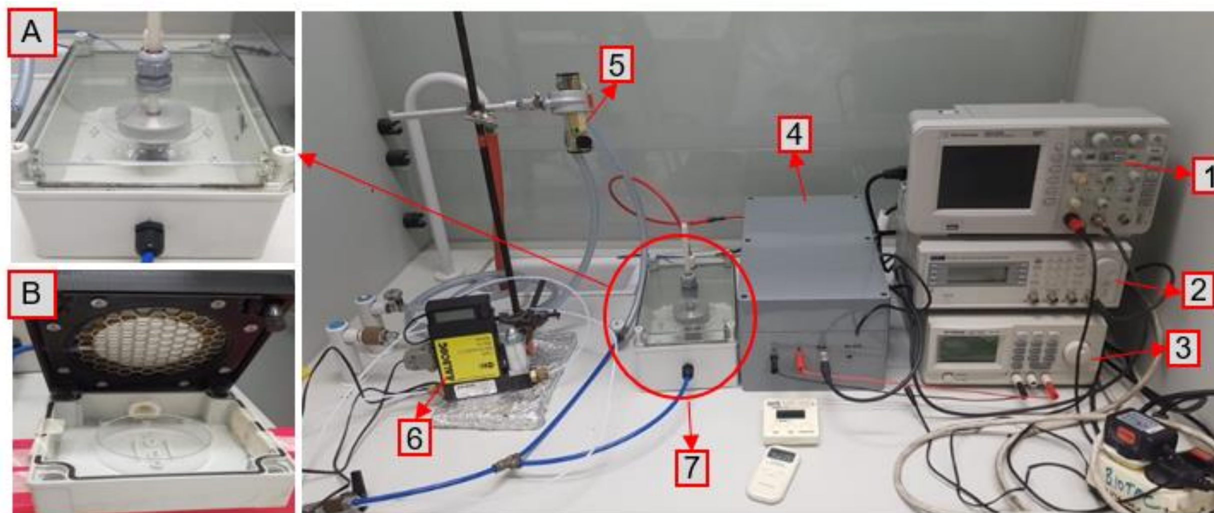


Figure 7: The plasma set-up with: (1) oscilloscope, (2) function generator, (3) DC power supply, (4) plasma power source, (5) helium flow meter, (6) oxygen glow meter, (7) plasma reactor, which can either be the (A) DBD or (B) SBD reactor

6.3.2. Treatment procedure

Prior to treatment, the lid from the petri dish is removed and the petri dish is put inside the reaction chamber (only one petri dish can be placed in the reaction chamber). When using the DBD reaction chamber, the dish is placed in between the two electrodes. When using the SBD reaction chamber, it is placed inside, at a distance of 1.0 cm below the electrode. When the reaction chamber is closed, the background air must be removed by means of flushing with the required feeding gas mixture. The DBD and the SBD reaction chambers are flushed for 4 and 2 minutes, respectively. After the flushing procedure, the plasma power source is energized to generate the plasma. The samples were treated up to 30 minutes. During the treatment, the plasma outputs i.e., voltage (peak to peak), current, and plasma power, are monitored. The values of these output parameters for each combination of feed gas and electrode type (when an input voltage of 13.88 V is applied) are shown in Table 2.

Table 2: The values for the plasma output parameters for each combination of feed gas and electrode type (standard deviations were calculated based on 4 repetitions)

Electrode	Oxygen percentage (v/v)	Voltage (peak to peak) (kV)	Plasma power (W)	Current (A)
DBD	0.0	4.0 ± 0.2	4.1 ± 0.1	0.298 ± 0.008
	0.5	5.1 ± 0.1	5.27 ± 0.05	0.384 ± 0.003
	1.0	6.2 ± 0.3	6.0 ± 0.3	0.44 ± 0.02
SBD	0.0	2.34 ± 0.08	2.1 ± 0	0.1525 ± 0.0005
	0.5	2.65 ± 0.05	2.40 ± 0.09	0.180 ± 0.002
	1.1	2.80 ± 0.01	2.6 ± 0	0.189 ± 0.001

6.4. Cell recovery and microbiological analysis

The cell density of the biofilm after a certain treatment time was determined by means of plate counting on both general and selective media. Before the viable plate counting procedure can be performed, the cells inside the biofilm must be recovered. This was done by adding 2 mL of sterile PBS into the petri dish and scraping the cells loose by using a cell scraper (blade width 20 mm, Carl Roth, Karlsruhe, Germany). The obtained suspension (of cells in PBS) will be used to make a serial decimal dilution. This is done by diluting the obtained suspension five consecutive times by transferring 100 µL into an Eppendorf containing 900 µL of sterile saline solution (0.85% (w/v) NaCl, Sigma-Aldrich, Missouri, US). Before a dilution is made, the Eppendorf is vortexed to homogenize the suspension. For each dilution, including the non-diluted sample, three drops of 20 µL are plated on general and selective agar plates. The general and selective agar plates for *Salmonella* contain TSB supplemented with 14 g/L technical agar and Xylose Lysine Deoxycholate-agar (XLD, Merck & Co, New Jersey, US), respectively. In the case of *Listeria,* these contain BHI supplemented with 14 g/L technical agar and PALCAM-agar (VWR international, Pennsylvania, US), respectively. After plating the dilutions, the petri dishes were incubated for 1 day at 37°C for *Salmonella* or at 30°C for *Listeria*. After the incubation period, plates which contained between 10 and a 100 CFU per drop of 20 µL, were counted.

6.5. Modelling, parameter estimation, and estimation of sublethal injury

Experimental data were collected in Matlab (The Mathworks inc.) and were fitted with the predictive model developed by Geeraerd et al. (2000). The model describes a microbial inactivation curve consisting out of three phases i.e., (i) shoulder, (ii) logarithmic order of destruction, and (iii) a tail (see Section 5.1). The model is given by the following mathematical function:

$$N_t = [(N_0 - N_{res}) \cdot e^{(-k_{max} \cdot t)} \cdot \frac{e^{(-k_{max} \cdot t_l)}}{(1 + e^{((-k_{max} \cdot t_l) - 1)} \cdot e^{(-k_{max} \cdot t)})} + N_{res}]$$

with *N(t)* [CFU/mL], the number of bacterial cells that survived after a treatment with a duration *t* [min], N_0 [CFU/mL] is the initial amount of bacterial cells, N_{res} [CFU/mL] stands for the residual population, k_{max} [1/min] defines the maximum specific death rate, and t_l [min] is the duration of the initial lag phase (Pérez-Rodríguez & Valero, 2013).

