OCCURRENCE AND CHARACTERISATION OF RESIDUAL CONTAMINATION AND BIOFILMS IN FOOD PROCESSING ENVIRONMENTS AND POULTRY DRINKING WATER SYSTEMS

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VOORWOORD

If you can't fly, then run.
If you can't run, than walk.
If you can't walk, then crawl,
but by all means, keep moving.

Martin Luther Kinf Jr.

VOORWOORD

Laat ik één ding meteen duidelijk stellen, ik heb me heel erg geamuseerd tijdens de 4 jaar waarin dit onderzoek werd uitgevoerd. Ik ben heel blij dat ik de stap naar het onbekende heb durven zetten, want daardoor heb ik 4 jaar lang kunnen doen wat ik graag doe en dat is onderzoeken, nieuwe dingen leren, zelfstandig werken maar ook samenwerken met fantastische mensen en mezelf beter leren kennen. Look's like we made it! En ik schrijf WE, omdat het natuurlijk niet enkel mijn eigen verdienste is dat dit werk is geworden wat het is geworden.

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SUMMARY

SUMMARY

Despite a regular cleaning and disinfection (C&D), residual bacterial contamination and biofilms still occur in food companies and primary animal production environments. In case pathogenic and spoilage organisms remain, these could lead to early food spoilage, foodborne illness and pose a threat for animal and human health. Further knowledge concerning the occurrence, composition and characteristics is needed to have a better estimate on the impact of this residual contamination. Therefore, this research aimed at gaining insights in the presence and characteristics of residual bacterial contamination and biofilms in different food sectors and in primary animal production, more specifically the drinking water system (DWS) in broiler houses.

In the first part of the research (chapters 3 and 4), biofilms and residual bacterial contamination in eight food companies of different sectors were mapped and investigated. To do so, there was a need for a suitable sampling method that allowed detection and/or quantification of microorganisms and biofilm matrix components. Two surface sampling methods were tested, the sponge stick method and the scraper-flocked swab method, whereby the latter method was evaluated as the most suitable. Sampling with this method provided results of the bacterial load and chemical composition of surface contamination after C&D. Bacterial enumerations were on average 3.62 ± 1.20 log CFU/100 cm^2 but reached up to 7.23 log CFU/100 cm^2. Respectively 20%, 15% and 8% of the surfaces investigated for biofilm matrix components were contaminated with low quantities of proteins, carbohydrates and uronic acids. On 17% of the investigated surfaces, both microorganisms and at least one of the chemical compounds were found, which is an indication for the presence of biofilm. The presence and the degree of residual contamination is highly variable by food sector, food company, sampling point and even sampling time. Genera that were most abundant in the residual bacterial contamination on food contact surfaces after C&D in the different food companies were *Pseudomonas*, *Microbacterium* and *Stenotrophomonas*, however 60% of the identified genera were company-specific. Of all the evaluated isolates, 88% had some kind of spoilage potential, with the ability to break down lipids as the most prevalent property. However it remains difficult to estimate the possible impact of these microorganisms on food safety and spoilage since it is not known whether they will be transferred from the surface to the food products, survive and grow in the food products and consequently cause spoilage.

In the second part of the research (chapters 5 and 6), the focus was on residual contamination and biofilm formation on the inside of the DWS in broiler houses after disinfection. Water quality in the DWS plays an important role in the general health and performance of broiler chickens since pathogens might be present. Conditions in the DWS of broilers are ideal for microbial biofilm formation. The presence of this contamination on the inside of the DWS was assessed in terms of bacterial load and chemical composition. Average bacterial counts of 6.03 ± 1.53 log CFU/20cm^2 were observed, ranging up to 9.00 log CFU/20cm^2 at some points. Proteins, carbohydrates and uronic acids were again found in low quantities in 58%, 14% and 5% of the samples, respectively. On 63% of the investigated surfaces, the presence of biofilm was suspected since microorganisms were detected in combination with at least one of the analysed chemical components. The most identified dominant species in the DWS were *Stenotrophomonas maltophilia*, *Pseudomonas geniculata* and *Pseudomonas aeruginosa*, which are opportunistic human pathogens. However at species level, most of the identified microorganisms were farm-specific. Almost all the isolates belonging to the three most abundant species were strong biofilm producers. Overall, 92% of all tested microorganisms were able to form biofilm in 96-well microtiter plates. Finally, the hypothesis that commensal bacteria in biofilms on surfaces could prevent attachment of pathogens such as *Salmonella* spp. was investigated. Since there is an increasing problem with *Salmonella* Java contamination on broiler chickens in Belgium, this pathogen was used to investigate its interaction with the commensal *Pseudomonas putida*, which is part of the natural microbiota in the DWS. Therefore, a new model that simulates biofilm formation on the inside of the DWS was developed and validated. In this model, *Salmonella* Java was evaluated as a strong biofilm former. However, when applied in the presence of *Pseudomonas putida*, biofilm formation by *Salmonella* Java was reduced due to competitive interactions indicating the potential of *Pseudomonas putida* as a biocontrol agent.

In conclusion, this research provided interesting new information for food companies to be used in their fight against unwanted contamination and for the development of more efficient C&D procedures. Also in primary animal production, new insights concerning the presence and composition of DWS contamination and its role in the prevention of pathogens were obtained.

SAMENVATTING

SAMENVATTING

Ondanks reiniging en ontsmetting (R&O) kunnen residuele bacteriële contaminatie en biofilms voorkomen in omgevingen zoals voedingsbedrijven en primaire dierlijke productie. Wanneer er bederf- en pathogene organismen achterblijven kunnen deze leiden tot vroegtijdig voedselbederf en voedsel-gerelateerde ziekten wat een risico kan vormen voor de dierlijke en humane gezondheid. Verdere kennis betreffende het voorkomen, de samenstelling en eigenschappen is nodig om de impact van deze residuele bacteriële contaminatie beter in te schatten. Daarom was het doel van dit onderzoek om inzichten te verwerven in de aanwezigheid en eigenschappen van residuele bacteriële contaminatie en biofilms in verschillende voedingssectoren en in de primaire dierlijke productie, meer specifiek in het drinkwatersysteem (DWS) in vleeskuikenstallen.

In het eerste deel van dit onderzoek (hoofdstukken 3 en 4) werden biofilms en residuele bacteriële contaminatie in acht voedingsbedrijven uit verschillende sectoren in kaart gebracht en onderzocht. Daarvoor was er nood aan een geschikte bemonsteringsmethode voor de detectie en/of kwantificatie van micro-organismen en biofilm matrix componenten. Twee oppervlakte bemonsteringsmethoden werden getest, de sponsstick methode en de schraper-flocked swab methode, waarvan de laatste geëvalueerd werd als de meest geschikte. Bacteriële tellingen waren gemiddeld 3.62 ± 1.20 log KVE/100 cm^2, maar konden oplopen tot 7.23 log KVE/100 cm^2. Respectievelijk 20%, 15% en 8% van de onderzochte oppervlakken bevatten lage hoeveelheden eiwitten, suikers en uronzuren. Op 17% van de onderzochte oppervlakken werden zowel micro-organismen als minstens één van de chemische componenten teruggevonden, wat een indicatie is voor de aanwezigheid van biofilm. De aanwezigheid en mate van residuele contaminatie is zeer variabel per voedingssector, bedrijf, locatie en zelfs tijdstip. Genera die het meest geïdentificeerd werden op voedings-contactoppervlakken na R&O zijn *Pseudomonas*, *Microbacterium* en *Stenotrophomonas*, hoewel 60% van de geïdentificeerde genera bedrijfsspecifiek waren. Van alle geëvalueerde isolaten hadden 88% enige mate van bederfpotentieel, waarbij de mogelijkheid tot vetafbraak het meest voorkwam. Toch blijft het moeilijk om de mogelijke impact van deze micro-organismen op voedselveiligheid en bederf in te schatten omdat het niet gekend is of deze zullen overgebracht worden van het oppervlak naar het voedingsmiddel, overleven en groeien in het voedingsmiddel en bijgevolg bederf veroorzaken.

In het tweede deel van het onderzoek (hoofdstukken 5 en 6) lag de focus op residuele contaminatie en biofilmvorming aan de binnenzijde van het DWS in vleeskuikenstallen na desinfectie. De aanwezigheid van de contaminatie aan de binnenzijde van het DWS werd geëvalueerd op basis van bacteriële belasting en chemische samenstelling. Gemiddelde bacteriële tellingen van 6.03 ± 1.53 log KVE/20cm^2 werden geobserveerd met maxima tot 9.00 log KVE/20cm^2 op sommige plaatsen. Eiwitten, suikers en uronzuren werden opnieuw in lage aantallen teruggevonden in respectievelijk 58%, 14% en 5% van de stalen. Op 63% van de onderzochte oppervlakken werd de aanwezigheid van biofilm vermoed door de simultane aanwezigheid van micro-organismen en chemische componenten. De meest geïdentificeerde dominante species in het DWS waren *Stenotrophomonas maltophilia*, *Pseudomonas geniculata* en *Pseudomonas aeruginosa*, welke opportunistische humane pathogenen zijn. Echter op species-niveau waren de meeste geïdentificeerde micro-organismen opnieuw bedrijfsspecifiek. Bijna alle isolaten behorend tot de drie meest abundante species werden geëvalueerd als sterke biofilmvormers. Over het algemeen hadden 92% van de geteste micro-organismen biofilmvormende eigenschappen in 96-well microtiterplaten. Tot slot werd de hypothese dat commensale bacteriën in biofilms de aanhechting van pathogenen, zoals *Salmonella* spp., zou voorkomen onderzocht. Aangezien er een toenemend probleem met *Salmonella* Java contaminatie in vleeskuikens heerst in België werd de interactie tussen deze pathogeen en de commensaal *Pseudomonas putida* (onderdeel van de natuurlijke microbiota in het DWS) onderzocht. Daarvoor werd er eerst een nieuw model ontwikkeld en gevalideerd die biofilmvorming aan de binnenzijde van het DWS simuleert. In dit model werd *Salmonella* Java geëvalueerd als sterke biofilmvormer. Echter, wanneer aangebracht in aanwezigheid van *Pseudomonas putida* werd biofilmvorming door *Salmonella* Java gereduceerd door competitieve interacties, wat het potentieel van *Pseudomonas putida* als biocontrole agens aangeeft.

In conclusie heeft dit onderzoek interessante nieuwe informatie verschaft voor voedingsbedrijven in hun strijd tegen ongewenste contaminatie en voor de ontwikkeling van meer efficiënte R&O procedures. Ook in de primaire dierlijke productie werden de eerste inzichten in de aanwezigheid en samenstelling van DWS contaminatie en de rol van commensale biofilms in de preventie van pathogeen-aanhechting verworven.

LIST OF ABBREVIATIONS

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A

ACP	Agar Contact Plate
AHL	N-acyl homoserine lactone
ATP	Adenosine Triphosphate
aw	Water Activity

B

BCA	Biocontrol Agent
BHI	Brain Heart Infusion

C

CAP	Cold Atmospheric Plasma
CDC	Centers for Disease Control and Prevention
CFU	Colony-Forming Units
C&D	Cleaning and Disinfection
CLSM	Confocal Laser Scanning Microscopy
CS	Countable Samples

D

DWS	Drinking Water System or drinkwatersysteem

E

eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic Acid
EPS	Extracellular Polymeric Substances

G

GHP	Good Hygiene Practices
GMP	Good Manufacturing Practices

H

HACCP	Hazard Analysis of Critical Control Points
HGT	Horizontal Gene Transfer

K

K1-K5	Broiler Companies 1 to 5
KIA	Kligler Iron Agar
KVE	Kolonie Vormende Eenheden

L

LAB	Lactic Acid Bacteria
LOQ	Limit of Quantification

M

MEA	Malt Extract Agar
MIC	Minimum Inhibitory Concentration
MTP	Microtiter Plate
MRS	de Man, Rogosa, Sharpe Agar
MST	Minimum Spanning Tree

O

OD	Optical Density

P

P1	*Pseudomonas putida* strain (weak biofilm former)
P2	*Pseudomonas putida* strain (moderate biofilm former)
P3	*Pseudomonas putida* strain (strong biofilm former)
PAB	Pseudomonas Agar Base
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
pH	Acidity

Q

Q1	First Quartile
Q2	Second Quartile (i.e. median)
Q3	Third Quartile
QQ	Quantile-Quantile
QS	Quorum Sensing

R

RCF	Relative Centrifugal Force
R&O	Reiniging en Desinfectie
rpm	Rounds Per Minute

S

S1	*Salmonella* Java Strain
S2	*Salmonella* Mbandaka strain
SAS	Statistical Analysis System Software
spp.	species (plural)
SS	Stainless Steel

T

TAC	Total Aerobic Count
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth

V

VBNC	Viable But Non Culturable
VRBGA	Violet Red Bile Dextrose Agar

X

XLD	Xylose Lysine Deoxycholate Agar

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CHAPTER 1: INTRODUCTION AND OUTLINE OF THE RESEARCH

1 INTRODUCTION AND OUTLINE OF THE RESEARCH

Microbial contamination occurs ubiquitous in all kinds of environments. In food industry and primary animal production, measures such as the performance of regular cleaning and disinfection (C&D) are taken to reduce the contamination level to acceptable levels. This is primarily done to prevent and eliminate pathogenic and spoilage organisms which can contaminate the produced food products or infect the animals. Ultimately this serves to prevent early food spoilage and foodborne illness and to reduce the threat for animal and human health. Despite C&D, residual bacterial contamination can still persist in the environment. Currently, there is a lack of knowledge concerning the occurrence, composition, characteristics and possible consequences of this residual contamination whether or not present as biofilms. This research therefore aims at gaining insight in the presence and characteristics of residual bacterial contamination and biofilms and their importance in the food industry and in primary animal production, more specifically in the drinking water system (DWS) of broiler houses. This was done in several studies which are discussed in the chapters following the literature review (**chapter 2**), schematically presented in Figure 1.1.

First of all, monitoring methods for biofilms in practice that include detection and/or quantification of microorganisms and matrix components are still missing. However, a suitable monitoring using efficient sampling method is necessary to investigate bacterial communities in different kinds of environments in the food production chain. Therefore in **chapter 3**, the aim was to select the most suitable method for microbiological and chemical detection and quantification of surface contamination after C&D out of two sampling methods.

Subsequently, the best sampling method could be used for monitoring and analysis of surface contamination after C&D in different types of food companies. **Chapter 3** provides information about the bacterial load and chemical composition of this residual surface contamination. Furthermore, it is important to gain information about the characteristics, importance and possible impact in food companies. This was the objective in **chapter 4** by identifying the dominant residual bacteria and evaluating their spoilage potential.

On the other hand, there is a lack of information concerning the presence, importance and composition of biofilms on the inside of the DWS in broiler houses. In **chapter 5**, results of

sampling the inside surfaces of the DWS are included in terms of bacterial load and chemical composition. Also, identification and evaluation of biofilm-forming capacities of the dominant bacteria are discussed.

Model systems are useful for the study of biofilms since they provide more controlled conditions than in practice. However, many models implement test conditions that differ a lot from reality whereby model results sometimes have limited significance to biofilms in practice. Therefore, the aim in **chapter 6** was to develop and validate a model to simulate biofilm formation on the inside of the DWS in broiler houses that approached practical conditions as good as possible. Specific parameters that were chosen to simulate field conditions were incubation temperature, contact surface, nutrient availability, flow conditions, inoculum strains and inoculum concentrations. This model was used to evaluate biofilm-forming capacity of multiple field strains in several inoculum densities.

The importance of biofilms in the protection of zoonotic pathogens such as *Salmonella* Java in the DWS of broilers is still unknown and was investigated in the newly developed biofilm model. Therefore, **chapter 6** also provides the results of dual-species biofilms of *Salmonella* Java and *Pseudomonas putida* (a frequently identified and harmless species in the DWS of broilers) to study their interaction and to evaluate the potential of *Pseudomonas putida* as a biocontrol agent.

Finally, a general discussion of the results obtained in the previous chapters is provided in **chapter 7**.

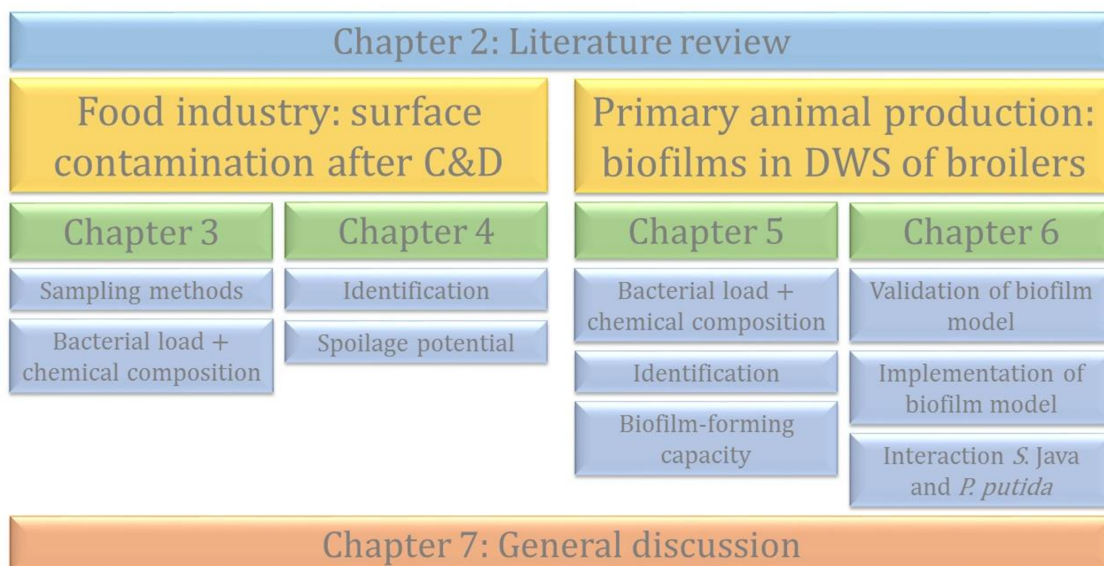

Figure 1.1: Schematic overview of the research.

CHAPTER 2: LITERATURE REVIEW

2 LITERATURE REVIEW

2.1 ELIMINATION OF MICROBIAL CONTAMINATION

2.1.1 Classic cleaning and disinfection (C&D)

In the food industry, microorganisms can enter and spread throughout the company in several ways. Incoming ingredients used in the preparation of the food products can be a source of microorganisms. Also packaging materials, water, air, rodents, insects and staff can introduce microorganisms in food companies (Marriott and Gravani, 2006). Once present in the company, these microorganisms can spread through process equipment, processing tools, staff, air, etc., leading to cross-contamination to other food products or other surfaces. Also in primary animal production, several routes for microorganisms to enter flocks and spreading inside it exist. Feed, water, equipment, staff, visitors and biological vectors such as vermin, birds and pets can carry all kinds of microorganisms, but also the animals themselves are a source of microbiota (Heyndrickx et al., 2002; Herman et al., 2003; Davies et al., 2004; Osman et al., 2010). These microorganisms, among which possibly pathogenic and spoilage organisms, that are present in food industry or primary production, can lead to early food spoilage or foodborne illness and pose a threat for animal and human health. Therefore, standard operating procedures, among which the performance of regular C&D, are developed and used in food industry and primary production to reduce the contamination level during food production or in the animal environment to acceptable levels.

Cleaning is an often underestimated step during sanitation of production environments, yet when this step is insufficiently performed, organic residues can prevent microorganisms from being further eliminated during disinfection. Especially in case of biofilms present on surfaces, cleaning plays a major role compared to disinfection. Cleaning generally starts with tidying the room and disassembly of processing equipment. Then, foreign materials are physically removed from processing surfaces and the environment by means of a dry cleaning with brushes and/or followed by a wet cleaning step using water under high pressure. This provides a first cleaning step before cleaning with a cleaning agent takes place. Four main factors determine an efficient cleaning. This is also called the circle of Sinner, including time, mechanical action, chemicals and temperature (Figure 2.1). The cleaning performance is a result of the sum of these factors

and in case one of the factors is reduced, the other three should compensate by increasing them. The factor time implies mainly a sufficient contact time between cleaning agent and the surface to be cleaned, which is commonly obtained by adding a foaming agent to the cleaner. Mechanical action can be obtained by manual scrubbing, creation of flow, inclusion of particles in the cleaning agent, etc. but it should not lead to damage of the surface. The type of cleaning agents has a major impact on the cleaning process, as they are designed for particular soils and conditions. For example, surfactants can be used to clean fatty soils. Alkali cleaners are useful for the elimination of fats or caramelised soils, whereas acids are more used to remove mineral deposits. In addition, components frequently added to cleaning agents are chelating agents (e.g. EDTA for mineral removal), solvents (to remove grease and oils), corrosion inhibitors and oxidizers (e.g. chlorine for the elimination of protein films). It is advisable to use the optimum concentration of a specific cleaning agent, as a lower concentration will lead to less effective cleaning and a higher concentration will not remove additional soil. Finally, optimal cleaning temperature depends on the type of soil and the used cleaning agent (Grinstead, 2009). After cleaning, a rinsing step with water is performed to remove the loosened dirt and residual cleaning agent. It is advised to eliminate most of the water to avoid dilution of the subsequently applied disinfection agent.

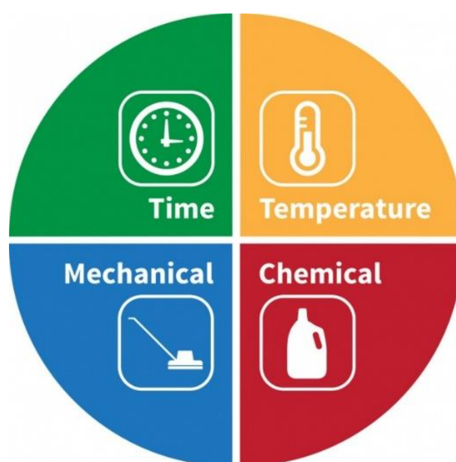

Figure 2.1: Circle of Sinner including four principle factors for successful cleaning (retrieved from http://www.europeancleaningjournal.com, consulted on May 3, 2018).

The use of disinfection is only useful in case a thorough cleaning has taken place in a previous step. Only then the present microorganisms can be reached and affected by disinfection agents. Moreover, the presence of remaining organic matter can inactivate several active agents in disinfection products. Disinfectants contain agents that inhibit the

growth of microorganisms ('-statics') or agents that kill them ('-cidals'). They mostly contain one or several active agents that are responsible for their mode of action and are selective for certain groups of microorganisms (Grinstead, 2009). Similar to antibiotics, that are used to treat bacterial infections, disinfectants can work against a broad or specific spectrum.

An overview of the most common used biocides in food processing environments with their main characteristics is given in Table 2.1.

Table 2.1: Overview of the most common used biocides in food processing environments with their main characteristics (adapted from Grinstead, 2009 and supplemented based on Mcdonnell and Russell, 1999).

Biocide class and active component	Target			Mode of action	Inactivation by organics	Particularities
	Bacteria	Fungi	Spores			
Oxidizers						
Hypochlorous acid	++	++	+	Oxidising, inhibition of DNA synthesis	Yes	Unstable, causes corrosion of surfaces
Chlorine dioxide	++	++	+	Oxidising, inhibition of DNA synthesis	Yes	Unstable gas, causes corrosion of surfaces
Iodine	++	++	++	Reacts with proteins, DNA, fatty acids	No	Stable, discolours surfaces
Ozone	++	++	+	Oxidising, reacts with amino acids, DNA	Yes	Unstable gas
Hydrogen peroxide	++	+	++	Oxidising, free radicals react with lipids, proteins, DNA	Yes	Low toxicity, only effective at high concentrations
Peroxyacetic acid	++	+++	++	Reacts with proteins	No	Low toxicity, effective at low concentration
Surfactants						
Quaternary ammonium compounds (cationic)	+	+	-	Disruption of cell membrane, bind to phospholipids and proteins	No	Stable, hard water limits efficacy
Acid anionic	+	+	-	Disruption of cell membrane	No	Used al low (<3) pH, also used as cleaning agent

Oxidizers are one of the most used classes of biocides in the food industry including halogens (e.g. chlorine, chlorine dioxide and iodine), peroxides (e.g. hydrogen peroxide) and peroxyacetic acid. The other broad class of biocides used by food processors is based

on surfactants. After disinfection, a rinsing step is again performed. C&D of loose parts of equipment is performed separately and afterwards the equipment is reassembled. An evaluation of the efficacy of the C&D procedure is generally performed and is discussed in section 2.7.

In primary animal production, C&D is an important aspect of the internal biosecurity to prevent or reduce the spread of pathogens within the flock. Not only the interior of animal houses should be cleaned and disinfected, but also the environment around the stables and equipment should be taken into account (Amass *et al.*, 2000). The general C&D procedure between two production rounds in animal houses exists of six steps which can be supplemented with two additional steps and an evaluation (Table 2.2) (Luyckx, 2016). The first part of the procedure includes cleaning to physically remove foreign material from the surfaces. A dry cleaning is generally the first step after the animals have left the farm, whereby most of the manure, dust and feed is removed using shovels, brushes and sometimes agricultural vehicles. Second, wet cleaning takes place where again the circle of Sinner plays an important role. In the optimal case, wet cleaning in animal houses starts with water under high pressure to remove loose organic materials. It was shown that an overnight soaking step with water caused a more efficient bacterial reduction (Luyckx et al., 2015b).

Table 2.2: Cleaning and disinfection procedure between production rounds in animal production (adapted from Luyckx, 2016).