To fit experimental data with the model, the parameters of the model need to be estimated. This was obtained via the minimization of the sum of the squared errors, using the *lsqnonlin* routine of the Optimization Toolbox of Matlab. The standard errors of these estimated parameters were determined based on the Jacobian matrix (these were achieved simultaneously with the parameter estimation). In addition, the Root Mean Square Error (RMSE) was added, as indicator for the goodness of the fit.

As mentioned in the previous section, treated biofilms were plated on both non-selective (general) and selective media. This makes it possible to determine the percentage of sublethal injury (%SI) as function of the time. The sublethal injury can be calculated by the following formula:

$$\%\mathrm{SI} = \frac{(\text{counts on general medium } - \text{ counts on selective medium counts})}{\text{counts on general medium}} \cdot 100\%$$

Theoretical data obtained from the Geeraerd et al. (2000) was used to calculate the %SI. By using the theoretical data, it is possible to calculate (estimate) the %SI at non-tested treatment times.

6.6. Statistical analysis

To determine whether there were significant differences amongst means of the estimated model parameters, an analysis of variance test was performed at a 95.0% ($\alpha = 0.05$) confidence level. To distinguish which means were significantly different from others, a post hoc test was performed, i.e., Fisher's Least Significant Difference (LSD) test. These analyses were performed using the Statistical Toolbox of Matlab (The Mathworks Inc., version R2015b).

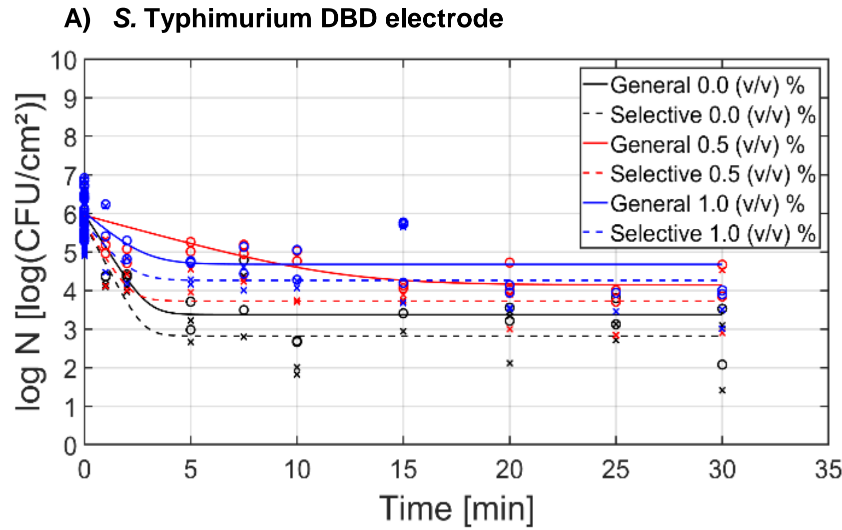
Different ANOVA tests were performed for each parameter of the Geeraerd et al. (2000) inactivation model, and for each microorganism separately. For each oxygen concentration and each medium type, a separate test has been performed to study the influence of the type of electrode (significant differences were indicated with a different capital letter). For each type of electrode and each medium type, a separate test has been performed to study the influence of the oxygen concentration (significant differences were indicated with a different small letter).

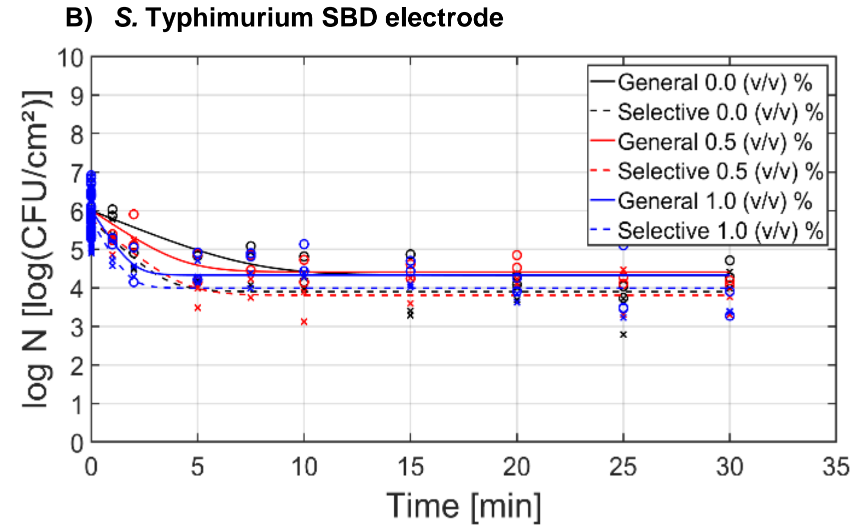
7. RESULTS AND DISCUSSION

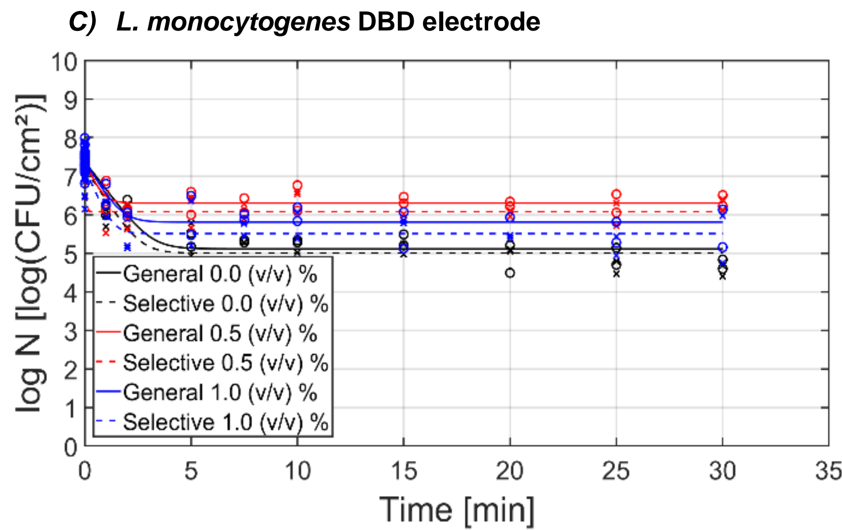
7.1. Inactivation curves and the corresponding parameters