Step	Category	Description
1	Cleaning	Dry cleaning
2		Wet cleaning: washing premises with water
3		Wet cleaning: soaking premises with cleaning product
4		Wet cleaning: rinsing premises with water
5		*Drying*
6	Disinfection	Disinfection of premises
7		*Rinsing with water*
8		*Vacancy*
9	Evaluation	Monitoring the hygiene status after C&D

In a third step, a cleaning agent is used to soak the premises and remove stuck dirt. The final step of cleaning includes the removal of the loosened dirt and residual cleaning product with water. After cleaning, the animal houses are left to dry in the fifth step of the

C&D procedure. Then, a disinfection step is introduced by applying a disinfectant by means of surface covering, fogging or fumigation. Similar to the food industry, chlorine based components, peroxides and quaternary ammonium compounds are often found in disinfection products for the primary sector. Additionally, alcohols and aldehydes are also used. After disinfection, an optional rinsing step with water can be performed to remove residual disinfectant followed by a vacancy period (Luyckx, 2016).

In case C&D is not performed properly, there is a chance for residual bacterial contamination and biofilms to persist in the environment. This can serve as a nutrient source for new microbial growth and lead to cross-contamination to other surfaces or to the produced food products coming into contact with these surfaces, possibly causing early food spoilage, foodborne illness and threatening animal and human health.

2.1.2 Innovative cleaning and disinfection methods

Because of insufficient C&D, microbial tolerance or resistance profiles and other phenomena causing failure of classic C&D such as biofilm formation, novel methods have been developed to eliminate contamination in certain environments. Some of those methods are discussed below.

The use of enzyme cocktails is suggested as an addition to classic C&D. In this way, residual organic compounds or extracellular polymeric substances (in case of biofilm) surrounding the microbial cells can be disrupted and subsequently applied disinfectants can better reach the cells (Johansen et al., 1997; Lequette et al., 2010; Sen et al., 2014; Stiefel et al., 2016; Timmerman et al., 2016). Since these substances are mostly composed by different kinds of biopolymers (such as proteins, carbohydrates, lipids, etc.), a mix of enzymes should be used that catalyse different chemical reactions and to affect the surface contamination as good as possible. Moreover, enzymes could interfere with molecules involved in the quorum sensing (QS) system (this is discussed in more detail in 2.3.3) of microbial cells, disturbing among others biofilm formation. Examples are acylhomoserine lactonases and acylases that degrade the ubiquitous signal molecule N-acyl homoserine lactone (AHL) in Gram-negative bacteria (Rendueles and Ghigo, 2012). An additional advantage of the use of enzymes is that they are non-corrosive, safe, easy to handle and less aggressive for the environment (Timmerman *et al.*, 2016). However, there

are still some doubts about the impact on food quality and spoilage of residual enzymes on food processing equipment and its value in most niches still has to be proven.

As an alternative for disinfection, competitive exclusion bacteria are already on the market for application in animal houses. For this method, safe probiotic bacteria are applied into the environment with the intention of creating a stable and healthy microbiota whereby negative bacteria such as pathogens should be outcompeted. Yet, (Luyckx et al., 2016b) showed that using a competitive exclusion protocol including the application of *Bacillus* spp. spores could not reduce the infection pressure to the same degree as when performing classic C&D in pig nursery units. In the food industry on the other hand, Zhao *et al.* (2006) used strains with anti-listerial activity in floor drains in a poultry plant and a reduction or even elimination of *Listeria* was observed. However, this method can not be applied on food contact surfaces unless complete harmlessness towards the produced food products and human health can be demonstrated.

The use of cold atmospheric plasma (CAP) is still under development. CAP is an ionised gas containing photons, atoms, ions, electrons and high energy species (mostly OH, H, O and NO radicals) that is capable to initiate chemical reactions and moreover is non-thermal due to the lower power that is used to produce it compared to thermal plasma. These initiated chemical reactions lead to the formation of reactive oxygen and nitrogen species which are highly bactericidal (Ziuzina et al., 2015; Jovicic et al., 2017). Also, molecules involved in the QS system of microbial cells can be a target for CAP, leading to altered biofilm formation and virulence profiles (Ziuzina *et al.*, 2015). These characteristics could be interesting for the removal of microbial contamination and biofilm on surfaces. Promising results were obtained under lab conditions concerning the removal of oil, milk and egg contamination from surfaces and even for inactivation of microorganisms on surfaces (Jovicic *et al.*, 2017). Yet, process parameters such as speed of the plasma jet, height between the jet and the surface, treatment time and also the type of microorganisms are very important for the efficacy of the technique (Ziuzina et al., 2015; Jovicic et al., 2017). However, the devices to apply this technique in practice such as in food processing environments or primary animal production sites are not yet on the market.

Disruption of the QS system is suggested as a new anti-infective strategy for biofilms. Compounds that interfere with the production or detection of signal molecules of this system (e.g. halogenated furanones produced by algae or synthetic antagonists of the signal molecules) could be applied and are called QS inhibitors. They interfere with the QS mechanism by reducing the expression of genes responsible for the production of quorum sensing molecules or by blocking receptor sites. Another strategy is to inactivate the signal molecules and hereby disrupting the QS system (Defoirdt et al., 2010; Høiby et al., 2011; Li and Tian, 2012). This method is called quorum quenching. Enzymes that are able to do this are already discovered in different classes of bacteria (Defoirdt et al., 2010; Zhang et al., 2017). QS interfering compounds were also found in nature for example in garlic extracts, plant essential oils and ginseng (Høiby *et al.*, 2011; Koh *et al.*, 2013). It is believed that, because it does not pose a risk to bacterial growth or a strong selective pressure for the organism, QS inhibition has an unlikely chance for resistance development. However, variations in signal molecules and signal receptors may cause a difference in bacterial fitness and consequently lead to a risk for resistance (Defoirdt et al., 2010; Li and Tian, 2012). In addition, Allen *et al.* (2014) suggest that, since QS has an influence on a broad range of cell functions, QS inhibitors will promote selection for resistance. More research to understand the evolutionary risks of the application of this type of molecules needs to be conducted before it could be included in current C&D methods and products.

Another recent approach to control and eradicate bacteria and biofilms is the use of bacteriophages. These viruses are only harmful for the infected bacteria and express their antimicrobial activity by lysis of the host cells at the end of their multiplication cycle. Moreover, some phages possess enzymes that can hydrolyse parts of the biofilm matrix or even the cell wall of bacteria. However, it has to be determined whether bacteriophages can be implemented in current C&D procedures in terms of temperature and compatibility with disinfectants. Also, more research concerning safety, large-scale production and application in practice is necessary (Gutiérrez et al., 2016).

Power ultrasound is a kind of energy in the form of sonic waves with frequencies ranging from 20 to 100 kHz and sound intensity of 10 to 1000 W/cm^2 which can be applied for the cleaning of several humid surfaces such as processing equipment and food surfaces. The main action is cell death caused by physical action, but also the production of reactive

compounds such as hydrogen peroxide can promote bacterial disruption. Physical action is obtained by the formation, growth and implosive collapse of gas bubbles or also called cavitation (Martin and Feng, 2009; Freitas Brilhante de São José et al., 2014). This technique could have its potential in eradication of biofilm from loose parts of production lines but is not practically applicable for large food processing equipment.

Most of the discussed innovative C&D methods are not yet applicable in practice. In addition, despite all efforts either through classic C&D or alternative methods, it is possible that, due to several factors, residual bacterial contamination or biofilm is still present on surfaces. These problems are addressed in the next sections.

2.2 RESIDUAL MICROBIAL CONTAMINATION

After C&D, residual bacterial contamination can still be present on surfaces. This can be due to mal performance of the C&D procedures. On the other hand, it is possible that the C&D procedures are not ideal for the corresponding surfaces and types of contamination. In addition, notwithstanding optimal C&D, still limited residual bacterial contamination will be present and sterile surfaces will never be obtained. To achieve the best result, contact time, temperature and concentration of the applied C&D agents together with the application of mechanical action have to be validated for each location. In case these factors are insufficient and they are not compensated by the other factors, the C&D procedure will not be effective enough to reduce contamination to a sufficient level. Suboptimal concentration of the disinfectants can be the result of residual water on the surface (diluting the disinfecting agents) or the presence of organic matter (interacting with the disinfecting agents) leading to ineffective elimination of the present microorganisms (Ruano et al., 2001; Moustafa Gehan et al., 2009).

Another factor determining the efficacy of C&D is the variation in susceptibility of different groups of microorganisms. In increasing order of relative resistance, microorganisms can generally be classified as followed: enveloped viruses, Gram-positive bacteria, large non-enveloped viruses, protozoa, yeasts, moulds, Gram-negative bacteria, fungal spores, protozoal cysts, small non-enveloped viruses, mycobacteria, protozoal oocysts, bacterial endospores and prions (Fraise et al., 2012). Microorganisms can have resistance properties that are naturally present (i.e. intrinsic resistance), resistance that is acquired through gene mutation or genetic elements (i.e. acquired resistance) and

resistance that is induced by a specific signal or social activities (i.e. adaptive resistance) (Mcdonnell and Russell, 1999; Fernández et al., 2011).

Intrinsic resistance is an inherited property of bacteria which enables them to circumvent the action of disinfectants or antibiotics. It can for example implement the production of enzymes, efflux pumps or an altered cell permeability, which is for example the case for spores and Gram-negative bacteria by means of a spore cortex and outer membrane, respectively (Mcdonnell and Russell, 1999). Also, biofilm formation is an example of intrinsic resistance. This mode of growth will be discussed in section 2.3. Acquired resistance to disinfectants can occur by spontaneous mutations or by acquisition of genetic material on transferable elements such as plasmids or transposons by horizontal gene transfer (HGT) (Mcdonnell and Russell, 1999; Aarestrup et al., 2008). Finally, adaptive resistance is a less studied mechanism and is a not so well understood phenomenon. Environmental conditions, such as subinhibitory concentrations of an antimicrobial and social activities like biofilm formation, lead to induction of resistance to one or more antimicrobial agents. This increased resistance is generally reversible when the trigger is removed and is therefore difficult to detect (Fernández *et al.*, 2011).

2.3 BIOFILM ASPECTS

2.3.1 General characteristics of biofilm

The definition of biofilm has evolved during the years and many researchers have different opinions on this definition as they include different characteristics of this phenomenon. Donlan and Costerton (2002) called biofilms microbial derived sessile communities characterised by cells that are irreversibly attached to a substratum or interface or to each other and embedded in a matrix of extracellular polymeric substances (EPS) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription. Microscopic observations showed that biofilms contain high cell numbers surrounded by fibrous, hydrated exopolysaccharide matrix (Costerton *et al.*, 1994). Cells typically represent less than 10 to 15% of the total biomass, while the matrix material occupies approximately 85 to 90% (Donlan and Costerton, 2002; Flemming and Wingender, 2010). Because of the numerous advantages (mainly elevated levels of protection against harmful environmental conditions), the biofilm mode of growth is the predominant growth form of microorganisms (Donlan and Costerton, 2002).

Biofilms generally demonstrate higher antimicrobial tolerance (temporary property) and resistance (permanent inherited or aqcuired property) compared to planktonic cells. This is caused by different characteristics but molecular mechanisms underlying this phenomenon are still not completely understood. One of the characteristics involved in biofilm tolerance is the reduced penetration of antimicrobials through the EPS matrix (because of interaction with or sorption to it) whereby target cells are not reached. On the other hand, cells are observed to have heterogeneous but generally low growth rates in biofilms, leading to an increased tolerance to antimicrobials. Another characteristic of biofilms is the initiation of the general stress response, leading to physiological changes that protect the cells from various environmental stresses such as temperature shocks, starvation, pH changes and antimicrobial agents. Finally, persister cells (a subpopulation of cells within a biofilm that entered a dormant, highly protected, spore-like state) can also account for the observed tolerance. When cells form biofilm, a biofilm-specific phenotype is activated because of an altered gene-expression profile compared to planktonic cells. This can include the expression of genes for resistance to antimicrobials (such as genes encoding for efflux pumps or enzymes for modification of the drug or target site), leading to enhanced survival when these antimicrobials are applied. Resistance genes can also be acquired by enhanced HGT in biofilms (Costerton et al., 1999; Mah and O'Toole, 2001; Stewart, 2002; Lewis, 2010).

2.3.2 Occurrence of biofilm

Microorganisms form biofilms preferably in environments with high shear rate (Donlan and Costerton, 2002). These environments can be natural or industrial and surfaces upon which biofilms grow can be biotic or abiotic. Examples that are visible to the naked eye are biofilms in rivers, where the interaction between algae and bacteria leads to the river's self-purification and even to the removal of organic matter in wastewaters (Tien et al., 2011). Though, in most situations biofilm is not directly visible. Biofilms can grow on plant (e.g. *E. coli* biofilm formation on sprouts and fruit) or animal tissue (e.g. multi-species biofilms in chronic wounds) which could eventually cause disease development or death for the host and possibly consumer (Yaron and Römling, 2014; Omar et al., 2017). Examples of biofilm present in the human body are dental plaque (Marsh, 2006), mucosal biofilms of *Pseudomonas aeruginosa* in the pulmonary tract of cystic fibrosis patients (Høiby et al., 2010b) and biofilm formation by staphylococci on implants (Arciola et al., 2015). On the other hand, biofilm can be found in more industrial environments such as

surfaces in food processing companies (Chmielewski and Frank, 2003) or in primary production such as animal houses (Zimmer et al., 2003). These last two cases will be discussed into more detail in further sections.

2.3.3 Biofilm development

The development of biofilm is a complex process, in which five stages can be distinguished: a) initial/reversible attachment, b) irreversible attachment, c) early development of biofilm architecture, d) maturation and e) detachment and dispersal. These stages are visualised in Figure 2.2 and will be shortly discussed.

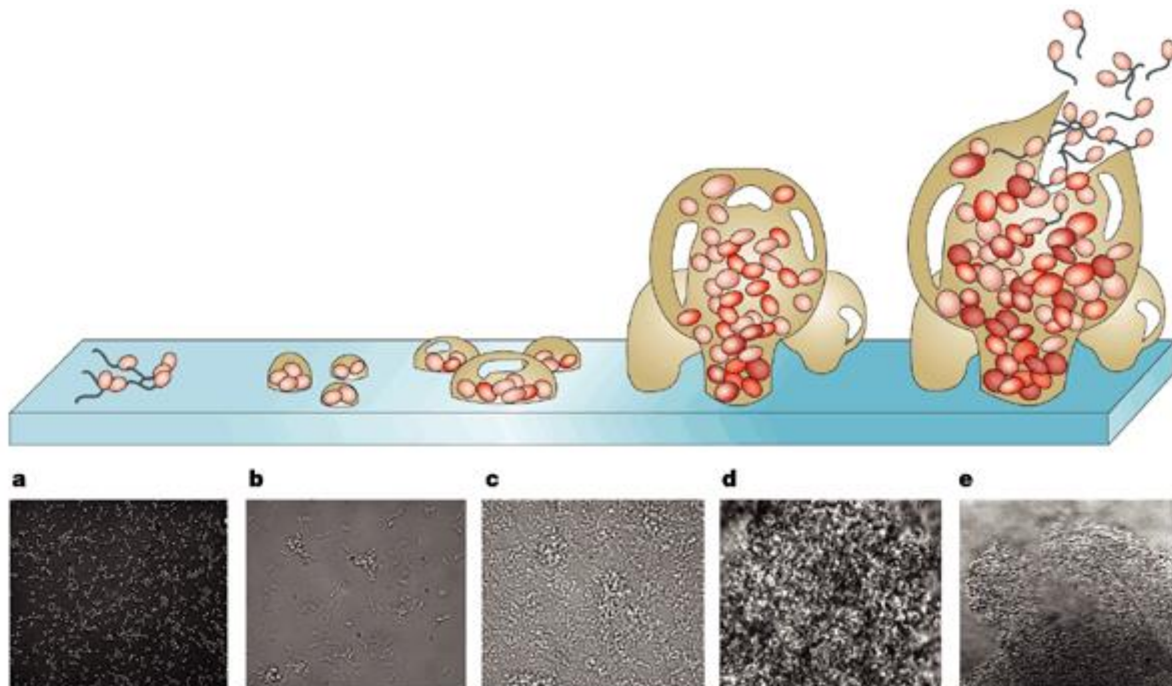

Figure 2.2: Schematic and photo micrographic imaging of different stages in biofilm development (adapted from Davies, 2003).

Transition from planktonic to sessile state of microorganisms is driven by environmental signals such as pH, temperature, nutrient availability, etc. and varies for different microorganisms (Chmielewski and Frank, 2003; Cloete et al., 2009). Biofilm formation starts with the attachment of planktonic microorganisms (stage a) that are in close proximity to surfaces or to each other. Various cell wall structures such as flagella, pili and outer membrane proteins are involved in this active initial attachment as they help to overcome repulsive electrostatic and hydrophobic forces between the surface and the organism (Cloete *et al.*, 2009; Frank, 2009). Conjugative plasmids would, according to Ghigo (2001), play a role in biofilm development as they can encode for pili and

consequently promote bacterial attachment. Also, attachment can be passively driven by gravity, diffusion and fluid dynamics (Chmielewski and Frank, 2003). The forces between organisms and surfaces (electrostatic and van der Waals forces) are weak during this stage whereby attachment is still reversible (Cloete *et al.*, 2009). It is possible that the surface on which microorganisms attach is preconditioned with a film of organic molecules and charged ions (also called fouling), which may influence the affinity with the organisms (Chmielewski and Frank, 2003; Cloete et al., 2009).

In a second stage, a more stable/irreversible attachment (b) is reached by the interaction of the surface with motility structures, cell wall structures and excreted exopolymers of the microorganisms. Forces involved are dipole, ionic, hydrogenic or hydrophobic interactions (Chmielewski and Frank, 2003; Cloete et al., 2009). Once irreversible attachment is achieved, a range of phenotypic and genotypic changes take place. Transcription of these specific genes is required for the further development of the biofilm.

The cell number increases by a combination of growth and recruitment of planktonic cells. When present in high numbers, bacteria regulate their collaborative activities and physiological processes by a mechanism called quorum sensing (QS), whereby signal molecules are produced, detected and responded to. In case cells are in close proximity to each other i.e. when they are in a community, the QS system is activated and signal molecules, called autoinducers, are produced and detected by a sensor in other bacterial cells. This causes expression of certain genes, influencing processes such as spore formation, programmed cell death, virulence, antibiotic production, etc. Moreover, the QS system plays a role in the formation and development of biofilms (Li and Tian, 2012). Autoinducers, for example N-acylhomoserine lactones (AHLs) produced by many Gram-negative bacteria or modified oligopeptides produced by Gram-positive bacteria, accumulate because the number of cells at a particular location increases. Once the critical level of autoinducers is reached, a number of genes show an altered expression level (Frank, 2009). These genes could influence the production of EPS components but also provide the typical biofilm phenotype involving tolerance and resistance, growth rate, virulence, etc. Increased cell numbers together with the secretion of EPS components leads to the development of the specific biofilm architecture (stage c). Biofilms usually exhibit a high level of organisation (Frank, 2009). The introduction of confocal light

scanning microscopy showed that microorganisms grow in microcolonies separated by less dense regions of the matrix with high permeable channels (Costerton *et al.*, 1994). These channels facilitate diffusion of nutrients, gases and metabolic waste products (de Beer et al., 1994).

If conditions are favourable, microcolonies will further develop into macrocolonies during the maturation stage (d) as a result of increasing cell numbers and EPS production. Several biofilm structures have been observed, among which the mushroom-like pillars formed by pseudomonads. A mature biofilm exhibits a higher metabolic and physiological heterogeneity compared to microcolonies (Cloete *et al.*, 2009).

Finally, the biofilm reaches a critical mass and dynamic equilibrium at which the outer layers begin to generate planktonic cells. Enzymes produced by the embedded cells can contribute to the detachment process, together with a local imbalance in EPS and nutrient level (Chmielewski and Frank, 2003; Cloete et al., 2009). Also, QS contributes to the dispersal of cells.

Detachment and dispersal of planktonic cells from the biofilm (e) can also be the result of external processes such as erosion and sloughing by fluid dynamics and shear effects. Detachment can result in the continual dispersal of single cells (erosion) or rapid loss of large cell aggregates (sloughing) (Cloete *et al.*, 2009; Frank, 2009). Cells can also detach as a result of human intervention like for example the performance of C&D. This final step in biofilm development is necessary for colonisation of new surfaces (Cloete *et al.*, 2009).

2.3.4 Biofilm composition

Different kinds of microorganisms can be present in biofilms. Most interest goes to bacterial biofilms because of their large medical and economic impact (Costerton et al., 1999; Stewart, 2002; Thomas et al., 2005; Høiby et al., 2011; de la Fuente-Núñez et al., 2013; Nya, 2015). More specifically, *Pseudomonas aeruginosa* biofilm research is abundant and consequently this organism is often used as model organism for biofilm formation (Hoyle et al., 1992; Drenkard, 2003; McDougald et al., 2008). In principle, all bacteria can occur in biofilms. Besides bacteria, other prokaryotic organisms i.e. archaea were observed in biofilm associations in more extreme environments such as thermal springs, crater lakes and caves (Fröls, 2013). Fungal biofilms are a well-recognised problem in clinical settings, of which *Candida* biofilms are the most widely studied ones

(Fanning and Mitchell, 2012; Costa-Orlandi et al., 2017). Also, algal biofilms were observed on moist surfaces (Leadbeater and Callow, 1992). Biofilms observed in nature are mostly mixed communities of the above mentioned groups of microorganisms.

The EPS matrix produced by the microbial cells is the basis for the three-dimensional structure of the biofilm. EPS plays an important role in colonisation or adhesion to surfaces, it provides the embedded cells with water and nutrients, it serves as a protective barrier against environmental stresses such as mechanical action, antimicrobial agents, antibiotics, oxidation, etc. (Flemming and Wingender, 2010; Flemming, 2016). Moreover, EPS keeps the embedded cells in close proximity whereby interaction and cell communication is more intense and HGT is enhanced (Donlan, 2002; Cloete et al., 2009; Flemming and Wingender, 2010; Savage et al., 2013; Van Meervenne et al., 2014).

The EPS matrix consists of many different types of biopolymers and this depends on several factors such as the present microorganisms, experienced shear forces, temperature, available nutrients, etc. Polysaccharides are a large part of the EPS matrix. Another important part are extracellular proteins. Enzymes, for example, can be present and degrade biopolymers in the matrix to provide nutrients for the cells or to promote detachment of the cells for colonisation of other surfaces. On the other hand, non-enzymatic proteins which are cell-surface associated or extracellular have a more structural function in the EPS matrix as they are involved in the formation and stabilisation of the polysaccharide network. Also, extracellular DNA (eDNA) is present in the EPS matrix which is not only a remnant of lysed cells but also plays a structural role during adhesion. The presence of lipids provides a more hydrophobic character to the biofilm matrix. As the EPS matrix creates a highly hydrated environment for the embedded cells, water is also a major component (Flemming and Wingender, 2010). Unfortunately it is not easy to investigate the EPS composition and to provide a complete biochemical profile of this part of the biofilm as it is so diverse (Flemming et al., 2007).

2.3.5 Biofilm formation by *Salmonella* and *Pseudomonas*

The presence of *Salmonella* is a well-known problem in both food industry and primary production (especially poultry). If *Salmonella* is present in the production environment of food companies, it is possible that this pathogen will be transferred to the food products and will finally reach the consumer causing foodborne disease (Figure 2.3). The genus *Salmonella* can be divided into two species i.e. *Salmonella enterica* and *Salmonella*

bongori. *Salmonella enterica* contains more than 2500 serotypes clustered in six subspecies, which highlights the great diversity within this genus. The most frequent serotypes in relation to foodborne illnesses in the European Union are *Salmonella enterica* subsp. *enterica* serotype Enteritidis and *Salmonella enterica* subsp. *enterica* serotype Typhimurium or short *Salmonella* Enteritidis and *Salmonella* Typhimurium. *Salmonella* can be divided into host-restricted, host-adapted and unrestricted serotypes. Host-restricted serotypes cause systemic diseases in only one species (e.g. *Salmonella* Typhi in human), host-adapted serotypes cause disease in more than one species (e.g. *Salmonella* Dublin in cattle but also in humans) and unrestricted serotypes occur in a wide range of species (e.g. *Salmonella* Enteritidis and *Salmonella* Typhimurium) (Uzzau et al., 2000). Another distinction can be made between typhoidal and non-typhoidal *Salmonella* serotypes. Non-typhoidal (e.g. *Salmonella* Enteritidis and *Salmonella* Typhimurium) mostly have a broad host-specificity and cause gastroenteritis worldwide, while few *Salmonella* serotypes (among others *Salmonella* Typhi and Paratyphi B) cause enteric fever which is a life-threatening, systemic disease mostly occurring in the developing world (Gal-Mor et al., 2014).