Figure 8 represents the inactivation curves for biofilms developed by *S.* Typhimurium (A and B) or *L. monocytogenes* (C and D) exposed to CAP treatment. These inactivation curves were obtained by fitting the experimental data with the Geeraerd et al. (2000) model (see Section 6.5). Figure 8A and 8B show the results when biofilms of *S.* Typhimurium are treated using the DBD and SBD electrode, respectively. Figure 8C and 8D show the same for biofilms developed by *L. monocytogenes.* The results obtained for the different types of feed gasses (i.e., pure helium and helium with an addition of 0.5% and 1% (v/v) oxygen), are shown in black, red, and blue, respectively. The experimental data is represented in the graphs with the following symbols: O, for counts on the general medium and X, for counts on the selective medium. The fit with the Geeraerd et al. (2000) model is given by a solid curve for the general medium and by a dashed curve for the selective medium. The corresponding inactivation parameters of the Geeraerd et al. (2000) model, i.e., the initial cell density (log N_0), the inactivation rate (k_{max}), the cell density in the tail (log N_{res}), and the corresponding statistical analysis are summarized for all tested conditions in Table 3 (*S.* Typhimurium) and Table 4 (*L. monocytogenes*). These two tables also show the overall log reduction (log N_0 - log N_{res}) with the corresponding statistical analysis and the Root Mean Square Errors (RMSE) for each tested condition.

When in the following paragraphs significant differences between the usage of different electrode types or different oxygen levels are discussed, and the type of medium is not specified, then these differences account for both media types. In addition to this, only $\log_{10}$ reductions will be mentioned for the general media, but the same can be concluded for the $\log_{10}$ reductions obtained for the selective media.







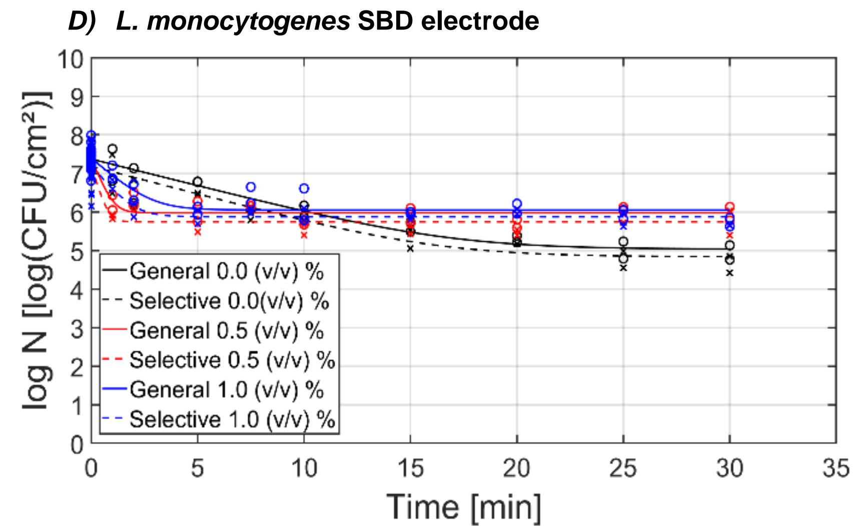


Figure 8: Graph A and B represent the inactivation curves for S. Typhimurium *biofilms, using the DBD and SBD to generate the CAP respectively. Graph C and D show the same for biofilms developed by L. monocytogenes. Each graph shows the logarithm of the cell density [log(CFU/cm^2)] as function of the treatment time [min]. The counts on the general medium are represented by an 'O', and the counts on the selective medium are given by an 'X'. The Geeraerd et al. (2000) fit of these counts is, respectively, given by a solid and a dashed curve. The three colours represent the results of the different feed gases that were tested, i.e., black for the pure helium, red for the helium with addition of 0.5% (v/v) oxygen, and blue for the helium with addition of 1% (v/v) oxygen.*

Table 3: Estimated inactivation parameters of the Geeraerd et al. (2000) model, overall log reduction, and RMSE value for all tested conditions for S. Typhimurium *biofilms.*

Electrode Type	Oxygen pct. ((v/v) %)	Medium	[1]Log$_{10}$ N_0[2] (log(CFU/cm^2))	[1]k_{max}[2] (1/min)	[1]Log$_{10}$ N_{res}[2] (log(CFU/cm^2))	[1]Log$_{10}$-reduction[2] (log(CFU/ cm^2)	RMSE
DBD	0.0	General	$^{A}6.0 \pm 0.1^{a}$	$^{B}2.4 \pm 0.6^{b}$	$^{A}3.4 \pm 0.2^{a}$	$^{B}2.6 \pm 0.2^{a}$	0.5533
		Selective	$^{A}5.74 \pm 0.09^{a}$	$^{B}2.4 \pm 0.6^{a}$	$^{A}2.8 \pm 0.2^{a}$	$^{B}2.9 \pm 0.2^{a}$	0.6532
	0.5	General	$^{A}5.95 \pm 0.08^{a}$	$^{A}0.35 \pm 0.09^{a}$	$^{A}4.1 \pm 0.2^{b}$	$^{A}1.8 \pm 0.2^{b}$	0.4932
		Selective	$^{A}5.7 \pm 0.1^{a}$	$^{A}2.2 \pm 0.7^{a}$	$^{A}3.7 \pm 0.2^{b}$	$^{A}2.0 \pm 0.2^{b}$	0.6021
	1.0	General	$^{A}6.02 \pm 0.09^{a}$	$^{A}1.1 \pm 0.6^{a}$	$^{B}4.7 \pm 0.2^{c}$	$^{A}1.3 \pm 0.2^{c}$	0.5510
		Selective	$^{A}5.8 \pm 0.1^{a}$	$^{A}1.6 \pm 0.8^{a}$	$^{A}4.3 \pm 0.2^{c}$	$^{A}1.5 \pm 0.2^{c}$	0.6964
SBD	0.0	General	$^{A}6.02 \pm 0.08^{a}$	$^{A}0.5 \pm 0.2^{a}$	$^{B}4.3 \pm 0.2^{a}$	$^{A}1.7 \pm 0.2^{a}$	0.4656
		Selective	$^{A}5.8 \pm 0.1^{a}$	$^{A}1.2 \pm 0.5^{a}$	$^{B}3.9 \pm 0.2^{a}$	$^{A}1.9 \pm 0.2^{a}$	0.4600
	0.5	General	$^{A}6.01 \pm 0.08^{a}$	$^{B}0.9 \pm 0.3^{a}$	$^{A}4.4 \pm 0.2^{a}$	$^{A}1.6 \pm 0.2^{a}$	0.4510
		Selective	$^{A}5.76 \pm 0.09^{a}$	$^{A}1.0 \pm 0.4^{a}$	$^{A}3.8 \pm 0.2^{a}$	$^{A}2.0 \pm 0.2^{a}$	0.5526
	1.0	General	$^{A}6.02 \pm 0.09^{a}$	$^{A}2.0 \pm 0.7^{b}$	$^{A}4.3 \pm 0.2^{a}$	$^{A}1.7 \pm 0.2^{a}$	0.5242
		Selective	$^{A}5.75 \pm 0.09^{a}$	$^{A}2.2 \pm 0.8^{a}$	$^{A}4.0 \pm 0.2^{a}$	$^{A}1.8 \pm 0.2^{a}$	0.5514

[1] Effect of electrode type: for each oxygen percentage and medium, model parameters bearing different superscripts (no uppercase capital letters in common) are significantly different (P ≤ 0.05)
[2] Effect of oxygen percentage: for each electrode type and medium, model parameters bearing different superscripts (no uppercase small letters in common) are significantly different (P ≤ 0.05)

Table 4: Estimated inactivation parameters of the Geeraerd et al. (2000) model, overall log reduction, and RMSE value for all tested conditions for L. monocytogenes biofilms.

Electrode Type	Oxygen pct. ((v/v) %)	Medium	[1]Log10 N_0[2] (log(CFU/cm^2))	[1]k_{max}[2] (1/min)	[1]Log10 N_{res}[2] (log(CFU/cm^2))	[1]Log10-reduction[2] (log(CFU/cm^2))	RMSE
DBD	0.0	General	$^{A}7.38 \pm 0.05^{a}$	$^{B}1.7 \pm 0.3^{a}$	$^{A}5.11 \pm 0.08^{a}$	$^{A}2.3 \pm 0.1^{c}$	0.2911
		Selective	$^{A}7.20 \pm 0.07^{a}$	$^{B}1.9 \pm 0.4^{a}$	$^{A}5.0 \pm 0.1^{a}$	$^{A}2.2 \pm 0.2^{c}$	0.3838
	0.5	General	$^{A}7.40 \pm 0.05^{a}$	$^{A}3.1 \pm 1.3^{a}$	$^{B}6.30 \pm 0.07^{c}$	$^{A}1.09 \pm 0.08^{a}$	0.2495
		Selective	$^{A}7.21 \pm 0.07^{a}$	30 ± inf*	$^{B}6.08 \pm 0.09^{c}$	$^{A}1.1 \pm 0.1^{a}$	0.3664
	1.0	General	$^{A}7.40 \pm 0.05^{a}$	$^{B}2.1 \pm 0.5^{a}$	$^{A}5.80 \pm 0.08^{b}$	$^{B}1.6 \pm 0.1^{b}$	0.2937
		Selective	$^{A}7.22 \pm 0.07^{a}$	$^{A}3.1 \pm 0.9^{a}$	$^{A}5.5 \pm 0.1^{b}$	$^{B}1.7 \pm 0.2^{b}$	0.3906
SBD	0.0	General	$^{A}7.40 \pm 0.05^{a}$	$^{A}0.32 \pm 0.03^{a}$	$^{A}5.0 \pm 0.1^{a}$	$^{A}2.3 \pm 0.2^{b}$	0.2700
		Selective	$^{A}7.20 \pm 0.06^{a}$	$^{A}0.33 \pm 0.05^{a}$	$^{A}4.8 \pm 0.2^{a}$	$^{A}2.3 \pm 0.2^{b}$	0.3691
	0.5	General	$^{A}7.40 \pm 0.05^{a}$	$^{A}3.1 \pm 0.8^{b}$	$^{A}5.98 \pm 0.07^{b}$	$^{B}1.42 \pm 0.08^{a}$	0.2508
		Selective	$^{A}7.21 \pm 0.06^{a}$	4 ± 2^{b}	$^{A}5.74 \pm 0.09^{b}$	$^{B}1.5 \pm 0.1^{a}$	0.3458
	1.0	General	$^{A}7.40 \pm 0.05^{a}$	$^{A}1.1 \pm 0.3^{a}$	$^{B}6.05 \pm 0.07^{b}$	$^{A}1.35 \pm 0.09^{a}$	0.2586
		Selective	$^{A}7.22 \pm 0.06^{a}$	$^{A}1.6 \pm 0.5^{ab}$	$^{B}5.87 \pm 0.09^{b}$	$^{A}1.3 \pm 0.1^{a}$	0.3248

[1] Effect of electrode type: for each oxygen percentage and medium, model parameters bearing different superscripts (no uppercase capital letters in common) are significantly different (P ≤ 0.05)
[2] Effect of oxygen percentage: for each electrode type and medium, model parameters bearing different superscripts (no uppercase small letters in common) are significantly different (P ≤ 0.05)
* As no standard deviation was obtained, no statistical analysis was performed for this result

7.1.1. General observations

Based on the inactivation curves in Figure 8 and the estimated model parameters in Table 3 and Table 4, several general conclusions can be drawn independent of the tested CAP treatment conditions.