Understanding how *Salmonella* enters and survives along the food chain is important for the development of elimination strategies. It is believed that biofilm formation plays an important role in the persistence of this genus (Grimont and Weill, 2008; Gamazo et al., 2009). Concerning *Salmonella* biofilm formation, the expression of fimbrial genes (more specifically curli thin aggregative and type I fimbriae) is related to the biofilm-forming capacity of the strain as it promotes initial cell-surface and cell-cell interactions. A second factor concerns the EPS compound cellulose, playing also an important role in biofilm formation by *Salmonella*, which facilitates the long-term survival of the bacterial cells. A third factor is the presence of biofilm associated proteins, providing connections between bacterial cells (Gamazo et al., 2009; Steenackers et al., 2012). QS sytems that have already been described in *Salmonella* and have a role in the regulation of *Salmonella* biofilm formation are AHL, autoinducer-2 and autoinducer-3 signalling. There is a response to AHLs of other bacterial species by the transcription factor SdiA produced by *Salmonella*. The function of SdiA-regulated genes (among others resulting in the production of extracellular matrix proteins and the expression of plasmid-encoded fimbriae) suggests its role in *Salmonella* biofilm formation. Also autoinducer-2 and 3 are shown to be involved in the regulation of biofilm-specific genes (Steenackers et al., 2012). Several

reports have demonstrated biofilm formation by *Salmonella* on different types of abiotic surface materials used in several sectors such as farms, slaughterhouses, food processing industry, kitchens, etc… (Steenackers *et al.*, 2012). Of course, serotype or strain type plays an important role in the development of biofilms (Chia et al., 2009; Schonewille et al., 2012; Wang et al., 2013).

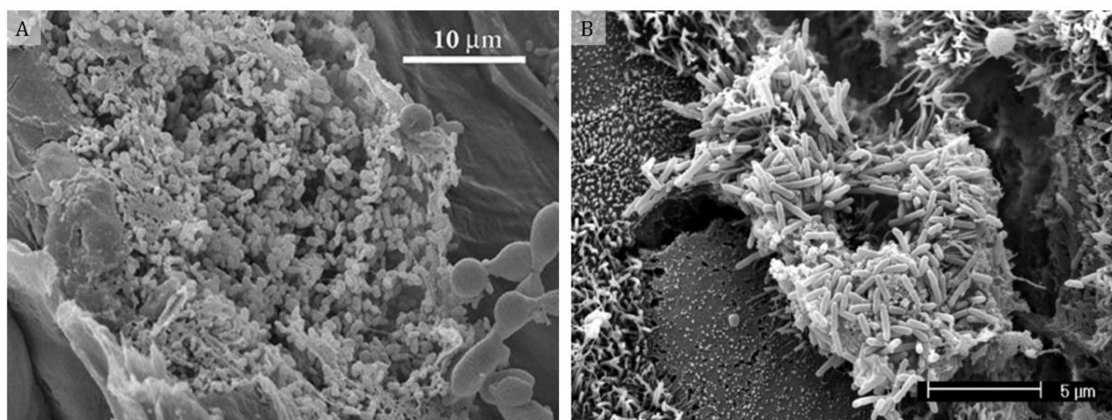

Figure 2.3: Scanning electron microscopy image of A) biofilm formation by *Salmonella enterica* serotype Poona on Cantaloupes (adapted from Annous *et al.*, 2009) and B) biofilm formation by *Pseudomonas aeruginosa* on epithelial cells (adapted from Woodworth *et al.*, 2008).

Also *Pseudomonas* is a known genus that is abundantly present in the environment. *Pseudomonas* spp. are the most identified species in the food processing sector and are of great importance concerning food spoilage. The genus *Pseudomonas* consists of many, some pathogenic, species that are metabolically versatile and often possess tolerance or resistance properties (Palleroni, 1993). The species *Pseudomonas aeruginosa* is considered an opportunistic human pathogen which may be the consequence of its resistance to antibiotics and disinfectants that limits competition with other bacteria. Moreover, this species is known for its good biofilm-forming properties (Figure 2.3). Mucoid and non-mucoid strains can be distinguished depending on the qualitative composition of excreted polysaccharides (among which alginates) in the EPS matrix. In *Pseudomonas aeruginosa* biofilms, eDNA plays an important role in protection, nutrition, motility and cell-to-cell interaction of the embedded cells. This is a consequence of the interaction with polysaccharides, leading to the formation of a protective and stable fiber network. Also extracellular flagella, pili and fimbriae are important matrix components in *Pseudomonas* biofilms as they have a motility and/or adhesive function (Stover et al., 2000; Meliani and Bensoltane, 2015; Rasamiravaka et al., 2015). *Pseudomonas* biofilms

are generally believed to produce much biomass leading to advanced mushroom- and pillar like structures (Korber et al., 2009). *Pseudomonas putida* has emerged as an often used laboratory work strain and as a model organism for food spoilage (Van Meervenne, 2014).

More information about the presence of *Salmonella* and *Pseudomonas* as residual bacterial contamination or biofilm in the food industry and in primary animal production is given in sections 2.5.1 and 2.6.1, respectively.

2.4 BIOFILM RESEARCH: AN OVERVIEW OF SOME MODEL SYSTEMS

A lot of the present knowledge about biofilms was obtained in biofilm model systems. There are a lot of *in vitro* and *in vivo* biofilm models to study their development, structure and characteristics under particular circumstances. Mostly, fixed parameters such as medium, incubation temperature, inoculum strain(s) and concentration, flow rate, attachment surface, etc. are chosen to investigate biofilm behaviour. Sometimes even antimicrobial agents or C&D protocols are applied to model biofilms to investigate their response to these stresses (Donlan and Costerton, 2002). On the other hand, some model systems are suitable to evaluate the effect of multiple values or a gradient of one parameter. Of course, biofilm models should provide repeatable and reproducible results.

One of the most used *in vitro* model systems for biofilm formation are 96-well microtiter plates (MTPs) (Coenye and Nelis, 2010; Elhariry et al., 2012; Wang et al., 2013). Also 6, 12 or 24 well MTP can be used wherein biofilms can form on the bottom or walls of the wells or on a coupon placed in the wells. MTP biofilm models are user-friendly, quick, cheap, closed and high-throughput systems (Coenye and Nelis, 2010). This model is consequently ideal for screening purposes and evaluation of the influence of multiple parameters (Niu and Gilbert, 2004). Yet, the main disadvantage of 96-well MTPs is that test conditions differ a lot from reality. A variation on traditional MTPs is the Calgary biofilm device wherein pegs are present on the lid of the MTP that perfectly fit into the wells of the MTP. Biofilms will grow on these pegs and can be easily transferred to new MTPs. This model is often used for the evaluation of antibiotic susceptibility and biofilm eradication by antimicrobial agents and is meanwhile commercially available (Ceri *et al.*, 1999).

Another very popular commercial *in vitro* model is the Modified Robbins device. A big advantage compared to MTPs is that flow conditions can be simulated and desired test materials upon which biofilms can develop can be assembled on plugs that fit into the device (Coenye et al., 2008). Flow cells are another example of plug flow reactors and are very suitable for real-time non-destructive monitoring of biofilm formation on the glass chambers using microscopy techniques (Coenye and Nelis, 2010). The Centers for Disease Control and Prevention (CDC) biofilm reactor consists of a glass vessel with moveable paddles upon which coupons of the desired materials can be assembled for biofilm formation. This is also an open system where flow conditions can be simulated and identical replicates can be obtained. Biofilm formation over time can be studied in this model and also C&D procedures can be easily tested. However, it is less suitable to test antimicrobial agents against biofilm formation because of the larger required volumes (Buckingham-Meyer et al., 2007; Nailis et al., 2009). Drip flow reactors are also flow displacement systems simulating environments close to an air-liquid interface with low shear and can for example be used to assess plasmid transfer between strains in a biofilm (Van Meervenne *et al.*, 2014).

Also model systems for *in vitro* biofilm formation on biotic surfaces are available wherein human cell lines are used for the study of for example mucosal, oral or epithelial biofilms. In this model, again the natural environment (microbiota) is not included (Dongari-Bagtzoglou, 2008). Besides the *in vitro* models, *in vivo* models are used for studies in very specific environments that may be representative for other situations. Examples are the *Caenorhabditis elegans* model (which is a model for molecular and developmental biology) or vertebrate animal models (Coenye and Nelis, 2010). Limitations for *in vivo* models are that ethical considerations have to be taken into account together with extra efforts needed for the maintenance of the animals.

There are also specific model systems available for biofilm simulation in drinking water systems, some of them described above and also useful for other applications. A distinction can be made between *in vitro* and *in situ* models wherein biofilm formation and biofilm removal can be investigated (Gomes et al., 2014).

It has been demonstrated that biofilm formation under laboratory (optimal) conditions differs from biofilm formation under practice (actual) conditions, indicating that laboratory tests sometimes have limited significance to the understanding of biofilm

formation in processing environments (Wang *et al.*, 2013). Yet, studying biofilms in their natural environment is not obvious since online monitoring of the desired characteristics is not always possible. Also, there will be no two identical biofilms under field conditions, which makes it difficult to draw general conclusions. Therefore, biofilm models are useful because conditions can be more controlled and repetitions can be observed. Every model system has its own advantages and disadvantages, and dependent on the research question, the most suitable biofilm model system can be implemented or an adapted or new model can be developed.

2.5 CONTAMINATION IN FOOD INDUSTRY

2.5.1 Presence of residual microbial contamination and biofilm

As previously mentioned, there are several entrance and dispersal routes for microorganisms in food companies. To reduce or eliminate these microorganisms as good as possible, standard operating procedures such as hazard analysis of critical control points (HACCP), good manufacturing practices (GMP) and good hygiene practices (GHP) including the application of C&D, are performed. Despite these measures, it is possible that residual bacterial contamination and biofilms still occur in food processing environments.

Because of their high nutrient and favourable moisture, pH and temperature conditions, food processing environments provide the ideal growth environment for a broad range of bacteria possibly resulting in biofilm formation (Chmielewski and Frank, 2003). Residual bacterial contamination can occur on every surface in the food company, but are of most importance on food contact surfaces since they can in this way reach the produced food products (Marriott and Gravani, 2006; Korber et al., 2009; Møretrø and Langsrud, 2017).

Concerning biofilm formation in food processing environments, the preconditioning of the surface with organic and inorganic molecules or components released from food products plays an important role in the initial attachment of the individual microbial cells. Also the last stage of biofilm development, i.e. the detachment and dispersal of the cells has a major impact in food companies because this leads to contamination of other surfaces and also contamination of the food products (Korber *et al.*, 2009). Unfortunately, few direct observations about the frequency, microbial composition and structure of residual bacterial contamination and biofilms in food processing environments are available.

The diversity of growth niches (nutrients, water availability, temperature, etc.) and variation in C&D frequency make it difficult to draw general conclusions about biofilm formation in several food sectors. Mostly surfaces in the processing environment rather than food contact surfaces are prone to the development of biofilms. Besides, also places that are difficult to reach and clean have a greater chance for biofilm formation. Surfaces that pose an increased risk for biofilm formation are dead ends in pipelines, corners, gasketed joints, hoses, exteriors or interiors of equipment, floor drains, etc. because they are difficult to clean or sometimes they are not included in the C&D procedure (Kumar and Anand, 1998; Frank, 2009; Korber et al., 2009). It must be emphasised that there is a tendency in the food industry to refer to every microbial surface contamination as biofilm, however, mostly these microorganisms do not get the opportunity to form a mature biofilm due to C&D (Frank, 2009).

The majority of the microorganisms identified in food processing environments after C&D are non-pathogenic (Møretrø and Langsrud, 2017). Yet, studies have reported the presence of *Salmonella* spp., *Escherichia coli* O157:H7, *Listeria* spp. (among which *Listeria monocytogenes*), *Bacillus* spp. and *Staphylococcus* spp. on food contact surfaces in several food processing plants after C&D (Vogel et al., 2001; Chmielewski and Frank, 2003; Rivera-Betancourt et al., 2006; Schlegelová et al., 2010; Møretrø and Langsrud, 2017). Floor drains seem to be an important source of *Salmonella* contamination in food processing environments (Rivera-Betancourt *et al.*, 2006). Residual bacteria or background flora on production surfaces are in most studies or quality systems represented by general enumeration data but often not identified. Therefore, their impact on food quality and safety is difficult to estimate. Møretrø and Langsrud (2017) gave an overview of the dominant residential bacteria on clean surfaces in different food sectors. This review indicates that bacterial diversity on surfaces in the food industry is high. Groups of bacteria with the highest prevalence in the study were identified as *Pseudomonas*, *Acinetobacter*, Enterobacteriaceae, spore-forming bacteria, *Staphylococcus* and lactic acid bacteria (LAB). Other genera that were also highly prevalent were *Aeromonas*, *Brochotrix*, *Microbacterium*, *Micrococcus*, *Neisseriaceae*, *Psychrobacter*, *Ralstonia*, *Rhodococcus*, *Shewanella*, *Sphingomonas*, *Stenotrophomonas* and *Vibrio*. Over all food sectors, Gram-negative bacteria dominate over Gram-positive bacteria. Carpentier and Chassaing (2004) reported the genera *Bacillus*, *Pseudomonas*,

Staphylococcus and *Stenotrophomonas* as being dominant on several sampling points in the food processing environment after C&D.

Pseudomonas spp. is the most frequently reported genus present on food processing surfaces after C&D. This genus is abundantly present in raw materials and water and is in this way introduced in food companies, where it can survive easily because of its high tolerance against low nutrient availability, temperature and other stress factors. Biofilm formation plays a role in the residential characteristics of this genus (Chmielewski and Frank, 2003; Korber et al., 2009; Møretrø and Langsrud, 2017). *Pseudomonas* biofilms in the food industry can be found on environmental surfaces (floors, drains, walls, disinfecting baths, etc.), food contact surfaces (conveyor belts, pipes, trolleys, etc.) and even on the food products themselves (spinach, lettuce, sprouts, etc.). Since the genus *Pseudomonas* consists of many different species, there is also a wide diversity among these species in food companies (Meliani and Bensoltane, 2015; Møretrø and Langsrud, 2017).

The occurrence of *Acinetobacter* is similar to *Pseudomonas* and its characteristics are also comparable (Møretrø and Langsrud, 2017). Enterobacteriaceae are commonly isolated from food contact surfaces and are sometimes used in monitoring programs as an indicator for faecal contamination, although many genera in this group are also present in the natural environment (Møretrø and Langsrud, 2017). Several genera that are part of the LAB group were mainly but not exclusively identified in dairy companies. LAB can be used as starter culture for food fermentation but they can also be introduced in food companies by other sources (Møretrø and Langsrud, 2017). *Staphylococcus* is one of the most identified Gram-positive genera identified in the food processing environment and often enters the company through raw materials or staff (Møretrø and Langsrud, 2017). Spore-forming bacteria such as *Bacillus* spp. are frequently introduced in the processing environment as they are abundantly present in raw materials and in natural environments (e.g. soil). The spore form of this genus is resistant to industrial environmental stresses such as heat treatment, desiccation and disinfection (Møretrø and Langsrud, 2017). Moreover, the species *Bacillus cereus* produces hydrophobic spores that attach even more readily on stainless steel surfaces than their vegetative counterparts (Ryu and Beuchat, 2005). In very specific (extremely dry, acid, hot) environments, fungi and yeasts can be the dominant microorganisms (Chmielewski and Frank, 2003; Korber

et al., 2009; Møretrø and Langsrud, 2017). It is possible that the presence of certain, at first sight harmless, strains can enhance the survival or attachment of pathogenic strains such as *Listeria monocytogenes* and *E. coli* O157:H7 (Carpentier and Chassaing, 2004; Habimana et al., 2010a). On the other hand, the presence of biofilm could also negatively affect the attachement of pathogens. This is due to naturally occurring interactions between different species (Mitri and Foster, 2013). It is assumed that surface contamination mostly consists of multiple species. However, knowledge about identification and characteristics of whole microbial communities on food contact surfaces is still limited. Most research focuses on the detection and quantification of pathogens on food contact surfaces, but information about the presence, quantity, identity and properties of other bacteria, among which spoilage organisms, is still highly needed. In addition, concerning biofilms that are present in food companies, there is no information available on the chemical composition of the EPS matrix. Research about the microbiological and chemical composition of residual contamination would provide important input for food companies to optimise their C&D procedures to further reduce the risk for foodborne illness and early food spoilage.

The type of surface material also has an influence on the presence of residual bacteria or biofilms. Materials that are often used in the food industry are stainless steel, different types of plastics and rubber (Kumar and Anand, 1998; Faille and Carpentier, 2009). Requirements for materials in contact with food products are that they have to be food grade, inert and hygienic (Faille and Carpentier, 2009). In clinical settings it was already demonstrated that implant material has a strong influence on infection susceptibility (Rochford et al., 2012) and also in food industry, differences in surface properties influence attachment and biofilm formation by some species (Faille and Carpentier, 2009; Gutiérrez et al., 2016). It is recommended to use smooth, high-polished surfaces and avoid scratches and crevices as this makes initial attachment of bacteria more difficult and prevents the arise of protective microniches for bacteria (Korber *et al.*, 2009).

2.5.2 Possible impact

The presence of residual bacterial contamination or biofilms can have several, mostly undesired, consequences. Microbiological contamination on sanitised surfaces can be transferred to the manufactured food and consequently lead to foodborne illness and early food spoilage. To cause foodborne illness, it is sufficient that pathogens or their

toxins are present on the food at their infectious dose (which is sometimes only a few bacteria or toxins) (Kothary and Babu, 2001). To cause food spoilage on the other hand, bacterial growth must occur to reach high levels of the spoilage bacteria and their spoilage products such as enzymes, off-flavours and off-odours (Møretrø and Langsrud, 2017). However, food preservation methods (heat treatment, low temperature, conservation, modified atmosphere packaging, additives, etc.) are applied to limit this growth.

The most reported gastrointestinal human pathogens in 2016 in the European Union were *Campylobacter* and *Salmonella* with 246.307 and 94.530 confirmed cases, respectively. However, the pathogen most related to foodborne outbreaks was *Salmonella* (responsible for 65.8% of all bacterial outbreaks resulting in 9.061 patients), followed by *Campylobacter* (28.3% of the bacterial outbreaks resulting in 4.606 patients) (European Food Safety Authority and European Centre for Disease Prevention and Control, 2017). Foodborne infection with *Listeria monocytogenes* is mostly caused by the consumption of insufficiently heated meat, seafood products or minimally processed foods and can lead to non-invasive gastroenteritis or invasive listeriosis (Allerberger and Wagner, 2009). Foodborne pathogenic *E. coli* (such as *E. coli* O157) can cause severe diarrhea but also hemolytic-uremic syndrome and is mostly related to the consumption of raw products such as fruits, vegetables, cattle meat and dairy products (Verhaegen, 2016; Yang et al., 2017a). *Bacillus cereus* is a toxin producing species which causes vomiting or diarrhea and is frequently found in dairy products, rice and pasta because of its tolerance to heat and other stress factors (Tewari and Abdullah, 2014; Møretrø and Langsrud, 2017). Also *Staphylococcus aereus* can produce toxins and cause foodborne intoxications (Schlegelová et al., 2010; Møretrø and Langsrud, 2017).

Pseudomonads are the most reported food spoilage organisms and are very problematic in aerobically stored food at low temperatures such as dairy, meat, fish and poultry. This group generally produces extracellular enzymes such as lipases and proteases, causing off-odours, off-flavours and rancidity. Especially heat-stable proteases produced by pseudomonads and post-processing contamination (meaning after a decontamination step such as pasteurisation) with this group of organisms are risks that can lead to spoilage (Chmielewski and Frank, 2003; Korber et al., 2009; Marchand et al., 2009b, 2012; Rawat, 2015; Stellato et al., 2015; Møretrø and Langsrud, 2017). Compared to pseudomonads, *Acinetobacter* spp. are of less importance in food spoilage (Møretrø and

Langsrud, 2017). Enterobacteriaceae are frequent dairy spoilers and occasional spoilers of modified-atmosphere packed and vacuum packed meat products and are the dominant spoilage organisms (over pseudomonads) for food products stored at higher temperature (Møretrø and Langsrud, 2017). LAB are important spoilers in cold stored foods packed at modified or vacuum atmosphere. The spoilage caused by LAB will typically result in souring, gas and slime formation and hydrogen sulphide production. Yet, sometimes they are deliberately added as starter culture inducing a fermentation process to obtain typical flavours and odours in cheeses and fermented sausages (Borch et al., 1996; Giaouris et al., 2014; Rawat, 2015; Møretrø and Langsrud, 2017). Spore-forming bacteria such as *Bacillus* spp. are important spoilers in pasteurised milk products by the production of extracellular enzymes (De Jonghe et al., 2010; Marchand et al., 2012; Rawat, 2015; Møretrø and Langsrud, 2017). Also yeast and moulds can lead to spoilage of products that are a suitable niche for these organisms, like fruit juices (Korber et al., 2009; Møretrø and Langsrud, 2017).

On the other hand, especially fouling and biofilm formation by these or other organisms can decrease heat transfer in for example heat exchangers, it can lead to mechanical blockage of tubes and it causes corrosion dependent on the type of surface material. Together with the costs of the energy loss and renewal of equipment, extra costs for the increased efforts and time that are necessary for sanitation have to be taken into account (Kumar and Anand, 1998; Trachoo, 2003).

2.6 CONTAMINATION IN PRIMARY ANIMAL PRODUCTION

2.6.1 Presence of residual microbial contamination and biofilm

Also in primary animal production, there are ways for microorganisms to enter and spread throughout the farm. The presence of animals, faeces, feed, water, etc. gives rise to high microbial numbers in the environment during housing. C&D between production rounds is performed to limit the infection pressure on newly arriving young animals and to prevent the persistence of foodborne pathogens. Of course, microorganisms are not fully eliminated.

According to (Luyckx et al., 2015a) the most accurate method for the evaluation of C&D in broiler houses was taking swab samples for enumerations of total aerobic count (TAC), *Enterococcus* spp. (as a hygiene indicator) and *E. coli* (as an indicator for *Salmonella* spp.) instead of agar contact plates. Locations that are still the most contaminated after C&D

are drain holes, floor cracks and drinking cups (Mueller-Doblies et al., 2010; Dewaele et al., 2012; Luyckx et al., 2015a). It is clear that the highest contaminated locations after C&D are places that are difficult to clean. Residual bacteria and dirt (which could serve as a nutrient source for the present microorganisms) can consequently evolve into a biofilm. Moreover, the fact that C&D is only performed in broiler houses after each production round (which is approximately six weeks for broilers) gives microorganisms more time to develop to a mature and strongly attached biofilm compared to biofilms in the food industry where C&D is usually performed at the end of every production day. The study of (Luyckx et al., 2015a)showed that C&D managed to decrease mean TAC on contaminated locations from 7.7 ± 1.4 to 4.2 ± 1.6 log CFU/625 cm^2 whereby the cleaning step provided the largest part of this decrease. In a successive study of Luyckx *et al.* (2016a), most dominant genera identified after C&D in broiler houses were *Brevibacterium*, *Microbacterium* and *Staphylococcus*. According to Mueller-Doblies *et al.* (2010), 68% of the evaluated turkey houses were still *Salmonella* positive after C&D. In Belgium, 0.27% of the broiler flocks ready for slaughter were positive for *Salmonella* Enteritidis or *Salmonella* Typhimurium and 2.41% were positive for *Salmonella* spp. in 2017 (Pierré, 2018). In a study of Marin *et al.* (2009) in broiler houses, the most prevalent serotype was *Salmonella* Enteritidis, whereof approximately 60% of the tested strains had biofilm-forming capacity. According to a study of van Asselt *et al.* (2009), the most identified serotype at the time of departure of broiler chickens from the broiler farm was *Salmonella* paratyphi B variant Java (hereafter named *Salmonella* Java), whereas this serotype was not abundantly present on samples taken before the chicks entered the farm. This indicates that *Salmonella* Java is mostly introduced at farm level whereafter it remains the predominant serotype until the end of the rearing. Besides the frequent identification of *Salmonella* Java in this Dutch study, its dominant presence was also determined in studies conducted in Belgium, Luxembourg and Germany but less in other European countries (van Asselt *et al.*, 2009). It was already described that *Salmonella* Java spreads rapidly within the flock and can persist easily (Van Immerseel *et al.*, 2004). Hygiene measures appear to be less effective against *Salmonella* Java compared to other *Salmonella* serotypes, which could explain the persistent character (van Pelt *et al.*, 2003). Another explanation could be that *Salmonella* Java has good biofilm-forming capacities and is thereby easily maintained in the stable environment. Yet, so far there is no evidence that supports this hypothesis and there is limited information available about specific

locations where this organism resides. The persistence of *Salmonella* Java in broiler houses and on broiler meat can cause a serious health threat for humans, especially because of the increasing antimicrobial resistance character of these strains (van Pelt *et al.*, 2003). In Belgium, an increasing trend in the prevalence of *Salmonella* Java was observed over recent years (Pierré, 2018). The presence of *Pseudomonas* spp. in primary production environments and especially in broiler production is less known. In the study of Luyckx *et al.* (2016a), only few non-pathogenic *Pseudomonas* strains were identified in broiler houses.