As mentioned in Section 5.1, the Geeraerd et al. (2000) model is a shoulder and tail type of inactivation model. However, the inactivation curves in Figure 8 do not show a shoulder phase, which implies that the bacterial cells within the biofilm are inactivated/injured as soon as the treatment with CAP starts. Therefore, all inactivation curves are fitted with the Geeraerd et al. (2000) model containing only a log-linear inactivation phase and a tail. The tailing phase is always present, which implies that a subpopulation is resistant to the CAP treatment. The existence of a resistant subpopulation can be explained either by a vitalistic or a mechanistic point of view (theory). The vitalistic theory states that there is genetic diversity within a population, and due to this genetic diversity, different levels of resistance against the treatment can be observed within a population. However, according to the mechanistic theory, which is based on a homogeneous point of view (no genetic differences within the population), the existence of a resistant subpopulation is explained by adaptation of some of the microorganisms to the treatment or by the effect of shielding. The effect of shielding is likely to occur within a biofilm, i.e., the matrix and the bacterial cells near the surface of the biofilm may serve as a shield (against the reactive species in the plasma) for the cells in the bottom layer of the biofilm (Bevilacqua et al., 2015).

In Table 3, Table 4, and Figure 8, it can be observed that the initial cell density (log N_0) of the *L. monocytogenes* biofilm is higher compared to the one obtained for the *S.* Typhimurium biofilm. However, when the initial cell densities are compared within one species, no significant differences can be observed between the different tested plasma conditions. This was to be expected, as the biofilms at t = 0 min are not treated yet, and thus, not influenced by the CAP treatment conditions. Consequently, it can be concluded that significant differences obtained for log N_{res} will also be found in the overall reductions, as these are calculated by log N_0 - log N_{res}.

It can be observed in Figure 8 that the log-linear inactivation phase of most of the inactivation curves ends before a treatment time of five minutes is reached. This implies that the final log reduction is achieved relatively fast. This is very important to stress out since this means that CAP treatment is a very low time-consuming process.

In Figure 8 as well as in Table 3 and 4, can be observed that for each tested condition, the cell density in the tail (log N_{res}) is lower for the selective medium compared to the general media. This indicates the presence of sublethally injured cells, as only these cells can grow on the general medium and not on the selective medium. In other words, the results of the general medium correspond with the total viable population, whereas the results of the selective medium correspond with the uninjured viable population. The (percentage of) sublethal injury is further discussed in Section 7.2.

Finally, in Table 3 and Table 4, there can be observed that the RMSE values are overall higher for *S.* Typhimurium biofilms compared to the ones obtained for *L. monocytogenes* biofilms. This is due to the higher variation in the results of the viable plate counting in the case of *S.* Typhimurium.

7.1.2. Effect of electrode type on CAP inactivation efficiency

In case of *S.* Typhimurium biofilms treated with CAP, significant differences for the inactivation parameters k_{max} and log N_{res} can be observed between the two tested electrodes (Table 3). However, this influence is dependent on the used oxygen level. Without addition of oxygen, the value of k_{max} is higher and the value of log N_{res} is lower when using the DBD electrode compared to using the SBD electrode. The lower value of log N_{res} in case of using the DBD electrode implies that a higher reduction was obtained using this electrode compared to when the SBD electrode was used. The differences between the k_{max} values can also be observed in Figure 8A and 8B, as k_{max} represents the slope of the log-linear inactivation phase. As the value of k_{max} is higher in case of the DBD electrode, the slope of the log-linear inactivation phase is steeper compared to when the SBD electrode is used. This implies that the final log reduction is achieved faster when the DBD electrode is used to generate the plasma. When helium with an addition of 0.5% or 1.0% (v/v) oxygen is used, no significant differences between the inactivation parameters for both electrodes can be observed.

In case of the *L. monocytogenes* biofilms, a significant difference for the value of k_{max} is observed when pure helium gas is used (Table 4). The value of k_{max} is higher in case of the DBD electrode. However, at this oxygen concentration, no significant influence of the electrode on the value of log N_{res} can be observed. This implies that due to the smaller value of k_{max} in case of the SBD electrode, a longer treatment time is needed to reach the same value of log N_{res} compared to the DBD electrode. This can be seen in Figure 8C and 8D as well, i.e., in case of the DBD electrode, a treatment time of approximately 5 minutes is needed to reach log N_{res} and in case of the SBD electrode, a treatment time of approximately 20-25 minutes is needed to reach the same value of log N_{res}. When helium gas with addition of 0.5% or 1.0% (v/v) oxygen is used, no significant differences in the value of k_{max} can be observed. However, when helium gas with addition of 0.5% (v/v) oxygen is used, a higher overall reduction (lower log N_{res}) was obtained using the SBD electrode, whereas in case of using helium gas with an addition of 1% (v/v) oxygen, the highest reduction was obtained using the DBD electrode. In other words, the effect of the electrode is dependent on the applied oxygen concentration.

The SBD electrode was expected to be less efficient than the DBD electrode. This hypothesis is based on the fact that in case of the SBD electrode, the biofilm is placed underneath the CAP generated field. This implies that the reactive species within the CAP need to be transported to the biofilm. During this transport, the reactive species in the plasma might collide with each other, creating less reactive species, resulting in a less efficient inactivation compared to the DBD electrode. In the latter case, the biofilm is placed within the CAP generated field (Niemira, 2012). Overall, the results for both treated *S.* Typhimurium and *L. monocytogenes* biofilms are in line with this hypothesis, except for the results for CAP treatment of the *L. monocytogenes* biofilms that were obtained using the SBD electrode in combination with 0.5% (v/v) oxygen (i.e. a reduction of 1.42 ± 0.08 (on the general media) was achieved by using the SBD electrode, whereas a reduction of 1.09 ± 0.08 (on general media) was obtained when using the DBD electrode).