Contamination of drinking cups in broiler houses after C&D can lead to infection of a new flock due to direct contact with the animals while drinking, but can also lead to contamination of the whole drinking water system (DWS). The water quality on broiler farms is regularly evaluated at the source and sometimes at the end of the drinking lines depending on the type of DWS (open or closed), but along the drinking lines (where the animals actually drink) often no assessment is done (Van Eenige et al., 2013; Vermeersch, 2016). Infected DWS with e.g. *Salmonella* spp., *Campylobacter jejuni*, *E. coli* and *Pseudomonas* spp. have been reported and can be of great importance for the flock's health (Pearson et al., 1993; Waage et al., 1999; Heyndrickx et al., 2002; Herman et al., 2003; Zimmer et al., 2003; Maharjan et al., 2016). Moreover, the combination of a convenient temperature, low flow rates and sufficient nutrients makes the DWS in broiler houses ideal for microbial numbers to increase and biofilms to form (Sparks, 2009). Unfortunately, sampling of surfaces on the inside of the DWS of broiler chickens is even less performed compared to water analysis. *Aeromonas* spp., *E. coli*, *Pseudomonas* spp and *Sphingomonas* spp. were previously described as biofilm-forming organisms in water systems of bovine and humans (Elhariry et al., 2012; Van Eenige et al., 2013; Liu et al., 2014; Mulamattathil et al., 2014; van der Wielen and Lut, 2016), but also *Salmonella* spp. and *Campylobacter* spp. are capable to form biofilms in poultry environments (Reeser et al., 2007; Zhao et al., 2011). However, current knowledge about the effective presence, importance and composition of biofilms on the inside of the DWS in broiler houses is still very limited. It was shown that coexistence of *Pseudomonas* with several pathogens enhances the survival of the pathogen in food processing environments (Habimana et al., 2010b; Hilbert et al., 2010; Culotti and Packman, 2015). Yet, knowledge is lacking about the importance of biofilm in the protection of zoonotic pathogens (e.g. *Salmonella* Java) by harmless bacteria (e.g. non-pathogenic *Pseudomonas* spp.) in the DWS of broilers.

2.6.2 Possible impact

There are a lot of infectious agents that can lead to poultry diseases. Examples are *Salmonella* Pullorum causing pullorum disease (systemic disease of poultry leading to poor growth, weakness and death), *E. coli* causing inflammation of the oviduct and ovary, and *Pseudomonas aeruginosa* causing inflammation of the eye, all followed by high mortality and economic losses for the farmer (Kebede, 2010; Nolan et al., 2017; Swayne, 2017a; b). The dispersal of these poultry pathogens is sometimes caused by vertical transmission (from mother animal to offspring) but mostly horizontal transmission (from animal to animal or by means of a vector) is responsible for a widespread infection. The environment plays an important role in this horizontal transmission (Heyndrickx et al., 2002; Herman et al., 2003)

Salmonella and *Campylobacter* are reported as responsible for the most reported foodborne outbreak in the European Union (see 2.5.2) and chicken meat is recognised as one of the main routes by which these pathogens can reach and infect humans. Therefore, it is of great importance to limit the presence of these pathogens in broiler houses and consequently on broiler meat as much as possible (Sparks, 2009). It was also acknowledged that biofilm formation as a reservoir for *Salmonella* in the farm environment is of great importance for food safety and consequently animal health (Schonewille *et al.*, 2012).

Especially as a consequence of biofilms in the DWS of poultry, animals can be under dosed due to the capture of drug particles (for broilers typically administered by the drinking water) in the biofilm matrix which can lead to risks for animal health and the development of resistant strains (Roberts et al., 2008; Høiby et al., 2010a). Concerning the development of resistant strains, biofilms are known as hotspots for plasmid transfer and consequently also for the transmission of resistance genes (Hennequin et al., 2012; Savage et al., 2013; Van Meervenne et al., 2014).

Additional costs in case of *Salmonella* or *Campylobacter* contamination of broilers (in respectively Belgium and the Netherlands) also have to be taken into account for logistic slaughter (meaning *Salmonella*- or *Campylobacter*-positive flocks have to be processed at the end of the day) and for extra efforts concerning a thorough C&D.

2.7 DETECTION AND SAMPLING OF SURFACE CONTAMINATION

Monitoring of surface contamination is an important tool to evaluate C&D procedures. In the food industry, the monitoring of surface contamination (among which residual bacteria and biofilms) is used to evaluate the hygienic state of surfaces and equipment and to map contamination routes through the production chain. In some primary production sectors, similar measures are taken. Most of the monitoring methods for surface contamination are either based on the enumeration and/or detection of microorganisms or on the quantification and/or detection of chemical compounds and they mostly concern offline methods, although an online monitoring should be recommended. Suitable monitoring methods should preferably provide *in situ* information about the adhesion and removal of contamination, should not interfere with the process, should be robust and reliable and should be applicable on different types of surface materials. It is obvious that monitoring should take place on locations where contamination is most likely to occur and which are representative for the overall plant (Pereira and Melo, 2009). In addition to monitoring or detection, sampling allows for a further characterisation of the surface contamination providing more information about its composition.

2.7.1 Detection methods for surface contamination independent of its composition

The most obvious way for the evaluation of surfaces for contamination is a visual inspection. This method gives no information about the composition of the surface contamination and in many cases, a false negative assessment will be given since surface contamination (especially microorganisms) is not always visible to the naked eye. Therefore, this method is mostly combined with other monitoring techniques.

Online monitoring methods that depend on different phenomena caused by surface contamination are available. However, one must be aware of what is exactly measured by each technique and how these measured quantities correlate with features of the contamination. Janknecht and Melo (2003) gave an overview of different kinds of monitoring systems. For most of the online monitoring methods, input signals are transmitted through the investigated surface. These input signals are modified by the contamination that is present and consequently, specific sensors detect the modified signal which gives us information about the surface contamination. Based on the scattering and absorption of light, turbidity can be a measure of deposits or contamination

on a surface. Surface contamination also leads to reduced heat transfer, which can be a measure for deposits in for example heat exchangers. Beside, increased roughness of the surface and reduced cross-sectional space of the flow passage is a consequence of surface contamination, which can be detected by measuring the pressure drop of the liquid. Detection of metabolic products, produced by microorganisms on surfaces, can be another possibility for the monitoring of microbiological surface contamination. Radiation signals are also used for detection and characterisation of surface deposits. Examples are bioluminescence (whereby the signal is spontaneously generated by the biomass itself), fluorescence (whereby absorption of photons from incoming radiation leads to the emission of radiation of another wavelength which is measured) and spectroscopy (whereby the absorbed radiation is measured) and are specific for the characteristics of molecules. Finally, measurement of electric signals and mechanical vibrations caused by surface contamination can be a method for its detection, quantification and characterisation (Janknecht and Melo, 2003; Pereira and Melo, 2009). These online monitoring methods could be applied in practice for the monitoring of surface contamination in closed systems (e.g. dairy processing lines, drinking water lines, etc.) but are less eligible for open systems (e.g. conveyor belts, tables, devices, etc.).

Commercial methods are known for the detection of residues or biofilm on surfaces in both closed and open systems (for example Bactoforce). For closed systems, deposits are determined by circulating a carbon solution through the system, followed by measurement of the amount of carbon that has reacted with the contamination present in the system. For open systems, locations where surface contamination is likely to occur (such as cracks, dead ends or surface defects) are visualised by a tracer solution followed by UV exposure.

Recently, more research has been done on the application of hyperspectral imaging for the detection of surface contamination and quality inspection of food products. It has for example been used for detection of faecal contamination on vegetables and poultry carcasses and also for detection of pathogenic biofilms on food processing surfaces. This type of research can lead to the further development of portable image devices for inspection of sanitation in several practice conditions (Jun et al., 2010; Yoon et al., 2011; Tewey et al., 2018).

2.7.2 Detection and enumeration of microorganisms

One of the most common ways for detection of microorganisms on surfaces is the application of agar contact plates (ACP). Using this method, an agar plate is directly pressed upon the investigated surface and incubated for enumerations of a group of microorganisms e.g. TAC, LAB or Enterobacteriaceae. This method is, among others, used in primary animal production for the determination of hygienogram scores for the evaluation of the C&D procedures (Maertens *et al.*, 2018) and in food companies for hygiene control because it is easy to use and no further lab processing is necessary. Secondly, swab samples can be taken to monitor surface contamination. Different types of swabs can be used to first sample the surface and subsequently microbial enumerations or detections (e.g. for pathogens) are performed in the laboratory. Swabbing allows to evaluate larger surfaces compared to ACP and multiple microbiological analyses can be performed on the same sample. Beside, the sampling technique is more sensitive compared to ACP. However, results require more effort and more time. A general drawback of culture-based methods is that only cultivable bacteria (which only account for a small percentage of the total number of microorganisms) will be observed under certain growth conditions which will lead to an underestimation of the actual number of microorganisms present. This can be overcome by applying nucleic acid-based techniques such as the polymerase chain reaction (PCR) for the determination of specific organisms or whole communities present on surfaces (Nivens et al., 2009). However PCR-based techniques do not distinguish between living and dead cells.

Another commercially available method that is suitable for the detection of microorganisms is Biofinder (Itram). Provided that there are at least 4 log units, this method visualises catalase positive bacteria by an enzymatic reaction causing a white foam. Also the BART test (Hach) is a commercially available kit for the detection of microorganisms. Dependent on the type of BART test, groups of microorganisms such as microalgae, acid producing bacteria, nitrifying and denitrifying bacteria, fluorescent pseudomonads, heterotrophic aerobic bacteria, iron related bacteria, sulphate reducing bacteria and slime-forming bacteria originating from surfaces or liquids can be detected. These last two methods have, however, not yet proven their value in scientific research. Beside, many other methods are commercially available.

2.7.3 Detection of chemical compounds

Chemical compounds such as proteins, carbohydrates, uronic acids, etc. can be traced as a hygiene control. Several commercial kits are available for the detection of proteins on surfaces. These on-site methods are mostly based on a colour reaction between peptide bonds and a substrate (e.g. copper sulphate in the 3M™ Clean-Trace™ Surface Protein Test Swabs). Similar kits are available for the detection of carbohydrates. Beside these on-site tests, sampling of the surface using swabs followed by analysis in the laboratory for different chemical compounds (e.g. proteins, carbohydrates, uronic acids, etc.) is also a possibility for the monitoring of surface contamination.

Another method that is suitable for surface contamination without bacterial enumerations is adenosine triphosphate (ATP) measurement. ATP is an energy molecule that is present in eukaryotic (among which plant and animal tissue) and prokaryotic (among which bacteria) living cells. ATP measurement systems are commercially available and are generally fast, providing results on site. Another advantage, compared to enumerations, is that all metabolically active cells are measured, independent of their cultivability. ATP measurement can be used for the evaluation of general surface contamination or biomass, however no direct correlation with bacterial cell counts exists (Chmielewski and Frank, 2003; Nivens et al., 2009; Pereira and Melo, 2009).

2.7.4 Detection of biofilm

A lot of the above mentioned monitoring methods for surface contamination only focus on one characteristic or one component (i.e. microorganisms or chemical compounds) of this contamination. However, in the search for biofilms it is necessary to detect microorganisms as well as chemical compounds that are part of the EPS matrix because these are essential components of this mode of growth. Since both components are not unique for biofilms, it is best to analyse them in parallel to exclude pure residual organic (fouling) or microbiological contamination. Moreover, it is advisable to apply monitoring methods after C&D since this approach will also include the residential characteristics of the contamination (Møretrø and Langsrud, 2017).

A non-destructive tool for visualisation of biofilm is the use of confocal laser scanning microscopy (CLSM) in combination with fluorescent dyes or fluorogenic substrates. This allows the simultaneous visualisation of microorganisms and EPS components on surfaces (Flemming and Wingender, 2010). However, this is a laboratory technique which

is not applicable in practice for the detection of biofilm in for example food companies or animal houses. A monitoring method for biofilms in practice that includes both microorganisms and EPS components is still missing and more research about this would be useful. Sampling should collect as much microbial cells as possible from the surface without damaging them and should preserve the composition of the EPS matrix.

CHAPTER 3: EVALUATION OF TWO SURFACE SAMPLING METHODS FOR MICROBIOLOGICAL AND CHEMICAL ANALYSES TO ASSESS THE PRESENCE OF BIOFILMS IN FOOD COMPANIES

Adapted from:

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Chemical characterisation was performed at University of Ghent, Department of Industrial Biological Sciences

3 EVALUATION OF TWO SURFACE SAMPLING METHODS FOR MICROBIOLOGICAL AND CHEMICAL ANALYSES TO ASSESS THE PRESENCE OF BIOFILMS IN FOOD COMPANIES

ABSTRACT

Biofilms are an important source of contamination in food companies, yet the composition of biofilms in practice is still mostly unknown. The chemical and microbiological characterisation of surface samples taken after cleaning and disinfection is very important to distinguish free living bacteria from the attached bacteria in biofilms. In this chapter, sampling methods that are potentially useful for both chemical and microbiological analyses of surface samples were evaluated.

In eight Belgian food companies surfaces were sampled after cleaning and disinfection using two sampling methods, i.e. the scraper/flocked swab method and the sponge stick method. Different microbiological and chemical analyses were performed on these samples to evaluate the suitability of the sampling methods for the quantification of EPS components and microorganisms originating from biofilms in food companies.

The scraper/flocked swab method was most suitable for chemical analyses of the samples as it gave no interference in the determination of the chemical components. For microbiological enumerations, the sponge stick method was slightly but not significantly more effective than the scraper/flocked swab method. In all but one of the food companies, at least 20% of the sampled surfaces showed more than 10^2 CFU/100 cm^2. Proteins were found in 20% of the chemically analysed surface samples, while carbohydrates and uronic acids were found in 15% and 8% of the samples, respectively. When chemical and microbiological results were combined, 17% of the sampled surfaces were found to be contaminated with both microorganisms and at least one of the analysed chemical components, leading them to be characterised as carrying biofilm. Overall, microbiological contamination in the food industry is highly variable by food sector and even within the company at various sampling points and sampling times.

3.1 INTRODUCTION

Biofilms are sessile communities of microorganisms surrounded by a matrix of self-produced extracellular polymeric substances (EPS). Biofilms can occur on almost every surface. EPS plays a fundamental role in the emergent properties of a biofilm (e.g., resource capture, social interactions, tolerance and/or resistance to desiccation and antimicrobial agents) and is mainly constituted of polysaccharides (e.g. uronic acids), proteins, lipids and extracellular DNA (Flemming et al., 2016). Due to these emergent properties, interest in biofilms as a source of contamination in food companies has grown (Wirtanen and Salo, 2016). Outbreaks of microorganisms such as *Salmonella* spp., *Escherichia coli, Pseudomonas* spp., lactic acid bacteria, Enterobacteriaceae and *Listeria monocytogenes* in food companies occur frequently (Wirtanen and Salo, 2005; Liao, 2006; Rivera-Betancourt et al., 2006; Lettini et al., 2012; Muhterem-uyar et al., 2015) and can lead to a reduced shelf life and foodborne transmission of diseases. Many of these outbreaks have been found to be associated with biofilms and can be mainly related to surfaces of the processing equipment (Chmielewski and Frank, 2003; Brooks and Flint, 2008).

Hygiene monitoring and biofilm sampling in food companies are often performed in tandem after cleaning and disinfection (C&D) of the food contact surfaces. Different sampling and monitoring methods, such as plating of swabs, sponge or wipe samples, agar contact plates or dip slides, can be used in food companies to monitor the microbiological contamination on surfaces (Salo et al., 2000; Wirtanen and Salo, 2005). Swabbing with a sponge stick is often applied in food companies because the method is easily applied and allows sampling of large surfaces as well as difficult to reach areas. Under laboratory conditions, biofilms are often harvested using a cell scraper (Hermans *et al.*, 2011). These methods focus on the detection and enumeration of microorganisms, however, biofilms consist of EPS and microorganisms, thus both components must be sampled and measured. As EPS plays a fundamental role in creating the unique properties of biofilms, knowledge about EPS composition can provide insights into these properties (Flemming et al., 2016). The microbiological results only give an indication of the contamination level and the identity of the organisms in biofilms, whereas the chemical characterisation provides information on the presence and composition of the EPS. The combination of microbiological and chemical characterisation on surface samples taken after C&D is

important to facilitate the distinction between free living bacteria and attached bacteria in biofilms.

Knowledge about biofilms in food companies is still lacking (Cappitelli et al., 2014) and requires further research into sampling, detection and composition. To the best of our knowledge, this is the first study to include both microbiological and chemical analyses of biofilms in food companies. In this study two types of sampling methods were tested for their suitability to determine the presence and quantity of chemical components situated in the EPS as well as the microbiological yield. The best sampling method was subsequently used for the monitoring and analysis of biofilms sampled after C&D in different types of food companies in Belgium.

3.2 MATERIALS AND METHODS

3.2.1 Biofilm sampling in food companies

In eight Belgian food companies (producers of oven foods (n=1), dairy products (n=2), meat products (n=2), baker's yeast (n=1), sauces (n=1) and egg products(n=1)) several surfaces were sampled after C&D. Only in the dairy industry, samples were taken after cleaning only because chemical disinfection is not performed. In all companies, sampling was mainly focused on the parts of production lines with food contact such as conveyor belts, pipelines, storage tanks and trolleys. Each company was sampled at least once but no more than three times. Between July 2014 - January 2016, a total of 174 surfaces were sampled. Depending on the available surface area (20 to 1200 cm^2 per sampling method) one or both of the sampling methods described below were used. First, surfaces where both sampling methods could be applied (n = 133) were used to evaluate the method's suitability for microbiological and chemical analyses in order to detect biofilms. Second, all sampled surfaces (n=174) were evaluated for microbiological and chemical contamination after C&D. Samples were transported to the lab under cooled conditions and analysed within 12h.

3.2.2 Sampling devices and sampling procedures

The first method for biofilm sampling, called "scraper/flocked swab method", was to scrape the surface with a cell scraper (VWR, Cat#7342602, Leuven, Belgium) followed by swabbing the same area with a flocked swab (Copan, Cat#552C, Brescia, Italy). The flocked swab was premoistened with ¼ Ringer's solution (Oxoid, Cat#BR0052, Basingstoke, Hampshire, England) before sampling when the sampling area was dry. In

case of a wet sampling area, the flocked swab was not premoistened. Scraping and swabbing were performed using the tip of the devices, covering the entire area with overlapping horizontal and vertical strokes. After sampling, the head of the cell scraper and the nylon tip of the flocked swab were removed from the breakable plastic applicator and placed together in a sterile stomacher bag containing 10 mL of ¼ Ringer's solution. The second biofilm sampling method, called "sponge stick method", was to swab with a sponge stick (3M microbiology, Cat#3MSSL100, Zwijndrecht, Belgium). Swabbing with a pre moistened sponge on stick was performed as described above. After sampling, the sponge stick was placed into the bag and the stick was bent to break it off, allowing the cellulose sponge to drop into the sterile plastic bag. Subsequently, 10 mL of ¼ Ringer's solution was added to the bag. The sampled areas for both methods were adjacent and had similar surface characteristics. The content of the stomacher bags containing the sampled material with 10mL diluent was homogenised in a stomacher (AES Laboratoire, Combourg, France) for 2 min. From each sample, one part of the solution was used for microbiological analyses. The remaining solution was collected and stored at -18°C until chemical analysis.

3.2.3 Microbiological characterisation

In consultation with each food company, sampling points and microbiological analyses were based on previously reported contamination problems. Appropriate 10-fold dilutions from the samples were made in sterile 0.1% w/v peptone water with 0.85% w/v salt (BioTrading, K110B009AA, Mijdrecht, the Netherlands). Total aerobic count (TAC) was determined by plating on Plate Count Agar (PCA, Oxoid, CM0325) and incubated at 30°C for 3 days. Other agar media used were Violet Red Bile Dextrose Agar (VRBGA, Bio-Rad, 356-4584, Marne-La-Coquette, France) for enumeration of Enterobacteriaceae (24h incubation at 37°C), Malt Extract Agar (MEA, Oxoid, CM0059) for counts of yeasts and moulds after 5 days of incubation at 25°C and de Man, Rogosa, Sharpe Agar (MRS, Oxoid, CM0361) for enumeration of lactic acid bacteria (LAB) under anaerobic incubation using AnaeroGen 3.5L (Oxoid, AN0035) in an airtight jar at 37°C for 3 days. Gram-negative bacteria were enumerated using PCA + 0,2% Crystal Violet incubated for 3 days at 30°C. *Pseudomonas* spp. were enumerated using Pseudomonas Agar Base (Oxoid, CM0559) with Pseudomonas CFC Selective Agar Supplement (Oxoid, SR0103) and incubation at 30°C for 2 days. For counts of mesophilic and thermophilic spores, samples were first heated for 10 min at 80°C, followed by plating on PCA and incubation for 3 days at 30°C

and 1 day at 55°C, respectively. Not all parameters except for TAC, were determined for all food companies. The lowest microbiological enumeration limit was 10 CFU/sampled area.

3.2.4 Chemical characterisation

First, protein, carbohydrate and uronic acid analyses were performed on blank premoistened (using physiological water) sponge sticks and flocked swabs (n=9) to evaluate the possible effect of the swab materials in the chemical analyses. Second, samples taken from surfaces in different Belgian food companies were chemically analysed as part of the combined microbiological-chemical evaluation. Before chemical analyses, an extraction procedure was performed to separate the EPS from the microorganisms as follows: after microbiological analyses, the remaining ¼ Ringer's fraction was sonicated (UP 400S, Hielscher, Germany) 3 times for 30s with an interval of 30s at an amplitude of 50% and a cycle of 0.5 in a water bath to disrupt the biofilm. After centrifugation (Savant, SFA13K) at 13 000 RCF for 10 min at room temperature, supernatant (containing EPS) was recovered and used for the chemical characterisation.

Total protein quantification was performed according to Bradford (1976) with bovine serum albumin (Sigma-Aldrich, Overijse, Belgium) as standard. In brief, 100 µL of sample was added into 96-well plate and mixed with 100 µL of Bradford Reagent (Sigma-Aldrich). After 5 min of incubation at room temperature, the absorbance was measured at 595 nm with a microplate reader (LabSystems Multiskan RC, Vantaa, Finland). The limit of quantification (LOQ) for proteins was 0.94 µg/mL.

The carbohydrate quantification was performed using a modified phenol-sulphuric acid method described by Masuko *et al.* (2005) with glucose (Sigma-Aldrich) as standard. In brief, 50 µL of the sample was added in 96-well plate and mixed with 30 µL of 5% phenol in water (Sigma-Aldrich), followed by adding 150 µL of concentrated H_2SO_4. This mixture was placed in a water bath at 90°C for 5 min, followed by incubation for 5 min at room temperature. The absorbance at 492 nm was measured using a microplate reader (LabSystems Multiskan RC). The LOQ for carbohydrates was 0.028 µmol/mL.

The uronic acid quantification was performed according to Blumenkrantz and Asboe-Hansen (1973) with minor adjustments and D-galacturonic acid as standard. In brief, 40 µL of the sample was added in a 96-well plate and mixed with 200µL of 0.125 M sodium

tetra borate in H_2SO_4 (Sigma-Aldrich). This mixture was placed in a water bath at 80°C for 1h. After cooling to room temperature, the absorbance was measured at 540 nm using a microplate reader (LabSystems Multiskan RC). Next, 40 µL of 2% m-hydroxydiphenyl (100 mg/mL in dimethyl sulfoxide) in H_2SO_4 (Sigma-Aldrich) was added to the 96-well plate, followed by incubation in the dark for 3 hours at room temperature. Finally, the absorbance was measured again at 540 nm. The LOQ for uronic acids was 1.41 µg/mL.

3.2.5 Statistical analyses

All statistical analyses for the chemical characterisation were performed using the software package SPSS Statistics v22. To test the interference of the swab material with the chemical characterisation (difference from 0), the Wilcoxon signed-rank test ($\alpha = 0.05$) was used. Statistical analyses on the obtained microbiological results were carried out using Statistical Analysis System software (SAS®, version 9.4, SAS Institute Inc., Cary, NC, USA). First, distribution of the log transformed enumerations per microbiological parameter was evaluated based on the histogram and QQ plot. For normally distributed data, the equality of variances for the two sampling methods was checked using Levene's test. For normally distributed microbiological parameters, the counts for the two sampling methods were compared for each parameter using a pooled t-test when variances were equal and a Cochran t-test when the variances were unequal. For data that were not normally distributed, a Kruskal Wallis test was used to compare enumerations between the two sampling methods. P-values ≤ 0.05 were considered significant. For the representation of the contamination level, values for microbiological and chemical analyses are represented by the first quartile, median and third quartile.

3.3 RESULTS AND DISCUSSION

3.3.1 Interference test of different swabs in the chemical analyses

No proteins could be detected in the blank swab solution regardless of swab material used (flocked swab or sponge stick, Figure 3.1). For the sponge stick, a significant quantity of carbohydrates and uronic acids was detected. For the flocked swab, no significant amounts of carbohydrates or uronic acids were detected.

The type of swabbing material can influence the sensitivity of the chemical analyses of the EPS, but no studies have been published about the chemical interference with different swab materials. EPS is comprised mainly of polysaccharides (including uronic acids) and proteins (Flemming et al., 2016). The sponge stick, composed of cellulose, released

carbohydrates and uronic acids from the swab into the swab solution after the extraction procedure and therefore interfered in the corresponding analyses. The nylon flocked swabs didn't release significant amounts of proteins, carbohydrates or uronic acids into the swab solution and is therefore a suitable sampling material for EPS.

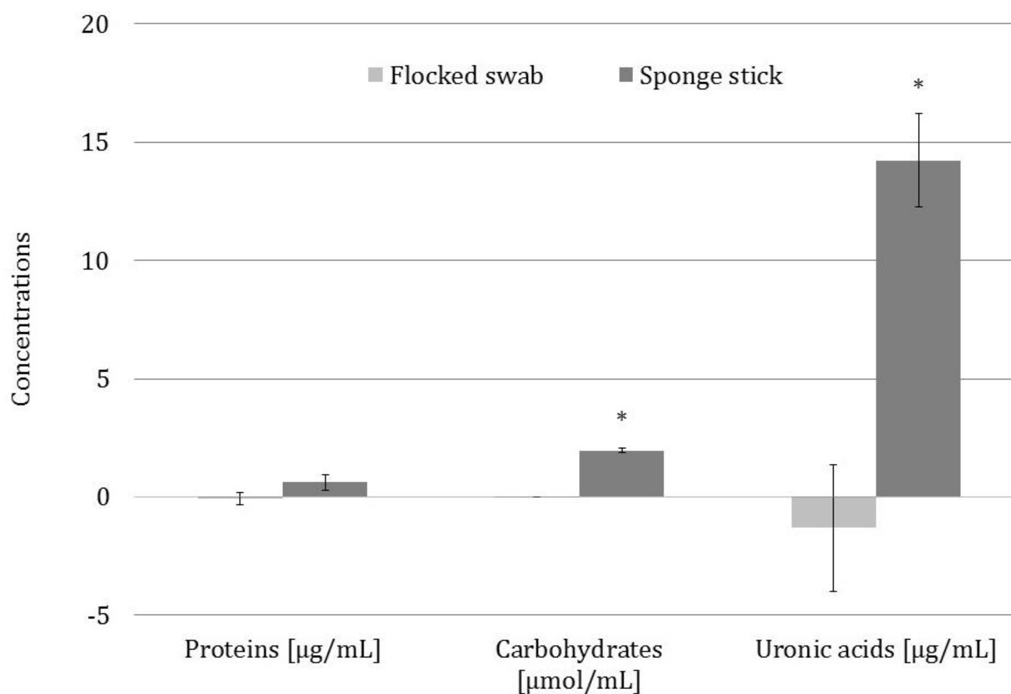

Figure 3.1: Comparison of the chemical analysis of components after extraction from blank flocked swabs (n=9) and sponge sticks (n=9). Asterisks indicate statistically significant differences (Wilcoxon signed-rank test versus 0, $\alpha = 0.05$).

3.3.2 Comparison of the microbiological counts using two biofilm sampling methods

In total, 133 surface samples were taken using two biofilm sampling methods after C&D in eight food companies. Using the sponge stick method, more surfaces were found to be contaminated as compared to the scraper/flocked swab method (Table 3.1). Using the scraper/flocked swab method, TAC could be determined on 54.9% of the sampled surfaces as compared to 59.4% for the sponge stick method. For all other investigated microbiological parameters, the sponge stick method also indicated more contaminated points compared to the scraper/flocked swab method. Mean TAC on contaminated surfaces after C&D sampled with the scraper/flocked swab method (n=73) was 2.01 $\pm$ 1.50 log CFU/100 cm^2 compared with 2.53 $\pm$ 1.68 log CFU/100 cm^2 (n=79) for the sponge stick method. Similar results, whereby higher mean counts on contaminated surfaces were found with the sponge stick method compared to the scraper/flocked swab method,

were found for *Pseudomonas* spp., Enterobacteriaceae and mesophilic spores. For all surfaces providing counts with both sampling methods, again higher mean counts for sponge sticks were observed for all investigated microbiological parameters (data not shown). For example, 55 sampling points provided counts for TAC with both methods with a mean count of 2.75 ± 1.82 log CFU/100 cm^2 for the sponge stick method and 2.30 ± 1.52 log CFU/100 cm^2 using the scraper/flocked swab method. However, none of these comparisons of microbiological enumerations were significant ($p > 0.05$).

Salo *et al.* (2000) assessed the yield of various cultivation based methods for surface hygiene control, i.e. contact plates, a dip slide and swabbing. They concluded that these three methods did not differ in terms of yield or precision for artificially contaminated stainless steel (SS) surfaces. Concerning different sampling swabs, Lahou and Uyttendaele (2014) observed no significant difference in the detection of *Listeria monocytogenes* on different types of artificially contaminated surfaces among swabs including sponge sticks. In field studies, (Luyckx et al., 2015a)indicated that swabs resulted in higher sensitivity in comparison to agar contact plates. The use of scrapers to sample biofilms in model systems has previously been described by Frank and Koffi (1990), Hermans *et al.* (2011) and Robijns *et al.* (2014), but the current study is the first to evaluate their efficacy in sampling bacteria from biofilms under field conditions.

A possible explanation why the scraper/flocked swab method reveals fewer microbiologically contaminated points and (non-significant) lower mean counts could be that the surface and the amount of absorbent material of the flocked swab is too small to capture all of the detached microorganisms. The results of this chapter indicate that the sponge stick had good sensitivity in detecting biofilms in the food industry, in contrast to Moore and Griffith (2002) who reported a sponge as less sensitive compared to other surface sampling methods. In their study, a sponge without a stick was used. The sponge stick used in our study may have facilitated handling, possibly allowing more mechanical force to be applied and leading to the capture of more detached microorganisms.

Table 3.1: Comparison of the percentage of surfaces generating counts and enumeration level for total aerobic count, lactic acid bacteria, *Pseudomonas* spp., Enterobacteriaceae, mesophilic and thermophilic spores after cleaning and disinfection in eight food companies using two biofilm sampling methods. Enumerations are shown by the mean and standard deviation.

Microbiological parameter	**Surfaces compared**	**Scraper/flocked swab method**		**Sponge stick method**		**P-value** [2]
		CS (%) [1]	Enumerations for CS (log CFU/100 cm^2)	CS (%)	Enumerations for CS (log CFU/100 cm^2)	
Total aerobic count	133	54.9	2.01 ± 1.50	59.4	2.53 ± 1.68	0.0530
Lactic acid bacteria	80	26.2	2.11 ± 1.30	32.5	1.88 ± 1.13	0.5854
Pseudomonas spp.	86	17.4	2.50 ± 1.69	22.1	2.96 ± 1.85	0.4549
Enterobacteriaceae	89	11.2	2.19 ± 1.91	13.5	2.50 ± 2.21	0.6437
Mesophilic spores	49	38.8	1.56 ± 1.03	53.1	1.81 ± 0.88	0.3760
Thermophilic spores	22	4.5	1.96 [3]	13.6	1.03 ± 0.30	/ [4]

[1] CS (%), proportion of countable samples given in percentage; [2] P-value, p-values ≤ 0.05 were considered as significant; [3] Only one value was obtained for the corresponding parameter; [4] /, No statistical analyses were performed on this microbiological parameter as there were too little data to compare.

3.3.3 Presence of biofilms in food companies in Belgium

In total, 174 surfaces were sampled after C&D in eight different food companies in Belgium. Of these, 133 surfaces were sampled using both sampling methods. Of the remaining surfaces, for practical reasons, 32 points were sampled using only the scraper/flocked swab method. Another nine surfaces were sampled using only the sponge stick method: for these samples, no results are available for chemical analyses due to chemical interference from the sampling sponge. Table 3.2 shows the results of the microbiological enumerations and the quantification of the chemical components after C&D in the eight food companies. At all of the food companies except for the producer of baker's yeast (where all results were not quantifiable), at least 20% of the sampled surfaces showed a high number (more than 10^2 CFU/100 cm^2) of microorganisms. Proteins were found in 20% of the chemically analysed surface samples (n=165), while carbohydrates and uronic acids were found on 15% and 8% of the sampled surfaces, respectively. When chemical and microbiological results were combined, 17% of the sampled surfaces in different food companies in Belgium appeared to be contaminated with both a high number of microorganisms and at least one of the analysed chemical components (Table 3.3). In these cases the surfaces were identified as carrying biofilm. The results show that microorganisms were mostly found in combination with either carbohydrates (13 of the 28 surfaces) followed by proteins (8 of the 28 surfaces). In one sample, microorganisms, proteins and carbohydrates were found, while in another sample microorganisms, proteins and uronic acids were found. Only one sample was found to contain microorganisms in combination with all three of the analysed chemical substances. For the microbiological results, 74% of the surfaces that were sampled twice or three times (n=53) showed similar results over time (36% stayed positive and 38% stayed negative, i.e. more or less than 10^2 CFU/100 cm^2). Of the surfaces that were repeatedly sampled and chemically analysed (n=47), 72%, 68% and 85% showed similar results (i.e. detectable or undetectable quantity) for respectively proteins, carbohydrates and uronic acids over time.

Table 3.2: Presence of biofilms in food companies in Belgium. The number of sampled points (n) together with the proportion of countable samples (%) and values (first quartile **- median** - third quartile) for microorganisms, proteins, carbohydrates and uronic acids are shown. Sampling points where both microorganisms (≥ 10^2 CFU/100 cm^2) and one or several chemical components were detected, are evaluated as biofilm.

Food company	**Microbiological analyses**			**Chemical analyses**							**Positive points for biofilm (%)**
	n	(%) [1]	Enumerations (log CFU/100 cm^2)	n	Proteins		Carbohydrates		Uronic acids		
					(%)	Quantity (µg/100 cm^2)	(%)	Quantity (µg/100 cm^2)	(%)	Quantity (µg/100 cm^2)	
Oven foods	11	45	3.56 – **3.81** – 4.78	11	36	3.16 – **7.53** – 66.35	9	108.10 *	0	/ [2]	18
	11	73	3.73 – **4.68** – 5.30	11	64	2.35 – **11.50** – 60.00	0	/	0	/	36
Baker's yeast	4	0	/	4	0	/	0	/	0	/	0
Dairy products 1	10	30	3.00 – **4.48** – 5.74	10	40	15.75 – **81.05** – 310.95	0	/	0	/	20
	13	15	2.35 – **2.44** – 2.53	13	15	13.0 – **28.9** – 44.8	0	/	23	2.6 – **30.7** – 87.1	0
Dairy products 2	6	50	2.09 – **2.20** – 2.83	6	0	/	0	/	17	20.9 *	17
Meat products 1	8	75	2.93 – **3.43** – 3.90	0							
	15	40	3.06 – **3.49** – 3.75 [3]	15	40	4.08 – **11.92** – 536.00	53	12.01 – **18.02** – 66.06	20	5.95 – **7.25** – 11.55	33
Meat products 2	20	30	2.95 – **3.57** – 3.70	19	0	/	0	/	0	/	0
	20	30	2.84 – **3.68** – 4.64	20	5	7.67 *	40	15.01 – **22.52** – 216.19	10	3.6 – **3.7** – 3.8	20
	17	41	2.10 – **2.27** – 2.38	17	12	1.04 – **2.25** – 3.47	0	/	0	/	0
Egg products	11	64	2.15 – **2.85** – 4.48	11	0	/	9	54.05 *	0	/	0
	11	64	3.54 – **4.09** – 6.48	11	45	24.2 – **27.1** – 82.4	9	360.32*	45	9.2 – **39.0** – 283.5	45
Sauces	5	40	2.93 – **3.99** – 5.05	5	20	120.7 *	60	54.05 – **432.38** – 75595.14	0	/	40
	5	80	2.76 – **3.82** – 5.10 [4]	5	20	5.8 *	60	36.03 – **72.06** – 90.08	0	/	60
	7	43	2.06 – **2.50** – 5.61	7	0	/	0	/	0	/	0

[1] (%), proportion of quantifiable samples given in percentage; [2] /, No values were countable or quantifiable for the corresponding parameter during sampling of the corresponding food company; [3] Values obtained for the corresponding parameter in the corresponding food company were normally distributed. Mean and standard deviation were 3.53±0.84 log CFU/100 cm^2; [4] Values obtained for the corresponding parameter in the corresponding food company were normally distributed. Mean and standard deviation were 3.93±1.49 log CFU/100 cm^2; *Only one value was obtained for the corresponding parameter in the corresponding food company.

Table 3.3: Overview of surfaces identified as carrying biofilm. The surface contamination on these locations is indicated in terms of presence (+) or absence (-) of at least 2.00 log CFU/100cm^2 and quantifiable amounts of proteins, carbohydrates and uronic acids.

Food Company	**Location**	**Microbiological analysis**	**Chemical analysis**		
			Proteins	Carbohydrates	Uronic acids
Oven foods	Flexible tube	+	-	+	-
	Conveyor belt	+	+	-	-
	Conveyor belt	+	+	-	-
	Connection tube (SS)[1]	+	+	-	-
	Flexible tube	+	+	-	-
	Connection tube (SS)	+	+	-	-
Dairy products 1	Pasteuriser (SS)	+	+	-	-
	Manifold (SS)	+	+	-	-
Dairy products 2	Steriliser (SS)	+	-	-	+
Meat products 1	Part of brining line (SS)	+	-	+	-
	Part of brining line (SS)	+	-	+	-
	Rinsing device (SS)	+	+	+	-
	Rinsing device (SS)	+	-	+	-
	Ice device (SS)	+	-	+	-
Meat products 2	Rack (SS)	+	-	+	-
	Knife a slicing line	+	-	+	-
	Conveyor belt	+	-	+	-
	Conveyor belt	+	-	-	+
Egg products	Storage tank (SS)	+	-	-	+
	Storage tank (SS)	+	-	-	+
	Part of filling line	+	+	+	+
	Part of filling line	+	+	-	+
	Conveyor belt	+	+	-	-
Sauces	Part of filling line (SS)	+	-	+	-
	Part of filling line (SS)	+	-	+	-
	Part of filling line (SS)	+	-	+	-
	Part of filling line (SS)	+	-	+	-
	Part of filling line (SS)	+	-	+	-

[1] SS, stainless steel

The results from microbiological and chemical analyses (and consequently the presence of biofilm) on samples taken from surfaces in different food companies during one to three sampling moments showed great diversity, ranging from no microbiologically or chemically contaminated surfaces (baker's yeast company) to several surfaces showing

high counts for microorganisms and proteins (two sampling times; oven food company) and many highly microbiologically contaminated surfaces and carbohydrates (two sampling times, sauce company). The presence of chemical components differed between companies and between multiple sampling times in the same company. Proteins were mostly found at the oven food company and at one of the dairy companies, while carbohydrates were predominantly found at the sauce company.

Literature contains no reports on field studies that include both microbiological and chemical analyses of biofilms in food companies. In the dairy industry, biofilm formation is a well-recognised problem as it can lead to problems with both hygiene and equipment functioning (Simões et al., 2010; Marchand et al., 2012). In our study, different places in one dairy company were contaminated with high numbers of microorganisms after cleaning, e.g. the inside of the pasteuriser (3.0 log CFU/100 cm^2) and manifolds (4.5 to 5.7 log CFU/100 cm^2). Schlegelová *et al.* (2010) reported that a conveyor belt surface in a cottage cheese processing company was contaminated with similar amounts of microorganisms after sanitation. Although no chemical disinfection step was performed in the sampled dairy companies, we found that the level of microorganisms, chemical components or biofilm was not different from other food sectors. This can be due to the use of higher temperatures in combination with a thorough cleaning step (60-85°C in dairy vs. 20-60°C in other food sectors) which can compensate for the lack of chemical disinfection. In the meat industry, biofilms can transmit pathogens and endanger product shelf life by transferring spoilage organisms (Giaouris *et al.*, 2014). According to Schlegelová *et al.* (2010), work table, the cutter and chicken transportation surfaces in two meat processing companies sampled after C&D have been shown to be the most contaminated surfaces with ca. 6.0 to 7.0 log CFU/sampled surface. Those surfaces were more contaminated than surfaces such as conveyor belts, hangers and small cutting material. In our study, samples taken in meat companies after C&D provided lower counts compared to the results of Schlegelová *et al.* (2010) with the most contaminated surfaces being the flake ice machine (5.0 log CFU/100 cm^2), a SS conveyor belt for ham (4.4 to 4.9 log CFU/100 cm^2) and a conveyor belt for pâté (4.0 to 4.6 log CFU/100 cm^2). High counts for microorganisms were mostly found in combination with carbohydrates. In the egg processing company, the most contaminated surfaces were the conveyor belt transporting intact eggs (4.5 to 6.5 log CFU/100 cm^2) and a scoop used for the removal of impurities in the separated egg flows (5.1 to 7.1 log CFU/100 cm^2). This high

microbiological contamination was not always found together with one of the analysed chemical components, however, leading these surfaces to be identified as free of biofilm. Over time, the results concerning the presence of biofilm in the egg processing company varied. Although no reports of sampling of biofilms in other egg processing companies could be found, it is known that different strains of *Salmonella enteritidis* can form biofilms (with cell numbers of 4.5 to 7.1 log CFU/cm^2) when grown in an egg-based medium under lab conditions (Yang et al., 2017b). Beside, Kuda *et al.* (2016) showed that egg components (especially egg yolk) provide protective effects for the growth of spoilage lactic acid bacteria on SS. In the oven foods company, several surfaces were highly contaminated with microorganisms (up to 6.2 log CFU/100 cm^2 for a flexible filling tube) after C&D. Biofilm presence on these surfaces did not persist over time. For all but one of the identified biofilm points, microorganisms were found in combination with proteins. Surfaces identified as carrying biofilm in the participating sauce company always consisted of microorganisms and carbohydrates. Enumerations for TAC provided counts up to 5.7 log CFU/100 cm^2 and the amount of carbohydrates retrieved after C&D was higher compared to other companies. For processors of sauces and oven food, also no reports concerning the frequency and composition of biofilms could be found.

When poor product quality is observed, the presence of biofilms in the food industry is often only presumed due to the lack of an accurate biofilm monitoring system. Generally, this estimation of biofilm occurrence is too low (Cappitelli *et al.*, 2014). Using conventional culturing methods following swabbing or scraping, the detachment of microorganisms and chemical components from the test surface is a limiting factor (Oulahal-Lagsir et al., 2000; Wirtanen and Salo, 2016). Cultivation, which is essential in the accurate determination of a biofilm, is sometimes hampered by the presence of damaged cells and viable but non culturable cells (Cappitelli et al., 2014; Wirtanen and Salo, 2016). When these limitations are taken into account, the results obtained from sampling in the different food companies in this study still underestimate the actual biofilm contamination. Yet in the present study, surfaces were identified as having an existing biofilm, and similar tests can also indicate emerging biofilm problems.

3.4 CONCLUSIONS

This study is the first to map biofilm presence in food processing environments based on microbiological and chemical analyses. The presence and composition of biofilms in a variety of Belgian food companies has now been better characterised. We can conclude that the nylon flocked swab is a suitable swab material for measuring the presence and chemical composition of the EPS from a biofilm as it gives no interference in the determination of proteins, carbohydrates and uronic acids (unlike the sponge stick). In contrast, the cellulose sponge stick method is slightly (but not significantly) more effective for capturing and cultivating microorganisms originating from biofilms. The results obtained by the scraper/flocked swab method will represent a small underestimation of the contamination (number of contaminated points and level of counted microorganisms) in practice. Microbiological contamination in the food industry varies by food sector and can even vary by company, sampling point and sampling time. Every sampled company, except for the baker's yeast company, revealed surfaces with high microbiological contamination after C&D. Chemical analyses of the samples provided information about the EPS composition, although these results always have to be combined with the microbiological results as these components do not occur exclusively in EPS but can also originate from product residues. In nearly all of the sampled companies, the results of a combination of microbiological and chemical evaluation revealed the presence of biofilms on one or more surfaces.

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CHAPTER 4: IDENTIFICATION AND SPOILAGE POTENTIAL OF THE REMAINING DOMINANT MICROBIOTA ON FOOD CONTACT SURFACES AFTER CLEANING AND DISINFECTION IN DIFFERENT FOOD INDUSTRIES

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Isolate identification and evaluation of spoilage potential was performed at ILVO, Technology and Food Science Unit

4 IDENTIFICATION AND SPOILAGE POTENTIAL OF THE REMAINING DOMINANT MICROBIOTA ON FOOD CONTACT SURFACES AFTER CLEANING AND DISINFECTION IN DIFFERENT FOOD INDUSTRIES

ABSTRACT

After cleaning and disinfection (C&D), surface contamination in the production environment of food companies can still be present. Microbiological contamination on cleaned surfaces can be transferred to the manufactured food and consequently lead to foodborne illness and early food spoilage. However, knowledge about the microbiological composition of this residual contamination after C&D and the importance in food spoilage in different food sectors is lacking. In this chapter, we therefore aimed to identify the remaining dominant microbiota on food contact surfaces in seven food companies after C&D and assessed the spoilage potential of the microbiota under lab conditions.

The dominant microbiota on surfaces that were still contaminated with 10^2 CFU/100 cm^2 or more after C&D was identified based on the 16S rRNA sequence. In addition, the potential to hydrolyse proteins, lipids and phospholipids, fermentation of glucose and lactose, production of hydrogen sulphide and the degradation of starch and gelatin was evaluated.

Genera that were most abundant among the dominant flora on food contact surfaces after C&D were *Pseudomonas*, *Microbacterium*, *Stenotrophomonas*, *Staphylococcus* and *Streptococcus*. *Pseudomonas* spp. were identified in five of the participating food companies and 86.8% of these evaluated isolates showed spoilage potential in the laboratory tests. *Microbacterium* and *Stenotrophomonas* spp. were identified in five or six of the food companies, respectively and all these tested isolated had spoilage potential.

This new information is useful for the concerned food industries in their quest to characterise surface contamination after C&D, to identify causes of microbiological food contamination and spoilage and to determine the possible need for a more thorough C&D.

4.1 INTRODUCTION

Standard operating procedures, among which regular cleaning and disinfection (C&D), are developed and used in food industry to eliminate foodborne pathogens and to reduce the contamination level with spoilage organisms in the production environment to acceptable levels. Despite these measures, surface contamination after C&D in food processing facilities still occurs (Schlegelová *et al.*, 2010; Simões *et al.*, 2010). Microbiological contamination on cleaned surfaces can be transferred to the manufactured food and, when containing pathogens, consequently lead to foodborne illness (Kusumaningrum et al., 2003; AlZaabi and Khan, 2017; Adator et al., 2018; Dantas et al., 2018). The most reported foodborne outbreaks in the European Union in 2016 were caused by *Salmonella* (responsible for 65.8% of all bacterial outbreaks) and *Campylobacter* (28.3%) resulting in 9.061 and 4.606 patients respectively (European Food Safety Authority and European Centre for Disease Prevention and Control, 2017). On the other hand, early food spoilage can be caused by residual spoilage organisms (or their spores) on food contact surfaces. Examples are *Bacillus* spp. and *Pseudomonas* spp. as known spoilage organisms in dairy products (De Jonghe *et al.*, 2010; Marchand *et al.*, 2012; Rawat, 2015; Stellato *et al.*, 2015) or lactic acid bacteria (LAB) causing a shorter shelf life for meat products (Borch *et al.*, 1996; Giaouris *et al.*, 2014; Rawat, 2015). Moreover, the presence of biofilm can cause or maintain contamination on surfaces or food products with pathogenic or spoilage microorganisms (Lindsay and von Holy, 2006; Brooks and Flint, 2008; Giaouris et al., 2014; Kuda et al., 2016).

In chapter 3, residual contamination on food contact surfaces after C&D was mapped and characterised in terms of bacterial counts and chemical composition. This showed that bacterial contamination after C&D of food contact surfaces ranged from <0.22 to 7.23 log CFU/100 cm^2 with an average of 3.62 ± 1.20 log CFU/100 cm^2 on contaminated surfaces. However, knowledge about the microbiological composition of this residual contamination after C&D and the importance in food spoilage in different food sectors is lacking.

Therefore in the present chapter, the remaining dominant bacteria on food contact surfaces after C&D in different food companies were collected and further characterised. The isolates were identified by 16S rRNA sequencing and their occurrence in the different companies was studied. Since bacteria in surface contamination can be transferred to the

produced food products, also the spoilage potential of these isolates was assessed under lab conditions to estimate their possible impact on food spoilage.

4.2 MATERIALS AND METHODS

4.2.1 Sampling and microbiological contamination of surfaces in food companies

Samples were collected from several surfaces in seven Belgian food companies (producers of oven foods (n=1), dairy products (n=2), meat products (n=2), sauces (n=1) and egg products(n=1)) after C&D or after cleaning in the dairy companies. Mainly parts of the production lines with food contact, such as conveyor belts, pipelines, storage tanks and trolleys, were sampled during two sampling moments. In total 174 surface samples were collected between July 2014 - January 2016 and subsequently evaluated for their microbiological load and chemical composition. These results are available in chapter 3. In that study, enumerations for total aerobic count (TAC) on plate count agar (PCA; Oxoid, CM0325) ranged from <0.22 to 7.23 log CFU/100 cm^2. For three food companies (dairy company 1, meat company 2 and the egg processing company), enumerations for *Pseudomonas* spp. on Pseudomonas Agar Base (PAB; Oxoid, CM0559) with Pseudomonas CFC Selective Agar Supplement (Oxoid, SR0103) were also performed and counts were in the same range as for TAC. Proteins were found in 20% of the chemically analysed surface samples (n=165), while carbohydrates and uronic acids were found in 15% and 8% of the analysed samples, respectively. When chemical and microbiological results were combined, 17% of the sampled surfaces in different food companies in Belgium appeared to be contaminated with both a high number of microorganisms (10^2 CFU/100 cm^2 or more) and at least one of the analysed chemical components which was, as mentioned in chapter 3, an indication for the presence of biofilm.

4.2.2 Microbiological characterisation of surface contamination

4.2.2.1 Isolate collection

An ad random selection (n=45) of microbiological contaminated food contact surfaces (TAC of 10^2 CFU/100 cm^2 or more) discussed in chapter 3 were further characterised in the present chapter by selecting the dominant flora. In case enumeration of *Pseudomonas* spp. was also performed on these samples, dominant flora on PAB were also collected (n=16). The plates with the highest serial 10-fold dilutions where growth occurred represented the dominant microbiota. Based on morphology, two to seventeen colonies

were selected from PCA and one to eight colonies were selected from PAB for each of the contaminated surface samples. Colonies were streaked and incubated on new PCA plates minimally three times to obtain pure cultures. The pure cultures were inoculated in Brain Heart Infusion (BHI; Oxoid, CM1135, Basingstoke, Hampshire, England) with 15% glycerol (Merck, 8.18709.1000, Darmstadt, Germany), incubated for two days at 30°C and kept at -80°C.