7.1.3. Effect of oxygen percentage on CAP inactivation efficiency

When biofilms of *S.* Typhimurium are treated with CAP using the DBD electrode, statistical differences between the values of log N_{res} are observed for the different oxygen percentages. When the oxygen percentage in the helium feed gas increases, the value of log N_{res} increases

(which implies lower overall reductions). This can be observed in Table 3 as well as in Figure 8. In case of the SBD electrode, no significant differences between the values of log N_{res} are observed for the different oxygen percentages. For both electrodes, the value of k_{max} does not seem to be affected by the different oxygen percentages that were tested.

In case of *L. monocytogenes* biofilms treated with CAP using the DBD electrode, statistical differences between the values of log N_{res} are observed for the different oxygen percentages (Table 4). The highest value of log N_{res}, and thus the lowest overall reduction, was obtained by using 0.5% (v/v) oxygen in the helium feed gas. The second highest value of log N_{res} was obtained by using 1.0% (v/v) oxygen and the lowest log N_{res} was achieved using pure helium gas. In case of the SBD electrode, the lowest log N_{res} was also achieved by using pure helium gas. However, between the usage of 0.5% and 1.0% (v/v) oxygen, no statistical differences were obtained for the value of log N_{res}. The same conclusion regarding the parameter k_{max} can be made as in case of *S.* Typhimurium, as for both electrodes, it does not seem to be affected by the different oxygen levels that were tested.

Higher reductions (lower values of log N_{res}) were expected when gas mixtures of oxygen and helium were used. This hypothesis is based on studies which performed tests on planktonic cells (Section 4.3) (Bourke et al., 2017). However, the results in this thesis show the opposite effect when biofilms are treated with CAP, i.e., the highest reductions were obtained by using pure helium gas (i.e., general medium log-reductions obtained by using helium with an addition of 0.0%, 0.5% and 1.0% (v/v) oxygen range (depending on the used electrode and microorganism) between 1.5-2.6, 1.01-2.0, and 1.1-1.9 respectively). This opposite effect is hard to explain since the inactivation mechanism of CAP is still under investigation. However, there is a high possibility that the extracellular matrix is responsible for this effect, as it may act as a shield against (some of) the reactive species in the plasma. The inactivation mechanism could be further investigated by means of performing a variety of tests such as (i) optical emission spectroscopy to identify the intensity of CAP species, (ii) scanning electron microscopy to visualise damage to the cells/matrix, (iii) studying the membrane integrity by analysing the amount of intercellular proteins in the supernatant, (iv) studying DNA damage (e.g., by using SYBR-green), and (iv) detecting ROS after the treatment using fluorescent probes (e.g., dichlorodihydrofluorescein) (Han et al., 2016).

7.1.4. Optimal combination of electrode type and oxygen percentage

In case of the *S.* Typhimurium biofilms, the highest reduction was achieved by using the DBD electrode in combination with pure helium gas. Using this combination, an overall log reduction of 2.4 ± 0.2 (on the general medium) was obtained within a treatment time of approximately 4-5 minutes (Figure 8A).

In case of the *L. monocytogenes* biofilms, the highest reduction was achieved by both the DBD and SBD electrode in combination with pure helium gas. By using these combinations, an overall log reduction of 2.3 ± 0.2 (on the general medium) was achieved. However, in case of the DBD electrode, this reduction was achieved faster (within 4-5 min) compared to when the SBD electrode was used (20-25 min). Due to the shorter treatment time that is needed to achieve the final log reduction in case of the DBD electrode, this electrode would be preferred.

These obtained log reductions are not high enough to assure food safety. For example, pasteurisation and sterilization processes aim for 6 or 12 log reductions, respectively, to assure food safety. Compared to these processes, CAP treatment on his own is insufficient. On the

positive side, the (relatively low) final reduction is achieved within only a few minutes. This is promising as this would imply only short periods of equipment downtime if this technology were to be used in the food industry. However, before this technology can be realised on industrial scale, higher reductions need to be achieved (within a similar or shorter treatment time as of now). To achieve higher reductions, higher voltage levels could be tested, although, this suggestion is based on studies performed on planktonic cells, which reported to achieve higher reductions when using higher voltage levels (Han et al., 2016). Consequently, there are no guarantees that the same effect will be found when biofilms are treated. There is also the possibility to include a pre- or post-treatment step. For example, Helgadóttir et al. (2017) reported that higher reductions were achieved when biofilms were treated with vitamin C for 15 minutes prior to the CAP treatment. Besides vitamin C, relatively low concentrations of chemical agents such as hydrogen peroxide or other antimicrobial agents could be tested as a pre- or post-treatment step. Nonetheless, when including these additional treatments, the overall advantage of using CAP technology decreases.

Based on the above-mentioned results, it can be concluded that similar overall reductions, within a similar treatment time, were obtained for both species when using the DBD electrode in combination with 0% (v/v) oxygen in the feed gas. This indicates that the treatment under these conditions is evenly effective against gram-positive and gram-negative species. But to confirm this indication, a larger variety of gram-negative and gram-positive species should be tested. It should be mentioned that although similar log reductions for *L. monocytogenes* and *S.* Typhimurium biofilms are obtained, there is still a difference in the value of N_{res}. This is due to the difference in initial cell densities between *L. monocytogenes* and *S.* Typhimurium biofilms. Consequently, remaining *L. monocytogenes* cells would still pose a higher risk for contamination of food products.