Table 4.1: Isolates collected from Plate Count Agar (PCA) and Pseudomonas Agar Base(PAB)with Pseudomonas CFC Selective Agar Supplement originating from contaminated surfaces after C&D in different food companies during one to three sampling occasions.

Food company	Sampled surfaces (n) [1]	Surfaces where isolates were collected (n) [2]	Isolates collected from PCA (n)	Isolates collected from PAB (n)
Oven food company	11	4	30	/ [3]
	11	8	65	/
Dairy company 1	10	4	49	7
	13	2	6	/
Dairy company 2	6	3	10	/
Meat company 1	8	3	11	/
	15	5	38	/
Meat company 2	20	6	62	24
	20	6	28	22
	17	0	/	/
Egg processing company	11	4	32	4
	11	7	35	12
Sauce company	5	2	21	/
	5	3	20	/
	7	0	/	/

[1] Microbiological enumerations and chemical analysis of the sampled surfaces in each food company were previously described in chapter 3; [2] An ad random selection of the samples with counts for TAC of ≥ 10^2 CFU/100 cm^2 were used for identification of the dominant flora; [3] No isolates were collected for this parameter in the corresponding food company.

A total of 407 isolates were collected from PCA and 69 from PAB originating from surface samples after C&D (Table 4.1). Isolates were classified as originating from samples in three bacterial abundance classes. For isolates collected from PCA, the class of less than 3 log CFU/100 cm^2 represented low bacterial numbers, the class of 3 to 5 log CFU/100 cm^2 represented medium numbers and the class of more than 5 log CFU/100 cm^2 represented high numbers. Isolates collected from PAB were classified as less than 2 log CFU/100 cm^2

(low numbers), 2 to 4 log CFU/100 cm^2 (medium numbers) or more than 4 log CFU/100 cm^2 (high numbers).

4.2.2.2 Identification of the isolates

From each isolate, except for those that could not be cultivated after storage at -80°C (35 out of 476 isolates), DNA was collected according to Strandén *et al.* (2003). DNA extracts were stored at 4°C and used on the same day for $(GTG)_5$ PCR based on Calliauw *et al.* (2016) for clustering of the isolates. PCR amplifications were performed in an automated thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems Europe, the Netherlands) with an initial denaturation (7 min at 95°C) followed by 30 cycles of denaturation (1 min at 95°C), annealing (1 min at 40°C) and extension (8 min at 65°C) and a final extension (16 min at 65°C). PCR products were separated using the QIAxcel Advanced System (QIAGEN Benelux B.V., Venlo, the Netherlands) and QIAxcel DNA High Resolution Kit (QIAGEN Benelux B.V., 929002) and clustering of the obtained fingerprints using BioNumerics version 7.6 software package (Applied Maths, Sint-Martens-Latem, Belgium) was performed according to (Luyckx et al., 2016a). Out of the 441 isolates included in the $(GTG)_5$ fingerprint clusters, 327 were selected for identification based on the occurrence of their pattern and as representatives for visually defined clusters. For clusters with two or three isolates, one isolate was selected to identify the complete cluster. For clusters with four or more isolates, a minimum of two isolates were selected for identification. These were the outer isolates of the cluster possibly supplemented with an isolate in the middle to represent the largest possible diversity. The 16S rRNA gene was amplified for identification of the selected isolates using universal bacterial primers 16F27-1 (pA, 5'-3' sequence: AGA GTT TGA TCC TGG CTC AG) and 16R1522 (pH, 5'-3' sequence: AAG GAG GTG ATC CAG CCG CA), according to Brosius *et al.* (1978). The microbial DNA (± 25 ng/µL) was used as a template in the 50 µL PCR reaction containing 1x PCR buffer II (Applied Biosystems Europe, N8080153, the Netherlands), 1.5mM $MgCl_2$ (Applied Biosystems Europe, N8080153), 0.03U AmpliTaq® DNA Polymerase (Applied Biosystems Europe, N8080153), 0.1mM of each deoxynucleotide triphosphate (GE Healthcare Europe, GE28-4065-58, Diegem, Belgium) and 1.0µM of the primers (Eurogentec, Seraing, Belgium). PCR amplifications were performed in an automated thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems Europe) with an initial denaturation (1 min at 95°C) followed by 30 cycles of denaturation (15s at 95°C), annealing (15s at 63°C) and extension (30s at 72°C) and a final extension (8 min at 72°C).

PCR products were separated in the same way as for $(GTG)_5$ PCR fragments except that a different method (OM500) was used. In case no PCR product could be visualised, the annealing temperature during amplification was changed to 57°C. When non-specific bands were amplified, PCR reaction was performed again with bacterial primers 16F358 (*gamma, 5'-3' sequence: CTC CTA CGG GAG GCA GCA GT) and 16R1485 (MH2, 5'-3' sequence: TAC CTT GTT ACG ACT TCA CCC CA) providing a 1169bp DNA fragment. PCR products were sequenced with forward and reverse primers by Macrogen Europe (Amsterdam, the Netherlands). Sequence reads of 500bp or more were used for further analysis in EZtaxon (Kim et al., 2012). The species in the database with the highest similarity (minimally 98.5%) and completeness was used to identify the isolates to the putative species level. When different species with the same similarity and completeness level occurred for an isolate, identification was performed to the genus level only. In total, 16S rRNA sequencing led to the identification of 281 of the 327 isolates. Together with the $(GTG)_5$ fingerprint results, 382 out of 476 isolates could be identified to the genus or species level.

4.2.2.3 Evaluation of the spoilage potential of the isolates

For isolates collected from PCA and PAB, that were used for 16S rRNA gene sequencing and of which identification was successful, spoilage potential was evaluated under laboratory conditions. All of these isolates originating from seven different food companies were evaluated for their potential to hydrolyse proteins, lipids or phospholipids. Additionally, other spoilage tests were performed depending on the type of products that are manufactured in each company and this is summarised in Table 4.2. Isolates collected in the egg processing company were also evaluated for their potential to produce hydrogen sulphide. The ability to ferment lactose was evaluated for isolates collected in the two dairy companies, the oven food company and the sauce company. Glucose fermentation was assessed for isolates collected in the two meat processing companies and the sauce company. Moreover, isolates originating from the oven food company and the sauce company were evaluated for their potential to degrade starch and isolates from the meat processing companies were checked for their ability to break down gelatin.

To evaluate the spoilage potential, the selected isolates were re-cultivated on PCA plates from the glycerol stocks at -80°C. For each spoilage test and each batch of tested isolates, a positive control was included. For the evaluation of proteolysis, isolates where streaked on PCA plates with 17% (v/v) skimmed milk and incubated at 30°C for three days. Isolates were evaluated as positive for proteolysis when a clear zone appeared around bacterial growth. Lipolysis was evaluated by cultivating the isolates on Tributyrin Agar (Oxoid, PM0004C) and incubation for three days at 30°C. A positive result could be observed as a clear zone around the colonies. Nutrient agar (Oxoid, CM0003) with 8% (v/v) egg yolk (Oxoid, SR0047) was used for the evaluation of phospholipolysis based on Price *et al.* (1982). Inoculated plates were incubated at 30°C for three days and afterwards a positive result was indicated by a precipitation zone. For the evaluation of hydrogen sulphide production, tubes with Kligler Iron Agar (KIA; Oxoid, CM0033) were used. The isolates were smeared on the surface of the KIA slope and stabbed in the butt with a micro loop. Tubes were incubated at 30°C and evaluated after 24h and 48h. A positive result was visible as the appearance of a black discoloration. Glucose and lactose fermentation were evaluated using Bromcresol purple broth (Sigma-Aldrich, 36408-500G, Overijse, Belgium) tubes with 0.7% (w/v) glucose (Oxoid, LP0071) or lactose (Oxoid, LP0070) respectively and Durham tubes were inserted. Tested isolates were inoculated in the tubes and incubated for 48h at 30°C. Sugar fermentation took place when the medium turned from purple to yellow. Fermentation was accompanied by gas formation in case gas had accumulated in the Durham tube. The ability to degrade starch was evaluated by inoculating the isolates on starch agar containing 3g Lab-Lemco Powder (Oxoid, LP0029), 10g potato starch (Sigma-Aldrich, S4501) and 12g bacteriological agar (Oxoid, LP0011) dissolved in 1L distilled water. After two days of incubation at 30°C, bacterial growth was scraped of and lugol solution (Sigma-Aldrich, L6146) was poured over the plates to visualise the result. A positive result was visible as a clear (orange) zone at the site of inoculation. Finally, the degradation of gelatin was checked using a medium containing 120g gelatin (Oxoid, LP0008), 3g Lab-Lemco Powder and 5g bacteriological peptone (Oxoid, LP0037) dissolved in 1L distilled water and divided into test tubes per 5mL. Tubes were inoculated by stabbing the isolate with a micro loop into the butt of the tubes. Inoculated tubes were incubated for seven days at 37°C together with a blank (non-inoculated) tube. Before interpretation of the result, test tubes were first placed at 7°C

until the medium in the blanc tube was solidified. In case the isolate produced gelatinase, the medium in the test tube had become liquid.

General results of the spoilage tests were evaluated in Microsoft Excel (2016). The similarities in spoilage potential of several groups of isolates (e.g. based on the originating company) were calculated in Bionumerics version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms were calculated using the Dice coefficient for binary data and isolates were clustered using UPGMA. Also, minimum spanning trees (MSTs) were generated for these groups of isolates.

4.3 RESULTS

4.3.1 Identification of microorganisms present on surfaces in food processing companies after C&D

4.3.1.1 Identification of isolates originating from the oven food company

Among the Gram-positive bacteria (n=34) collected from PCA, nine families were found represented by 14 genera (Figure 4.1). Most of the identified Gram-positive bacteria were present in medium or high numbers, except for *Bacillus* spp. and *Paenibacillus validus*. The most abundant Gram-positive genus was *Microbacterium* (n=14). The identified Gram-negative bacteria (n=39) belonged to seven families represented by 12 genera. The most abundant Gram-negative genus was *Pseudomonas* (n=18).

4.3.1.2 Identification of isolates originating from two dairy companies

Gram-positive bacteria originating from PCA (n=42 and n=8 for dairy 1 and dairy 2, respectively) belonged to eight families and 12 genera (Figure 4.1). For dairy company 1, the most identified Gram-positive bacteria belonged to the genera *Streptococcus* (yet in low numbers), *Microbacterium*, *Solibacillus* and *Dermacoccus*. For dairy company 2, all isolates were present in low numbers and were identified as *Bacillus* spp. or *Microbacterium lacticum*. Twelve Gram-negative bacteria were identified in dairy company 1 collected from PCA, represented by five families and an equal number of genera. The most abundant genus was *Brevundimonas*. No Gram-negative bacteria were identified in dairy company 2. Also in dairy company 1, seven isolates were identified from PAB (Figure 4.2). It always concerned *Stenotrophomonas rhizophila* or *Pseudomonas hunanensis* in low numbers.

Classes

- < 3 log CFU/100cm^2
- 3-5 log CFU/100cm^2
- > 5 log CFU/100cm^2

Gram	Family	Species	Oven food company	Dairy company 1	Dairy company 2	Meat company 1	Meat company 2	Egg processing company	Sauce company
Gram-positive	*Bacillaceae*	*Bacillus simplex*		2					
		Bacillus spp.	2		2				
		Lysinibacillus fusiformis		1					
		Exiguobacterium indicum	2						
	Carnobacteriaceae	*Carnobacterium mobile*				2			
	Corynebacteriaceae	*Corynebacterium testudinoris*						2 *	
	Dermacoccaceae	*Calidifontibacter indicus*		2					
		Dermacoccus abyssi		2					
		Dermacoccus nishinomiyaenis		3					
	Enterococcaceae	*Enterococcus faecalis*	1						
	Lactobacillales	*Lactobacillus plantarum* subsp. *plantarum*	2						
	Listeriaceae	*Brochotrix thermospacta*				2			
	Microbacteriaceae	*Curtobacterium paraplantarum*	1						
		Leucobacter chromiiresistens					1		
		Microbacterium aurantiacum		1					
		Microbacterium flavum	6				4		
		Microbacterium foliorum	1						
		Microbacterium hydrothermale					1		
		Microbacterium kyungheense	4						
		Microbacterium lacticum		4	6				
		Microbacterium oxydans		1					
		Microbacterium proteolyticum					1		
		Microbacterium spp.	3				2	6	
		Plantibacter flavi		1					
		Pseudoclavibacter helvolus	2			1		4*	
	Micrococcaceae	*Arthrobacter protophormiae*					4		
		Kocuria rhizophila				2	2		
		Kocuria salsicia	2	3					
		Micrococcus aloeverae	1						
		Micrococcus yunnanensis		4 *					
		Micrococcus spp.							2
		Rothia marina					5	5 *	
		Rothia spp.	1				2		
	Nocardiaceae	*Rhodococcus* spp.	1						

Paenibacillaceae	*Paenibacillus validus*	2						
Planococcaceae	*Kurthia gibsonii*	2						
	Solibacillus isronensis		6					
Promicromonosporaceae	*Cellulosimicrobium funkei*		1					
Staphylococcaceae	*Staphylococcus hominis* subsp. *hominis*					1		
	Staphylococcus saprophyticus subsp. *saprophyticus*						6	
	Staphylococcus spp.						2	
	Staphylococcus warneri		1				10	
	Staphylococcus xylosus						5	
Streptococcaceae	*Lactococcus lactis* subsp. *cremoris*	1						
	Streptococcus australis		1					
	Streptococcus infantis							1
	Streptococcus mitis							4
	Streptococcus pseudopneumoniae						1	
	Streptococcus rubneri					1		
	Streptococcus salivarius subsp. *salivarius*		2				1	
	Streptococcus sanguinis		2					
	Streptococcus spp.		5			1		
Brucellaceae	*Ochrobactrum pseudogrignonense*					1		
	Ochrobactrum rhizosphaerae	2						
	Pseudochrobactrum kiredjianiae					1		
Caulobacteraceae	*Brevundimonas* spp.	2	5					
Comamonadaceae	*Acidovorax temperans*				1			
Enterobacteriaceae	*Citrobacter youngae*							1
	Enterobacter asburiae							1
	Enterobacter kobei	1						
	Hafnia paralvei	1						
	Klebsiella michiganensis				1			
	Lelliottia amnigena	1					1	
	Lelliottia nimipressuralis							2
	Pantoae vagans	1						
	Raoultella terrigena	2						
	Serratia marcescens subsp. *marsescens*	2						
	Serratia myotis	1						
	Serratia spp.						1	
Flavobacteriaceae	*Chryseobacterium nakagawai*					3		
	Chryseobacterium rhizoplanae					1		
	Chryseobacterium spp.						6	
	Chryseobacterium vietnamense					2		
	Elizabethkingia miricola	2						

	Family	Species							
Gram negative	*Lysobacteriaceae*	*Stenotrophomonas humi***					1		
		*Stenotrohomonas maltophilia***	4			8	6 *	3	1
		*Stenotrophomonas rhizophilia***		3			2 *		
		Stenotrophomonas spp. **						2	
	Moraxellaceae	*Acinetobacter gyllenbergii*							1
		Acinetobacter haemolyticus					1		
		Acinetobacter johnsonii							2
		Acinetobacter oryzae					1		
		Acinetobacter spp.					2		1
		Psychrobacter faecalis				1			
		Psychrobacter sanguinis	2						
		Psychrobacter spp.				2			
	Methylobacteriaceae	*Methylobacterium zatmanii*		2					
	Pseudomonadaceae	*Pseudomonas abietaniphila*							1
		Pseudomonas asturiensis	1					2	
		Pseudomonas coleopterorum							1
		Pseudomonas cremoricolorata				1			
		Pseudomonas fragi	5 *			1			1
		Pseudomonas geniculata							2
		Pseudomonas gessardii					2 *		
		Pseudomonas hibiscicola	2				2	1	
		Pseudomonas hunanensis				2	1		
		Pseudomonas indoloxydans				2			
		Pseudomonas koreensis				1			
		Pseudomonas libanensis	1						
		Pseudomonas lurida	2						
		Pseudomonas mosselii							1
		Pseudomonas rhizosphaerae							1
		Pseudomonas simiae							3
		Pseudomonas spp.	7			9	9		5
		Pseudomonas taiwanensis							1
	Rhizobiaceae	*Rhizobium larrymoorei*		1					
		Rhizobium nepotum					1		
		Rhizobium radiobacter					1		
	Shewanellaceae	*Shewanella xiamenensis*							1
	Sphingobacteriaceae	*Sphingobacterium anhuiense*					2		
		Sphingobacterium kitahiroshimense						1	
	Sphingomonadaceae	*Sphingomonas aquatilis*		1					

Figure 4.1: Family (based on http://www.bacterio.net/, verified on April 12, 2018), genera and species identity of isolates from PCA of surface samples after C&D collected in seven food companies. Different colors represent the magnitude of TAC enumerations of samples whereof the bacteria were isolated. * Indicates that the corresponding species was found during the first and second sampling round in the corresponding company. ** This species belongs to the family Xanthomonadaceae according to NCBI classification.

4.3.1.3 Identification of isolates originating from two meat companies

For Gram-positive bacteria from PCA, six families and ten genera represented seven isolates in meat company 1 and 25 isolates in meat company 2 (Figure 4.1). In meat company 2, the most identified Gram-positive bacteria belonged to the genera *Microbacterium* and *Rothia* which were not found in meat company 1. The identified Gram-negative bacteria belonged to nine families represented by 11 genera. In meat company 1, much more Gram-negative bacteria (n=29) were identified from PCA compared to Gram-positive bacteria (n=7). The most dominant Gram-negative bacteria identified in the meat companies belonged to the genera *Stenotrophomonas* or *Pseudomonas*. None of the identified microorganisms collected from TAC were found in high numbers in both of the meat companies. For meat company 2, isolates were also collected from PAB (n=33; Figure 4.2). These isolates were classified into four families represented by four genera. The most identified species on PAB was *Pseudomonas hunanensis.*

4.3.1.4 Identification of isolates originating from the egg processing company

In Figure 4.1, results of the identified Gram-positive (n=42) and Gram-negative (n=17) bacteria collected from TAC are shown. Gram-positive bacteria belonged to five families represented by six genera and this was also the case for the Gram-negative bacteria. Most of the identified Gram-positive bacteria were present in medium numbers and were identified as *Staphylococcus* spp. (n=23). For the Gram-negative bacteria, which mostly occurred in high numbers, *Stenotrophomonas* spp. and *Pseudomonas* spp. were identified the most. Also, different isolates (n=15) belonging to five families represented by five genera, were collected from PAB (Figure 4.2).

4.3.1.5 Identification of isolates originating from the sauce company

Isolates collected from TAC were mostly identified as Gram-negative bacteria (26 isolates compared to seven Gram-positive bacteria; Figure 4.1). The most identified Gram-positive bacteria belonged to the genus *Streptococcus* (n=5) whereas most Gram-negative bacteria were identified as *Pseudomonas* spp. (n=16) or *Acinetobacter* spp. (n=4).

Classes
< 2 log CFU/100cm^2
2-4 log CFU/100cm^2
> 4 log CFU/100cm^2

	Family	Species	Dairy company 1	Meat company 2	Egg processing company
Gram negative	*Comamonadaceae*	*Delftia acidovorans*			1
	Enterobacteriaceae	*Citrobacter youngae*		1	
		Serratia glossinae			3
		Serratia spp.			1
	Lysobacteriaceae	*Stenotrophomonas maltophilia***			3
		*Stenotrophomonas rhizophilia***	6*	3	
	Moraxellaceae	*Acinetobacter baumannii*		1	
		Acinetobacter oryzae			1
	Pseudomonadaceae	*Pseudomonas azotoformans*		2	
		Pseudomonas brenneri		2	
		Pseudomonas extremaustralis		2*	
		Pseudomonas fragi		1	1
		Pseudomonas gessardii		2	
		Pseudomonas hunanensis	1	5*	
		Pseudomonas indoloxydans		1	
		Pseudomonas japonica		3*	
		Pseudomonas lundensis			2
		Pseudomonas plecoglossicida			1
		Pseudomonas proteolytica		1	
		Pseudomonas salomonii		1	
		Pseudomonas songnenensis		4	
		Pseudomonas spp.		4	2

Figure 4.2: Family (based on http://www.bacterio.net/, verified on April 12, 2018), genera and species identity of isolates from PAB of surface samples after C&D collected in three food companies. Different colors represent the magnitude of PAB enumerations of samples whereof the bacteria were isolated. *Indicates that the corresponding species was found during the first and second sampling round in the corresponding company. **This species belongs to the family Xanthomonadaceae according to NCBI classification.

4.3.1.6 Similarities and differences in identification of isolates originating from different food sectors

Over all the sampled food companies, most of the identified isolates collected from PCA (n=327) belonged to the genera *Pseudomonas* (20.5%), *Microbacterium* (12.2%), *Stenotrophomonas* (9.2%), *Staphylococcus* (7.6%) and *Streptococcus* (5.8%). For isolates that were classified to the tentative species level (n=247), 8.9% were identified as *Stenotrophomonas maltophilia*, 4.5% as *Staphylococcus warneri* and 4.0% as *Microbacterium flavum*, *Microbacterium lacticum* or *Rothia marina*. The other identified species occurred in less than 4.0% of the isolates. Approximately the same number of Gram-positive (48.6%) and Gram-negative bacteria (51.4%) were identified from PCA. Isolates collected from PAB in the different food companies (n=55) were in 63.6% and 21.8% of the cases identified as *Pseudomonas* spp. and *Stenotrophomonas* spp., respectively. Isolates originating from PAB and identified to the species level (n=48) mostly concerned *Stenotrophomonas rhizophila* (18.8%), *Pseudomonas hunanensis* (12.5%) or *Pseudomonas songenensis* (8.3%).

Bacteria isolated from PCA belonged to 50 genera, whereof 60% were company specific. The genus *Stenotrophomonas* was found in all the food companies except for dairy company 2. *Pseudomonas* spp. were identified in all food sectors except for the dairy companies and *Microbacterium* spp. were also found in five out of seven food companies. The genera *Kocuria* and *Streptococcus* were identified in four food companies. Concerning isolates that were identified to the tentative species level, 84.7% were company specific. Three species (*Pseudoclavibacter helvolus*, *Pseudomonas fragi* and *Pseudomonas hibiscicola*) were identified in three different food companies and only one species (*Stenotrophomonas maltophilia*) was identified in five out of seven food companies.

4.3.2 Spoilage potential of microorganisms present on surfaces in food processing companies after C&D

In total, 278 isolates collected from PCA (n=229) and PAB (n=49) were evaluated for three (proteolysis, lipolysis and phospholipolysis) to six spoilage tests to evaluate the possible impact on food spoilage when these isolate end up in food products through cross-contamination from food processing surfaces. Results of the spoilage tests are presented in Table 4.2.

Table 4.2: Results of spoilage tests for isolates originating from different food companies and isolated from PCA or PAB. For each company, the number of evaluated isolates (n) is given together with the percentage of isolates that showed a positive result for each evaluated spoilage test. Also the percentage of isolates that showed a positive result for at least one of the evaluated spoilage tests is given for each company.

Company	**n[1]**	**Positive isolates (%)**								
		Proteolysis	**Lipolysis**	**Phospho-lipolysis**	**H_2S formation (n=51)**	**Glucose fermentation (n=126)**	**Lactose fermentation (n=124)**	**Starch degradation (n=71)**	**Gelatin degradation (n=102)**	**Minimally one spoilage test**
Oven foods	49	38.8	65.3	14.3	/ [2]	/	22.4	18.4	/	83.7
Dairy 1	46	45.7	84.8	6.5	/	/	13.0	/	/	91.3
	39 (TAC)	41.0	82.0	2.6	/	/	15.4	/	/	89.7
	7 (PAB)	71.4	100.0	28.6	/	/	0.0	/	/	100.0
Dairy 2	6	16.7	100.0	16.7	/	/	50.0	/	/	100.0
Meat 1	25	36.0	76.0	28.0	/	36.0	/	/	8.3 [3]	84.0
Meat 2	78	42.3	73.1	42.3	/	33.3	/	/	0.0	91.0
	51 (TAC)	39.2	68.6	33.3	/	33.3	/	/	0.0	94.1
	27 (PAB)	48.2	81.5	59.3	/	33.3	/	/	0.0	85.2
Egg processing	51	43.1	72.5	37.3	0.0	/	/	/	/	82.4
	36 (TAC)	38.9	72.2	22.2	0.0	/	/	/	/	83.3
	15 (PAB)	53.3	73.3	73.3	0.0	/	/	/	/	80.0
Sauces	23	56.5	69.6	43.5	/	65.2	8.7	4.5 [4]	/	100.0
All companies	278 [5]	42.5	74.1	28.8	0.0	39.7	17.7	14.1	2.0	88.5

[1] n, number of evaluated isolates collected from PCA. In case isolates for one company were collected from both PCA and PAB, results were separated by parameter and this in indicated by (TAC) or (PAB); [2] /, this spoilage test was not performed on isolates originating from the corresponding food company; [3] Only 24 isolates from meat company 1 were evaluated for gelatin degradation, [4] Only 22 isolates from the sauce company were evaluated for starch degradation, [5] This number of evaluated isolates only applies for spoilage tests to evaluate proteolysis, lipolysis and phospholipolysis. The other spoilage tests were evaluated for specific food companies only, mentioned in the column headings

4.3.2.1 Spoilage potential of isolates originating from the oven food company

In the oven food company, several spoilage profiles were observed. 18.4% of the evaluated isolates (n=49) were capable of hydrolysing proteins and lipids. 16.3% of the isolates were negative for all evaluated spoilage tests and 16.3% could only hydrolyse lipids. All isolates of the dominantly present *Microbacterium* spp. (n=10) could degrade lipids. Isolates with the strongest spoilage potential (positive for four out of five evaluated spoilage tests) were identified as *Bacillus* spp. or *Serratia* spp.