7.2. Sublethal injury

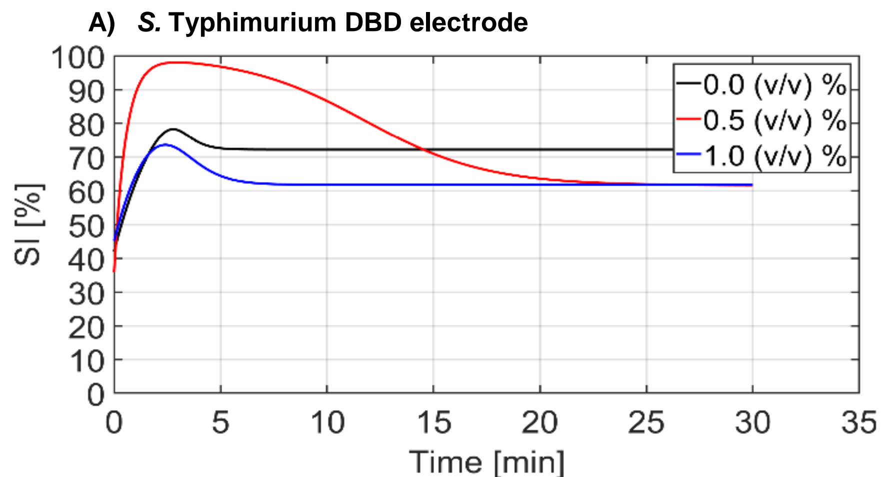
To investigate the sublethal injury, viable plate counting was performed on both general and selective media. The percentage of sublethal injury was calculated by using the theoretical data of the Geeraerd et al. (2000) model, which made it possible to estimate the percentage of sublethal injury at any given time within the tested treatment time range (0-30 min). Figure 9 shows the percentage of sublethal injury as function of the treatment time for all tested conditions.

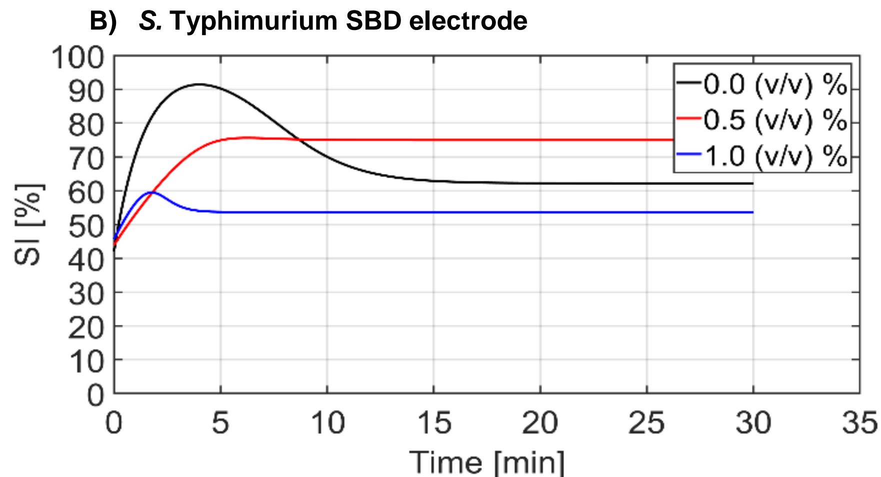
It appears that sublethally injured cells are already present before CAP treatment (t = 0 min). This initial percentage of sublethal injury is about 45% for *S.* Typhimurium biofilms (Figure 9A and 9B) and about 35% for *L. monocytogenes* biofilms (Figure 9C and 9D). To the best of the authors knowledge, no explanation can be found in literature concerning the presence of sublethally injured cells in (non-treated) biofilms. However, studies have shown that community life can be stressful for bacterial cells due to accumulation of waste products (e.g., acids), nutrient competition, and a non-uniform distribution of oxygen (Serra & Hengge, 2014). These stressing conditions are a potential explanation for the presence of sublethally injured cells in a biofilm. Based on this hypothesis, the difference in sublethal injury between *S.* Typhimurium biofilms and *L. monocytogenes* biofilms could be explained due to the higher (general) stress resistance of the latter species, which results in a lower percentage of SI. For example, Koutsoumanis & Sofos (2004) reported that *L. monocytogenes* is more acid resistant than *S.* Typhimurium. Thus, when waste products such as acids accumulate in the biofilm, *L. monocytogenes* will be less affected (less injured cells) at a certain concentration, compared to the *S.* Typhimurium biofilms.

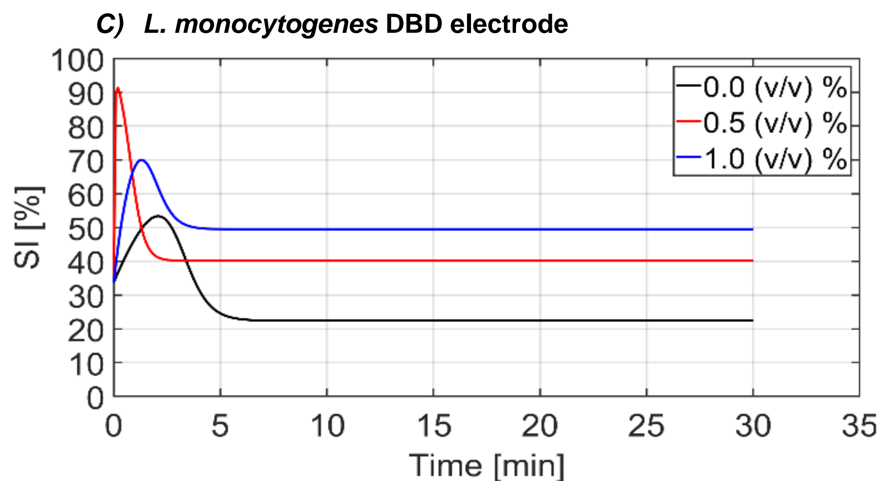
Most of the curves in Figure 9 show an increase in sublethal damage at the beginning of the treatment, followed by a maximum, after which the sublethal damage decreases again. At the end of this decrease, the SI remains at a constant value. The point of the maximum percentage of sublethal injury coincides with the transition from the log-linear inactivation phase to the tailing phase of the inactivation curves in Figure 9. During the tailing phase, the SI remains at a constant value. The general trend of the curves (increase-max-decrease) suggest a mechanism of injury accumulation, which eventually results in cell lysis (Noriega et al., 2013).