4.3.2.2 Spoilage potential of isolates originating from two dairy companies

Isolates collected in the dairy companies (n=46 for dairy 1 and n=6 for dairy 2) mostly showed two main spoilage profiles, i.e. 36.5% were able to hydrolyse only lipids (dominated by *Microbacterium* spp. isolates) and 32.7% were able to hydrolyse lipids and proteins (dominated by *Stenotrophomonas* spp. isolates; Figure 4.3). Only one strain (*Bacillus* spp.) collected from the dairy company 2 showed a positive result for all the evaluated (4) spoilage tests. Other organisms with high spoilage potential (positive for three out of four tests) were identified as *Cellulosimicrobium funkei* or *Stenotrophomonas rhizophila*.

4.3.2.3 Spoilage potential of isolates originating from two meat companies

Two main clusters with isolates (n=25 for meat 1 and n=78 for meat 2) that showed the same spoilage pattern were observed. The largest cluster with 20.4% of the isolates contained microorganisms that were able to hydrolyse proteins, lipids and phospholipids. The second largest group of isolates (17.5%) could only hydrolyse lipids. Both groups contained mainly *Pseudomonas* spp. and *Stenotrophomonas* spp. For meat company 1, isolates with the strongest spoilage potential (positive for three out of five tests) were *Brochotrix thermospacta*, *Kocuria rhizophila*, *Pseudomonas* spp. and *Stenotrophomonas maltophilia*. In meat company 2, isolates with positive results for four out of five spoilage tests (capable to ferment glucose and proteins, lipids, phospholipids) were identified as *Pseudomonas extremaustralis*, *Pseudomonas gessardii*, *Pseudomonas salomonii* or other *Pseudomonas* spp. (not identified to species level).

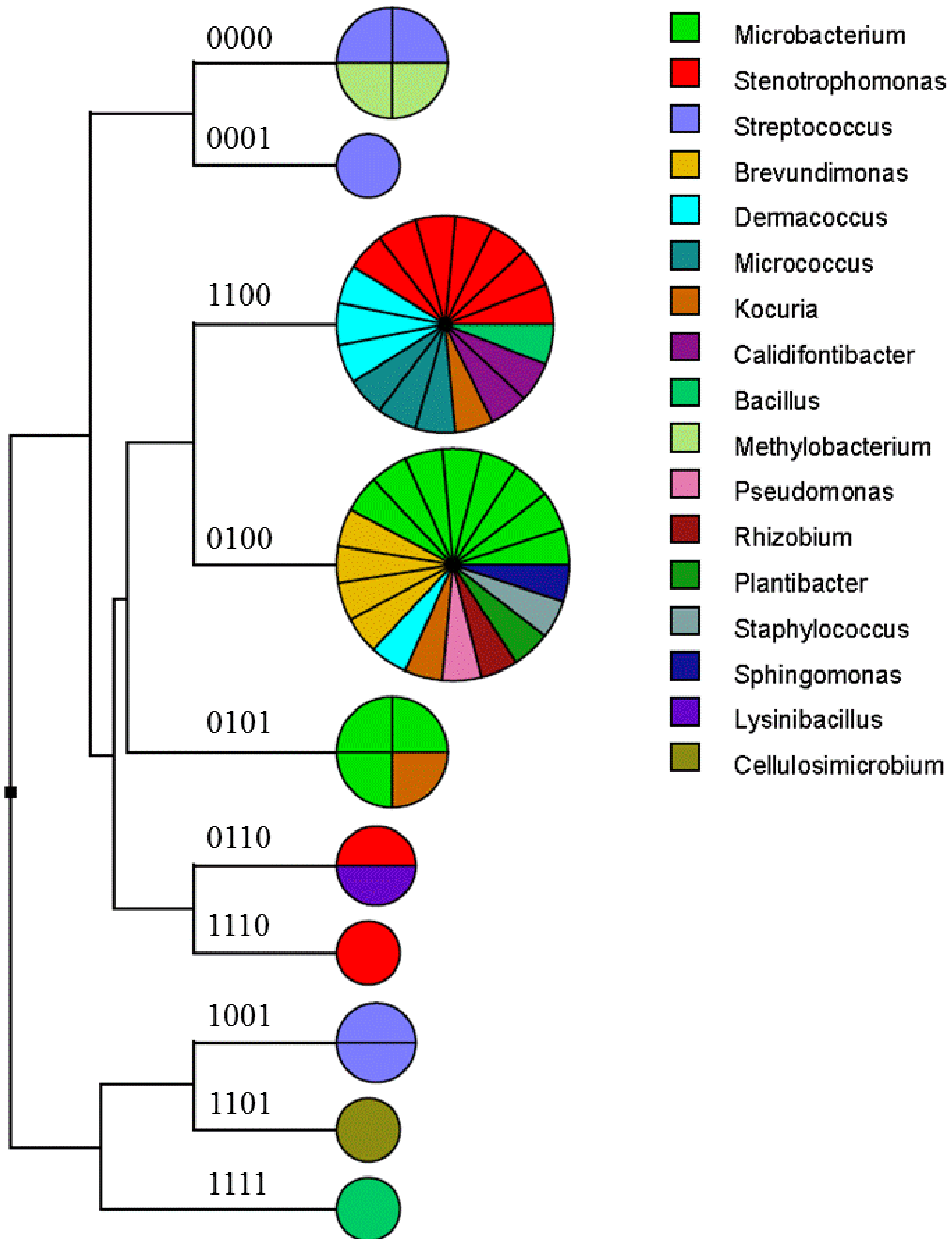

Figure 4.3: Clustering of spoilage results of isolates originating from two dairy companies based on UPGMA. The clustering is based on the result of four spoilage tests i.e. proteolysis, lipolysis, phospholipolysis and lactose fermentation respectively. The results for these tests are shown in this order on the branch of every cluster as '0' (in case the result was negative for the corresponding test) or '1' (in case the result was positive). Groups were made based on the identification on genus level of the tested isolates.

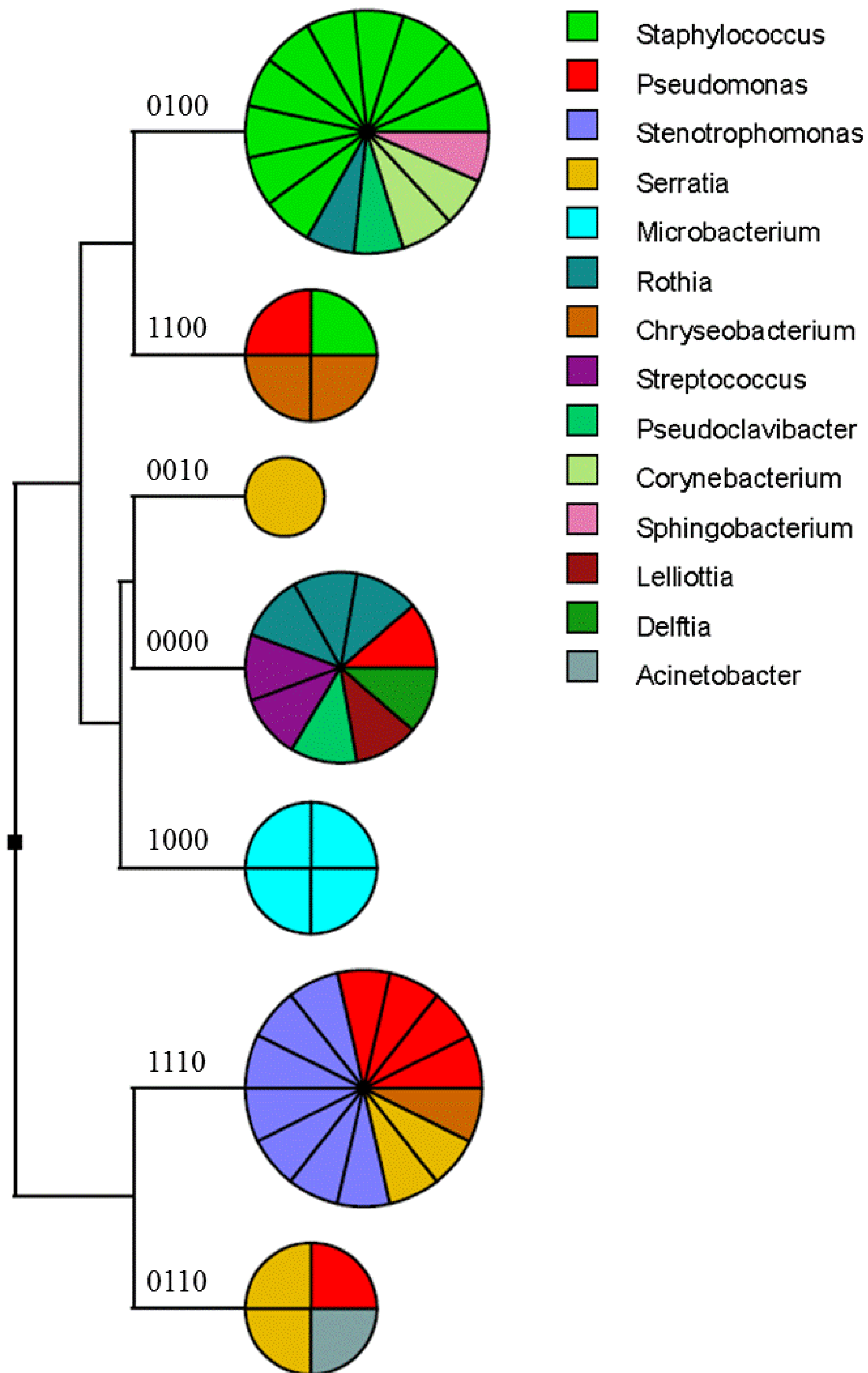

Figure 4.4: Clustering of spoilage results of isolates originating from an egg processing company based on UPGMA. The clustering is based on the result of four spoilage tests i.e. proteolysis, lipolysis, phospholipolysis and H_2S formation respectively. The results for these tests are shown in this order on the branch of every cluster as '0' (in case the result was negative for the corresponding test) or '1' (in case the result was positive). Groups were made based on the identification on genus level of the tested isolates.

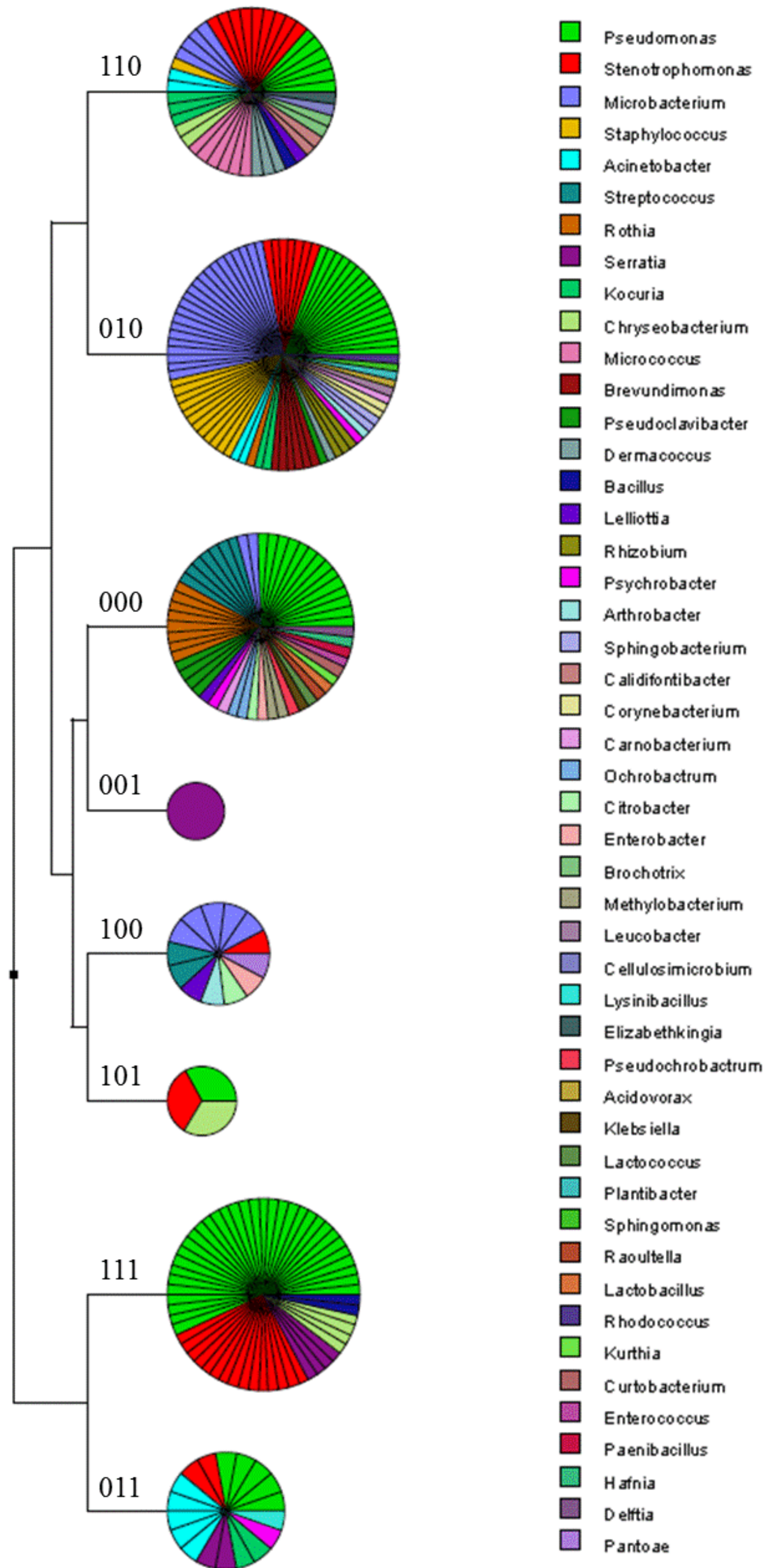

Figure 4.5: Clustering of spoilage results of isolates originating from all the food companies based on UPGMA. The clustering is based on the result of three spoilage tests i.e. proteolysis, lipolysis and phospholipolysis respectively. The results for these tests are shown in this order on the branch of every cluster as ‘0’ (in case the result was negative for the corresponding test) or ‘1’ (in case the result was positive). Groups were made based on the identification on genus level of the tested isolates.

4.3.2.4 Spoilage potential of isolates originating from the egg processing company

Isolates (n=51) were mainly clustered in two groups of spoilage potential (Figure 4.4). The largest group (29.4%) was able to hydrolyse lipids and was dominated by *Staphylococcus* spp. The second group (27.5%) was able to hydrolyse proteins, lipids and phospholipids and was dominated by *Stenotrophomonas* spp. (mostly *Stenotrophomonas maltophilia*) and *Pseudomonas* spp. but also *Chryseobacterium* spp. and *Serratia* spp. were part of this group. Also, 17.6% of the evaluated isolates showed a negative result for all the evaluated (4) spoilage tests.

4.3.2.5 Spoilage potential of isolates originating from the sauce company

The spoilage pattern of isolates (n=23) was very diverse, yet all the isolates showed a positive result for at least one of the tests. Isolates with the strongest spoilage potential (positive for four out of six tests) were identified as *Citrobacter youngae*, *Pseudomonas simiae* or other *Pseudomonas* spp.

4.3.2.6 Spoilage potential of isolates originating from different food sectors

Over all the sampled food companies, spoilage properties that were most prevalent for the evaluated isolates were lipolysis (206 or 74.1% of the tested isolates were positive), proteolysis (118 or 42.5%) and glucose fermentation (50 or 39.7%). Overall, 246 or 88.5% of the evaluated isolates showed a positive result for one or more spoilage tests varying from 82.4% (egg processing company, n=51) to 100% (dairy company 2, n=6 and sauce company, n=23). None of the evaluated isolates (all originating from the egg processing company) were capable to produce hydrogen sulphide. In general, isolates collected from PAB did not have more spoilage properties compared to PCA isolates, yet PAB isolates were more capable to hydrolyse proteins, lipids or phospholipids compared to isolates collected from PCA.

Four main clusters of spoilage potential profiles were visible in the MST considering the results for proteolysis, lipolysis and phospholipolysis for all isolates (Figure 4.5). 30.9% of the tested isolates could only hydrolyse lipids, 20.9% were positive for the three tests, 19.8% were negative for the three tests and 15.8% could hydrolyse proteins and lipids. In the largest cluster (with isolates that had only lipolytic potential), more than 50% of the isolates were collected from PCA and were identified as *Microbacterium* spp., *Pseudomonas* spp. or *Staphylococcus* spp. The cluster with isolates positive for the three spoilage tests was dominated by *Pseudomonas* spp. In general, isolates collected in all the

food companies and identified as *Bacillus* spp., *Serratia* spp., *Pseudomonas gessardii* and *Pseudomonas salomonii* had the highest spoilage potential which means positive for four out of four or four out of five evaluated spoilage tests.

Concerning spoilage potential of isolates identified among the most abundant genera (*Pseudomonas*, *Microbacterium*, *Stenotrophomonas*, *Staphylococcus* and *Streptococcus*), *Pseudomonas* spp. (isolated from PCA and PAB) were in 43.4% of the cases (n=76) able to hydrolyse proteins, lipids and phospholipids. Moreover, 80.3% of the identified *Pseudomonas* spp. produced lipases. Yet, 13.2% of *Pseudomonas* spp. showed no spoilage potential based on the performed tests. Concerning *Pseudomonas* species that were identified the most, *Pseudomonas hunanensis* (n=8) showed little spoilage potential while all the tested *Pseudomonas songenensis* strains (n=3) showed a positive result for lipolysis and phospholipolysis. Most of the isolates identified as *Microbacterium* spp. (66.7%) were able to hydrolyse lipids but not proteins nor phospholipids (n=33). Moreover, all *Microbacterium* strains showed some spoilage potential. Also, two strains collected from a dairy company and identified as *Microbacterium lacticum* were able to ferment lactose. Three main clusters of spoilage potential profiles were visible in the MST containing all evaluated *Stenotrophomonas* isolates (n=35). 42.9% of these isolates were positive for proteolysis, lipolysis and phospholipolysis, 25.7% were positive for proteolysis and lipolysis and 20% were only positive for lipolysis. 94.3% of the evaluated *Stenotrophomonas* strains (among which all *Stenotrophomonas maltophilia* strains) were able to hydrolyse lipids and all the strains showed a positive result for at least one of the evaluated spoilage tests. All but one of the evaluated isolates identified as *Staphylococcus* spp. (n=13) had the same spoilage potential when results for proteolysis, lipolysis and phospholipolysis are considered, i.e. only positive for lipolysis. The other *Staphylococcus* strain could also hydrolyse proteins.

4.4 DISCUSSION

4.4.1 Microorganisms originating from the oven food company

Dominant microorganisms identified on surfaces in the company that produces ready-to-heat products mostly concerned *Microbacterium* spp. and *Pseudomonas* spp. For this food sector, very little literature could be found where surface contamination was characterised microbiologically. Masegosa *et al.* (2013) found a contamination of 2.6 to 3.0 log CFU/g for TAC and 1.8 to 2.8 log CFU/g for Enterobacteriaceae on freshly cooked

ready-to-heat vegetable meals. Stratakos *et al.* (2015) found enumerations for TAC of 3.3 log CFU/g and low numbers of LAB on refrigerated ready-to-heat lasagne immediately after packaging. On chicken croquettes, significant amounts of LAB and Enterobacteriaceae were counted on the product at the time of packaging (Cordoba et al., 1999). Moreover, in the same study surface contamination reached up to 2.2 log CFU/cm^2 for LAB after processing, but levels of less than 2.0 log CFU/cm^2 for all the analysed microbiological parameters were observed on clean surfaces before processing. Several Enterobacteriaceae and LAB were also identified on surfaces in the oven food company in this study, which are a risk to be transferred to the processed food by cross-contamination (Cordoba *et al.*, 1999; Stratakos *et al.*, 2015). Cordoba *et al.* (1999) identified a *Xanthomonas maltophilia* (later renamed as *Stenotrophomonas maltophilia*) strain on the croquettes at the time of packaging, which is a species also identified on surfaces in the oven food company in this study producing croquettes as well.

Strains identified as Enterobacteriaceae or LAB in this study generally showed low spoilage potential (except for *Lelliottia amnigena*, *Serratia marcescens* subsp. *marcescens* and *Serratia myotis*) but were always capable to ferment lactose. The latter could consequently lead to acidification and a limitation of the shelf life of the oven food products often containing milk products. At the time of sampling, food products that were processed on the production line where on the surface *Lactococcus lactis* subsp. *cremoris* in high amounts (>5 log CFU/100 cm^2) was identified also showed high contamination with LAB one day after production (4.40 to 7.18 log CFU/g) and at the end of the shelf life (7.08 to 8.04 log CFU/g) (data not shown, personal communication, November 28, 2017).

4.4.2 Microorganisms originating from two dairy companies

In the dairy companies, identified isolates were mostly Gram-positive bacteria such as *Streptococcus* spp. and *Microbacterium* spp. These genera are, beside pseudomonads, part of the psychotropic flora in refrigerated raw milk (Coorevits, 2011; Marchand *et al.*, 2012; Porcellato *et al.*, 2018). Thermophilic *Streptococcus* spp. are on the other hand able to survive the pasteurisation process and it is known that they can form biofilm on dairy equipment (Marchand *et al.*, 2012). Gunduz and Tuncel (2006) reported several Gram-positive and Gram-negative biofilm-forming bacteria isolated at different sampling points in dairy processing plants. Among those, *Bacillus* spp. and *Staphylococcus* spp. were also identified in the dairy companies in our study. Schlegelová *et al.* (2010) reported that

Staphylococcus spp. was identified in samples originating from surfaces in a dairy plant after sanitation. Moreover, the species identified in this study in dairy company 1 (*Staphylococcus warneri*) was also identified on technical equipment in the study of Schlegelová *et al.* (2010).

It is known that *Pseudomonas* spp. are the predominant Gram-negative species that limit the shelf life of ultra-heat treated milk at 4°C, mainly through the production of heat-stable enzymes (Marchand et al., 2009a; Simões et al., 2010; Coorevits, 2011). It is therefore surprising that no *Pseudomonas* strains were isolated from PCA in the dairy companies and only one was identified on PAB originating from dairy company 1. This one strain concerned *Pseudomonas hunanensis* and was positive for lipolysis. Concerning genera that were most abundant on dairy equipment, isolates identified as *Microbacterium* spp. showed little spoilage (except for degradation of lipids and sometimes lactose fermentation). Yet, dairy company 2 reported a customer's complaint on food spoilage where *Microbacterium* spp., the dominant surface contaminant, was identified in the end product. Also *Bacillus* spp. are considered dangerous in dairy industry because of their capacity to form endospores which are resistant to the applied heat treatments during milk processing (Coorevits, 2011). Besides the production of toxins, this could result in the production of spoilage enzymes in the food products which is supported by the results of the spoilage tests performed with the *Bacillus* strains identified in the dairy companies in the current study.

4.4.3 Microorganisms originating from two meat companies

In refrigerated food processing environments such as meat companies, specific niches that select for cold-tolerant bacteria are created. In the study of Hultman *et al.* (2015), Pseudomonadales were identified on surfaces in different parts of the sausage processing plant. Genera that are part of this group and also isolated from meat contact surfaces in the present study were identified as *Acinetobacter*, *Psychrobacter* and *Pseudomonas*. The genus *Brochotrix* (mostly *Brochotrix thermospacta*) was earlier described as most abundant on raw meat (Borch *et al.*, 1996; Hultman *et al.*, 2015) and was in this study also identified on surfaces that come into contact with unprocessed meat. De Filippis *et al.* (2013) also found that *Pseudomonas* spp., *Psychrobacte*r spp. and *Brochotrix thermospacta* were the dominant microbiota on environmental swabs. Moreover, these organisms were found predominantly on spoiled beefsteaks (De Filippis *et al.*, 2013) and

previously described as known spoilage organisms on other meat products at different storage conditions (Doulgeraki et al., 2012). This is supported by the results of the spoilage tests performed on the concerning isolates in this study. *Stenotrophomonas* spp. (mostly *Stenotrophomonas maltophilia*) were often identified on surfaces in the two meat companies. This was not described before for the meat industry. Isolates identified as the latter genus showed varying (as in positive for one to three out of five evaluated tests) spoilage potential. Bacterial species mostly differed between the two meat companies, yet some species (*Kocuria rhizophila*, *Stenotrophomonas maltophilia* and *Pseudomonas hunanensis*) occurred in both companies, indicating their possible role as core species on surfaces after C&D and in biofilm formation.

Beside isolates collected from PCA and PAB, several isolates from PCA with 0.2% crystal violet for the enumeration of Gram-negative bacteria in meat company1 were evaluated for their spoilage potential (data not shown). Among these isolates, *Kluyvera* spp. (identified on a stainless steel funnel for raw meat with counts of 3.65 log CFU/100 cm^2) also showed high spoilage potential as it was positive for glucose fermentation and the degradation of phospholipids and gelatin.