Regarding the use of different electrodes, no general trends can be observed concerning their effect on the percentage of SI. When using different oxygen levels, also no general trend can be observed when the DBD electrode is used. However, there seems to be a general trend when different oxygen levels are used in combination with the SBD electrode (Figure 9B and 9D). For both microorganisms, the highest value of SI at the end of the treatment was achieved using 0.5% (v/v) oxygen in the feed gas, followed by using pure helium gas. Finally, the lowest percentage of sublethal injury was achieved using 1.0% (v/v) oxygen (in helium).

The residual percentages of SI for the treated *S.* Typhimurium and *L. monocytogenes* biofilms ranges between approximately 50-75% and 20-50%, respectively, depending on the tested conditions. It should be mentioned that these sublethally injured cells still pose a risk for the food safety as these cells might recover from their injuries. Thus, the presence of these cells should not be overlooked.







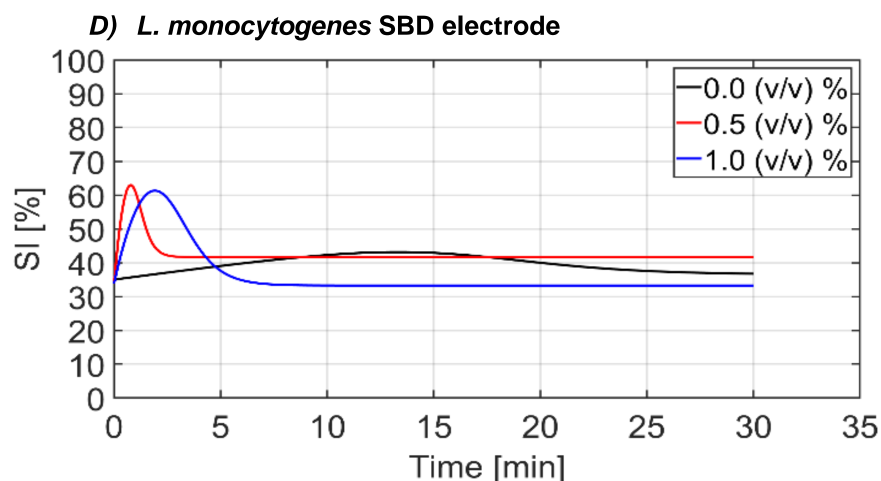


Figure 9: Graph A and B represent the percentage of sublethal injury [%] as function of the treatment time [min] (with CAP) for S. Typhimurium *biofilms, using the DBD and SBD to generate the CAP respectively. Graph C and D show the same for biofilms developed by L. monocytogenes.*

8. CONCLUSION

The goal of this research was to study the effect of cold atmospheric plasma on biofilms developed by *S.* Typhimurium and *L. monocytogenes*. To study this effect, two different electrodes were tested in combination with three types of feed gas mixtures. Viable plate counting was performed on treated biofilms and the results were fitted with the Geeraerd et al. (2000) predictive model. By plating on both media, it was also possible to investigate the sublethal injury. Based on the obtained results, the following main conclusions could be drawn.

None of the inactivation curves that were obtained by fitting the results of the viable plate counts with the inactivation model developed by Geeraerd et al. (2000) showed a shoulder phase, which implies that the cells are inactivated/injured as soon as the treatment starts. However, a tailing phase was always present, which means that there is always a more resistant sub-population remaining on the surface.

Regarding the use of different types of electrodes on the efficiency of the CAP treatment, there can be concluded that this difference in efficiency between these electrodes is dependent on the used feed gas composition. Namely, when pure helium gas was used, the DBD electrode was more efficient compared to the SBD electrode, whereas when pure helium gas was used with an addition of 0.5% or 1.0% (v/v) oxygen, no general trend can be observed. Concerning the use of different compositions of feed gases, it could be established that the usage of pure helium gas resulted in the most efficient CAP treatments. This implies that the most efficient CAP treatments were achieved when using the DBD in combination with pure helium gas, and this for both microorganisms. Using this combination, overall log reductions of 2.4 ± 0.2 and 2.3 ± 0.2 (on general media) were achieved within 5 minutes of CAP treatment for *S.* Typhimurium and *L. monocytogenes* biofilms, respectively. The fact that the (relatively low) final reduction is achieved within only a few minutes is promising, as this would imply short periods of equipment downtime, if this technology were to be used in the food industry. However, before this technology can be realised on industrial scale, higher reductions need to be achieved (within a similar or shorter treatment time as of now). To achieve higher reductions, higher voltage levels could be tested, and/or a pre/post-treatment step could be implemented. The similar log reductions that were obtained for both species, under the above-mentioned conditions, indicate that the treatment is evenly effective against gram-positive and gram-negative species. To confirm this indication, a larger variety of gram-negative and gram-positive species should be tested.

Based on the results of the sublethal injury, it can be concluded that sublethally injured cells are already present in the biofilm before CAP treatment is performed. During the treatment, a general trend of the percentage of sublethal injury as function of the treatment time could be observed, which suggests a mechanism of injury accumulation that eventually results in cell lysis. At the end of the treatment, there is still a residual percentage of SI for the treated *S.* Typhimurium and *L. monocytogenes* biofilms, which lays between approximately 50-75% and 20-50% respectively, depending on the tested conditions. These sublethally injured cells should not be overlooked, as these still pose a risk for the food safety.

For the use of different electrodes, as well as different oxygen levels, combined with the DBD electrode, the results for SI are inconclusive. However, when using the SBD electrode in combination with different oxygen levels, a general trend can be observed. Based on these results, it can be concluded that for both microorganisms the highest value of SI at the end of the treatment

was achieved using helium with 0.5% (v/v) oxygen, the second highest values of SI were achieved by using pure helium gas, and the lowest values were achieved by using helium with 1.0% (v/v) oxygen.

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FACULTY OF ENGINEERING TECHNOLOGY
TECHNOLOGY CAMPUS GHENT
Gebroeders De Smetstraat 1
9000 GHENT, Belgium
tel. + 32 92 65 87 09
iw.gent@kuleuven.be
WWW.IIW.KULEUVEN.BE