4.4.4 Microorganisms originating from the egg processing company

Microorganisms recovered from surfaces after C&D in the egg processing company were mostly classified as Gram-positive and identified as *Staphylococcus* spp. According to De Reu *et al.* (2008), *Staphylococcus* is the dominant genus on the eggshell. Due to cross contamination, this could be the main reason for identification of this genus in medium to high numbers on several surfaces in the egg processing company despite C&D. In addition, evaluated *Staphylococcus* isolates in this study had the ability to hydrolyse lipids. Genera that are also found frequently on the eggshell and moreover have been identified in rotten eggs are *Alcaligenes*, *Escherichia* and *Pseudomonas* (Mayes and Takeballi, 1983; Neira et al., 2017). This last genus was also identified in this study on surfaces that come into contact with the raw egg content and moreover, all but one of these isolates showed strong spoilage potential what manifested itself through degradation of lipids and also regularly degradation of proteins and phospholipids. However, the pasteurisation process should eliminate these genera and will mostly select for Gram-positive heat-tolerant bacteria such as streptococci, enterococci and *Bacillus* or other spore-forming genera (Baron and Jan, 2011). *Streptococcus* spp., *Bacillus* spp. and *Paenibacillus* spp. were

identified on different surfaces in the egg processing company (collected from PCA after heat treatment of 10 min at 80°C of the sample, data not shown) after C&D. Yet, these strains didn't show any positive result for the four evaluated spoilage tests. Bacteria that survive the pasteurisation process, together with contamination that occurs post-pasteurisation, will pose the greatest possibility of spoilage.

None of the isolates originating from the egg processing company that were evaluated for their spoilage potential had the ability to form hydrogen sulphide which results in a typical rotten egg smell. Besides isolates collected from PCA and PAB, several isolates from Violet Red Bile Glucose Agar for the enumeration of Enterobacteriaceae in the egg processing company were evaluated for their spoilage potential (data not shown). Among these isolates, *Yersinia nurmii* (identified on a conveyor belt for intact eggs with counts of 6.48 log CFU/100 cm^2) also showed high spoilage potential as it was positive for the degradation of proteins, lipids and phospholipids.

This company reported a customer's complaint about green discolouration of a pasteurised whole egg product. In this product, the presence of *Paenibacillus* spp. and *Sporosarcina* spp. was demonstrated. *Paenibacillus validus* was in this study identified on surfaces on the inside of storage tanks with raw egg products and also on a filling head for packaging after pasteurisation (isolated from PCA after heat treatment of 10 min at 80°C of the sample, data not shown). *Paenibacillus* spp. and *Sporosarcina* spp. are known as spore-forming genera, which could explain the survival of the pasteurisation process and the possibility to cause spoilage in end products (An et al., 2007; Grady et al., 2016).

4.4.5 Microorganisms originating from the sauce company

The contamination level in emulsified sauces (e.g. mayonnaise as produced by the participating sauce company) is mostly low (Smittle, 1977). Due to the low pH (maximally 4.5) and the addition of preservatives typical for such food products (such as sodium benzoate and and potassium sorbate), pathogens generally don't grow and spoilage organisms usually only consist of yeasts, moulds and lactic acid bacteria (mostly lactobacilli, Vermeulen, 2008). For the first time, isolates collected from food contact surfaces in a sauce company were identified: they were mostly Gram-negative bacteria from the genus *Pseudomonas*, while also a number of lactic acid bacteria, i.e. *Streptococcus* spp., were identified.

4.5 CONCLUSIONS

Generally, Gram-negative bacteria, more specifically *Pseudomonas* spp., Enterobacteriaceae and *Acinetobacter* spp. are the dominant species on food processing surfaces (Marchand et al., 2012; Møretrø and Langsrud, 2017). In dairy and dry production environments, mostly Gram-positive bacteria are found (Møretrø and Langsrud, 2017). This was also observed in this study as overall 56.8% of all the identified isolates collected in the seven food companies were Gram-negative bacteria, while 63.8% of the isolates collected in the dairy companies were Gram-positive. Moreover, *Pseudomonas* was the most identified genus (20.5% of all isolates collected from TAC) over all the food companies. In addition to the abundance of *Pseudomonas* spp. in food processing environments, this genus also plays an important role in spoilage of food stored at low temperatures (Møretrø and Langsrud, 2017). This was supported in this study since 86.8% of the evaluated *Pseudomonas* isolates showed some kind of spoilage potential in the laboratory tests. An important observation is that, besides *Pseudomonas*, the genera that were most identified were *Microbacterium* (12.2% of all isolates collected from PCA) and *Stenotrophomonas* (9.2%). These were not previously described as predominantly present on surfaces in food processing environments, but were in this study identified in five or six out of the seven food companies, respectively. *Microbacterium* and *Stenotrophomonas* were mostly present in medium (3 to 5 log CFU/100 cm^2) or high (> 5 log CFU/100 cm^2) numbers and always provided some spoilage in the lab tests.

Of the evaluated isolates in this study, 88.5% have to a more or less extent some spoilage potential. However, it remains to be further investigated whether these bacteria will (a) be transferred from the surface to the food product, (b) survive and grow in the food products (Borch *et al.*, 1996), and (c) consequently cause spoilage. It should also be noted that differences in expression level of these spoilage characteristics can be observed on strain level (De Jonghe *et al.*, 2010).

Microbial safety of food products depends on the quality of the raw ingredients but also depends strongly on hygienic practices in food processing companies. Surface contamination with microorganisms plays an important role in cross contamination to these end products and could eventually cause foodborne illness or early food spoilage. Unfortunately, a lot of food companies have little to no knowledge about the identity of

their microbial contaminants or spoilage organisms. In the current study, surface contamination was mapped for several food companies from different food sectors and the possible role in food spoilage was investigated. This knowledge is useful for the food companies to better understand their contamination and spoilage problems. Moreover, microorganisms present in a biofilm on a surface are difficult to eliminate, leading to persistent contamination. The presence of biofilm could possibly enhance the attachment of pathogenic bacteria (Habimana *et al.*, 2010). All these aspects highlight the importance of an optimised and effective C&D procedure which would be able to further eliminate the bacteria found in this study. Therefore, further research of our groups about the biofilm forming capacity of the residual bacteria after C&D is planned together with the development of optimal C&D protocols to provide more insights in the importance of biofilms in food contamination and food spoilage.

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CHAPTER 5: OCCURRENCE AND CHARACTERISATION OF BIOFILMS IN DRINKING WATER SYSTEMS OF BROILER HOUSES

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5 OCCURRENCE AND CHARACTERISATION OF BIOFILMS IN DRINKING WATER SYSTEMS OF BROILER HOUSES

ABSTRACT

Water quality in the drinking water system (DWS) plays an important role in the general health and performance of broiler chickens. Conditions in the DWS of broilers are ideal for microbial biofilm formation. Since pathogens might reside within these biofilms, they serve as potential source of waterborne transmission of pathogens to livestock and humans. Knowledge about the presence, importance and composition of biofilms in the DWS of broilers is largely missing. In this chapter, we therefore aim to monitor the occurrence, and chemically and microbiologically characterise biofilms in the DWS of five broiler farms.

The bacterial load after the application of disinfectants in DWSs was determined by enumerations of total aerobic flora (TAC) and *Pseudomonas* spp. The dominant flora was identified and their biofilm-forming capacity was evaluated. Also, proteins, carbohydrates and uronic acids were quantified to analyse the presence of extracellular polymeric substances of biofilms.

Despite the application of disinfectants in the water and the DWS, average TAC was 6.03 ± 1.53 log CFU/20cm^2. Enumerations for *Pseudomonas* spp. were on average 0.88 log CFU/20cm^2 lower. The most identified dominant species from TAC were *Stenotrophomonas maltophilia*, *Pseudomonas geniculata* and *Pseudomonas aeruginosa*. However at species level, most of the identified microorganisms were farm specific. Almost all the isolates belonging to the three most abundant species were strong biofilm producers. Overall, 92% of all tested microorganisms were able to form biofilm under lab conditions. Furthermore, 63% of the DWS surfaces appeared to be contaminated with microorganisms combined with at least one of the analysed chemical components, which is indicative for the presence of biofilm.

The three earlier mentioned dominant species are considered as opportunistic pathogens and could consequently be a potential risk for animal health. Additionally, the biofilm-forming capacity of these organisms could promote attachment of other pathogens such as *Campylobacter* spp. and *Salmonella* spp.

5.1 INTRODUCTION

Drinking water quality and the drinking water system (DWS) play an important role in the general health and performance of livestock, including broiler chickens (Maharjan *et al.* 2016). Drinking water for broiler chickens can be contaminated with chemical and microbiological components i.a. through the source or through the animals via the drinking cups. *Campylobacter jejuni*, *E. coli*, *Pseudomonas* spp. and *Salmonella* spp. are microorganisms frequently found in drinking water for broilers (Pearson *et al.* 1993; Waage *et al.* 1999; Herman *et al.* 2003; Zimmer *et al.* 2003; Maharjan *et al.* 2016). Waterborne transmission of pathogens to livestock and humans can occur and thereby cause a potential risk for animal and human health (Van Eenige *et al.* 2013; Sparks 2009).

The number of microorganisms can increase when conditions are favourable or when they attach to or form a biofilm on the inside of the DWS. The combination of a convenient temperature (average temperature of ±25°C in broiler houses), low flow rates and sufficient nutrients makes the DWS in broiler houses ideal for microbial numbers to increase and biofilms to form (Sparks, 2009). Biofilms are sessile communities of microorganisms, surrounded by a matrix of self-produced extracellular polymeric substances (EPS). *Aeromonas* spp., *E. coli, Pseudomonas* spp. and *Sphingomonas* spp. were previously described as biofilm-forming organisms in water systems of bovine and humans, but also *Salmonella* spp. and *Campylobacter* spp. are capable to form biofilms in poultry environments (Reeser et al., 2007; Zhao et al., 2011; Elhariry et al., 2012; Van Eenige et al., 2013; Liu et al., 2014; Mulamattathil et al., 2014; van der Wielen and Lut, 2016). Biofilm-forming capacities of microorganisms depend on several factors such as growth conditions, contact surface and species or strain type (Chia *et al.*, 2009; Elhariry *et al.*, 2012; Schonewille *et al.*, 2012; Wang *et al.*, 2013; Lianou and Koutsoumanis, 2013). Biofilms do not per se contain pathogens, but they can provide a place that is easy to attach for these kind of cells (Buswell *et al.* 1998; Trachoo *et al.* 2002). The presence and composition of biofilm in the DWS of broilers is still insufficiently known. The water quality on broiler farms is regularly evaluated at the source and sometimes at the end of the drinking lines depending on the type of DWS (open or closed), but along the drinking lines (where the animals actually drink) often no assessment is done (Vermeersch 2016; Van Eenige *et al.* 2013). Surfaces on the inside of the DWS of broiler chickens are even less or not sampled.

Disinfection of the water and DWS with oxidizers (for example chlorine or hydrogen peroxide), acids or a combination is often performed between flocks (Sparks, 2009), but does not guarantee the elimination of all the microorganisms present. For poultry, drinking water is preferred for medicine administration because of practical reasons (Vermeulen *et al.* 2002). Microorganisms present in biofilms are protected against disinfection products and medicine by the EPS matrix and by enzymes produced by the microorganisms themselves (Hoyle et al., 1992; Trachoo et al., 2002; Bridier et al., 2011; Bobinienė et al., 2012). Moreover, medicines (more specifically carrier substances) and additives (e.g. vitamins) administered by the drinking water can serve as a nutrient source for microorganisms and benefit biofilm formation (Van Eenige et al., 2013). On the other hand, animals can be sub-dosed due to the capture of medicine particles in the biofilm matrix, which can lead to risks for animal health and the development of resistant strains (Roberts *et al.*, 2008; Høiby *et al.*, 2010a). Concerning the development of resistant strains, biofilms are known as hotspots for plasmid transfer and consequently also for the transmission of resistance genes (Hennequin et al., 2012; Savage et al., 2013; Van Meervenne et al., 2014).

There is a lack of information concerning the occurrence, importance and composition of biofilms on the inside of the DWS of broiler chickens. Therefore, the aim of this study was to sample the inside of the DWS of broiler houses to assess the occurrence and chemical and microbiological characteristics of biofilms. Subsequently, the dominant bacteria were evaluated for their biofilm-forming capacities in an *in vitro* biofilm model system.

5.2 MATERIALS AND METHODS

5.2.1 Sampling on broiler farms

On five different Belgian broiler farms (K1-K5), surfaces on the inside of the DWS were sampled during vacancy, approximately 24 hours after the the application of disinfectants. More information about water disinfection that was performed by the farmers during production and DWS disinfection during vacancy is provided in Table 5.1. Sampling points include the end of the pipes, openings at the height of drinking cups, the inside of pressure regulators and water samples before entering the broiler house (thus without disinfection products). In the period July 2015 – October 2016, each broiler house was sampled once or twice (with a time interval of approximately one year) resulting in 85 surface and 7 water samples. A surface area of approximately 20 cm^2 was swabbed

using the tip of a flocked swab (Copan, Cat#552C, Brescia, Italy). After sampling, the nylon tip of the flocked swab was deducted from the breakable plastic applicator and placed in a sterile stomacher bag containing 10 mL of ¼ Ringer's solution (Oxoid, BR0052, Basingstoke, Hampshire, England). Samples were transported to the lab under cooled conditions. In the lab, sampled material in the 10 mL diluent was homogenised in a stomacher (AES Laboratoire, Combourg, France) for 2 min. From each sample one part of the diluent was used for microbiological analyses on the same day as sampling. The remaining part (approximately 7 mL) was collected and stored at -18°C until chemical analysis.

5.2.2 Microbiological characterisation of biofilm

5.2.2.1 Microbiological enumerations

For the surface samples, appropriate 10-fold dilutions were made in sterile 0,1% w/v Peptone Water with 0,85% w/v Salt (BioTrading, K110B009AA, Mijdrecht, the Netherlands) and spread plated. For the water samples, five times 1mL was pour plated. Enumerations of total aerobic count (TAC) and *Pseudomonas* spp. were performed on both types of samples. TAC was chosen to get an idea of the total microbial contamination. PAB on the other hand was chosen since *Pseudomonas* spp. are known to be abundantly present in natural waters (Mena and Gerba, 2009; Casanovas-Massana and Blanch, 2012) and are consequently expected on the inside surfaces of the DWS in broiler houses. TAC was determined by plating on Plate Count Agar (PCA; Oxoid, CM0325) and incubating at 30°C for three days. *Pseudomonas* spp. were enumerated using Pseudomonas Agar Base (PAB; Oxoid, CM0559) with Pseudomonas CFC Selective Agar Supplement (Oxoid, SR0103) and incubation at 30°C for two days. The lowest microbiological enumeration limit was 1.00 log CFU/20cm^2 for surface samples and one CFU/5mL for water.

5.2.2.2 Isolate collection

Samples with counts of 2.00 log CFU/20cm^2 or more were considered as originating from potential biofilm carrying surfaces as describes in chapter 3. From these samples, the dominant microbiota was collected for further identification. The plates with growth on the highest serial 10-fold dilutions represented the dominant microbiota. Based on morphology, 4 to 12 colonies were selected from PCA and 1 to 7 colonies were selected from PAB for each of the surface samples. Per water sample, 3 to 6 colonies and 1 to 3 colonies were selected from PCA and PAB, respectively. Colonies were streaked on new

PCA plates minimally three times to obtain pure cultures. The pure cultures were inoculated in Brain Heart Infusion (BHI; Oxoid, CM1135) with 15% glycerol (Merck, 8.18709.1000, Darmstadt, Germany), incubated for two days at 30°C and kept at -80°C. From surface samples, a total of 241 isolates were collected from PCA and 105 from PAB. From the water samples, 22 and 10 isolates were collected from PCA and PAB, respectively. Collected isolates were classified as originating from samples in three bacterial abundance classes. For isolates collected from PCA, the class of less than 4 log CFU/20cm^2 represented low bacterial numbers, the class of 4 to 7 log CFU/20cm^2 represented medium numbers and the class of more than 7 log CFU/20cm^2 represented high numbers. Isolates collected from PAB were classified as less than 4 log CFU/20cm^2 (low numbers), 4 to 6 log CFU/20cm^2 (medium numbers) or more than 6 log CFU/20cm^2 (high numbers).

5.2.2.3 Isolate identification

The procedure for isolate identification in this chapter is equal to the procedure described in chapter 4. From each isolate, except for those that could not be cultivated after storage at -80°C (38 out of 378 isolates), DNA was collected according to Strandén *et al.* (2003). DNA extracts were stored at 4°C and used on the same day for $(GTG)_5$ PCR for clustering of the isolates. Out of the 340 isolates included in the $(GTG)_5$ fingerprint clusters, 200 were selected for identification based on the occurrence of their pattern and as representatives for visually defined clusters. The 16S rRNA gene was amplified for identification of the selected isolates as described in chapter 4. PCR products were sequenced by Macrogen Europe (Amsterdam, the Netherlands) and further analysed in EZtaxon (Kim et al., 2012). In total, 16S rRNA sequencing led to the identification of 191 of the 200 isolates. Together with the $(GTG)_5$ fingerprint results, 330 out of 378 isolates could be identified to the genus or species level.

5.2.2.4 Evaluation of the biofilm-forming capacities of the isolates

The ability of a random selection of identified strains (n=169) to form biofilms in polystyrene microtiter plates was determined based on Peeters *et al.* (2008) with some modifications as described in the following section. Starting from an overnight liquid culture in Luria-Bertani broth (LB, Composition: 10g l-1 tryptone (Organotechnie, 19553, La Courneuve, France), 5g l-1 yeast extract (Organotechnie, 19512), 10g l-1 NaCl (VWR, 7647-14-5, Radnor, Pennsylvania) and 20g l-1 glucose (Tereos Syral, 14431-43-7,

Marckolsheim, France)) at 30°C, a 1:100 dilution was made in LB. For each strain, 16 wells of a round-bottomed polystyrene 96-well microtiter plate (Greiner Bio-One, 650 101, Kremsmünster, Austria) were inoculated with 100 µL of this dilution. As negative control 16 wells were filled with sterile LB medium. The microtiter plate was incubated at 30°C for 4h to allow for the adhesion of the microorganisms. After this, the liquid (containing non-adhered cells) was removed by inverting the microtiter plate and all the wells were rinsed once with 100 µL of sterile ¼ Ringer's solution (Biokar, BR00108, Beauvais, France). Fresh sterile LB medium was added to all wells and the microtiter plate was further incubated for 24h at 30°C. Subsequently, the liquid with culture was removed and all wells were washed three times with sterile ¼ Ringer's solution to remove non-adhered cells. The remaining biofilm was fixated with 150 µL of 99% methanol (Acros Organics, 268280025, Geel, Belgium) per well for 15 min. After this the microtiter plate was emptied and air dried. Then, 100 µL of crystal violet solution used for Gram staining (Merck, 109218, Darmstadt, Germany) was added to all wells for 20 min. The excess stain was removed by placing the microtiter plate under running tap water and the microtiter plate was air dried for 2h. Retained crystal violet was dissolved by adding 150 µL of 33% (v/v) glacial acetic acid (Merck, 100063). The absorbance was measured at 590nm using a microtiter plate reader (BioRad, 1681135, Hercules, CA, USA).

Based on the absorbance measured at 590nm after crystal violet staining, biofilms were classified into following categories as previously described by Stepanović *et al.* (2000): non biofilm producer, weak, moderate or strong biofilm producer. The cut-off OD (ODc) was defined as three standard deviations above the mean absorbance of the negative control. Strains were classified as follows: $ODstrain \leq ODc$ = no biofilm producer, $ODc < ODstrain \leq (2 \times ODc)$ = weak biofilm producer, $(2 \times ODc) < ODstrain \leq (4 \times ODc)$ = moderate biofilm producer and $(4 \times ODc) < ODstrain$ = strong biofilm producer.

5.2.2.5 Chemical characterisation of biofilm

Chemical analyses were performed on all surface samples collected in the five broiler farms during the first sampling round (n=43). Before chemical analyses, an extraction procedure was performed to separate the EPS from the microorganisms. Therefore, the remaining diluent part (¼ Ringer's fraction after microbiological analyses) was sonicated (UP 400S, Hielscher, Germany) 3 times for 30s with an interval of 30s at an amplitude of 50% and a cycle of 0.5 in a water bath to disrupt the bacterial clumps. After centrifugation

(Savant, SFA13K) at 13 000 RCF for 10 min at room temperature, supernatant (containing EPS) was recovered and used for the chemical characterisation. Protein, carbohydrate and uronic acid analyses were performed as described in chapter 3.

5.2.2.6 Statistical analysis

All values of the chemical analyses are the result of the average of three technical replicates and all values of the biofilm-forming capacities are the result of the average of sixteen technical replicates. Statistical analyses on the obtained microbiological enumerations and chemical results were carried out using Statistical Analysis System software (SAS®, version 9.4, SAS Institute Inc., Cary, NC, USA). Distribution of the log transformed enumerations per microbiological parameter and quantification of the analysed chemical components was evaluated based on the histogram and QQ plot. For the representation of the contamination level, the values for microbiological and chemical analyses are represented by mean and standard deviation for normally distributed values. First quartile (Q1), median (Q2) and third quartile (Q3) are calculated for values that did not follow a normal distribution.

5.3 RESULTS

5.3.1 Contamination of water samples on broiler farms

All broiler farms used well water as drinking water for the broiler chickens (Table 5.1). Water samples were taken at different broiler farms at the point just before entering the broiler house and before any disinfection product was administered. Table 5.1 shows the results of the bacterial load. TAC results varied from 6 to >300 CFU/mL while enumerations for *Pseudomonas* spp. varied from 2 to >300 CFU/mL. No identifications could be done of isolates originating from water samples taken at K1. The dominant microbiota of water samples from K2 collected from TAC were identified as *Bosea robiniae*, *Chryseobacterium scophthalmum* and *Rhizobium radiobacter* and the isolates collected from PAB were identified as *Delftia acidovorans* and *Pseudomonas peli*. *Chryseobacterium* spp., *Aeromonas media*. and other *Aeromonas* spp. were identified as the dominant total aerobic water flora on K3, while *Aeromonas salmonicida* and *Pseudomonas koreensis* were identified among the isolates collected from PAB. The water flora on K4 contained predominantly *Arthrobacter russicus* and *Pseudomonas* spp. (isolated from TAC).

Table 5.1: Bacterial load as total aerobic count (TAC) and *Pseudomonas* count (PSEUDO) of water samples taken at five broiler farms. Also, water disinfection during production and drinking water system disinfection during vacancy on broiler farms as performed by the farmers is shown.

Broiler Farm	**Type of water (number of samples)**	**Water contamination (CFU/mL)**		**Water disinfection during production**				**DWS disinfection during vacancy**			
		TAC	PSEUDO	Frequency	Disinfection product	Active compound	Applied concentration [1]	Frequency	Disinfection product	Active compound	Applied concentration
K1	Well water	>300	>300	2-3/week	DM CID (CID lines [2])	NaClO + KOH	0.00010%	1/round	CID 2000 (CID lines)	H_2O_2 + Acetic acid + Peracetic acid	0.050%
K2	Well water	>300	20	/ [3]	/	/	/	1/year	MS Oxy-Clean 1.0 (MS Schippers [4])	H_2O_2	Unknown
K3	Well water (2)	>300	30 and >300	Continuously	Di-O-Clean (MS Schippers)	ClO_2	Week 1, 2, 3: 0.030% Week 4, 5, 6: 0.015%	1/round	Huwa-San Veterinary Applications (Sanac[5])	H_2O_2	0.027%
K4	Well water	59	2	Daily	Top Clean Aqua (Topturn [6])	H_2O_2	0.20%	1/round	CID Clean (CID lines)	H_2O_2	1.0%
K5	Well water (2)	6 and 102	3 and 33	Week 1 and 2	CID Clean (CID lines)	H_2O_2	0.010%	1/round	CID Clean (CID lines)	H_2O_2	2.0%

[1] Applied concentration of the disinfection product (not of the active compound); [2] CID lines, Ieper, Belgium; [3] /, No water disinfection applied; [4] MS Schippers, Arendonk, Belgium; [5] Sanac, Wervik, Belgium; [6] Topturn, Bergeijk, the Netherlands

Pseudomonas libanensis (isolated from PAB), *Bacillus thuringiensis* and *Pseudomonas poae* (isolated from TAC) were identified as the dominant flora in one of the water samples taken at K5. The dominant flora of the other water sample taken on this farm mainly consisted of *Aeromonas* spp., *Bacillus* spp., *Chryseobacterium rhizosphaerae* and *Pseudomonas extremorientalis* isolated from TAC and *Pseudomonas granadensis* isolated from PAB. Disinfection products (mostly based on hydrogen peroxide or chlorine) were regularly applied in the drinking water in all farms during production, except in farm K2.

5.3.2 Surface contamination in DWS of broiler houses

In total, 85 surfaces on the inside of the DWS were sampled after the application of disinfectants (Table 5.1). In all broiler farms, disinfection started by filling the drinking lines during vacancy with a disinfection product (always based on hydrogen peroxide as active component). Afterwards, a rinsing step with water was performed. All farms performed this procedure after each production round, except for farm K2 where disinfection is only performed once a year. The applied concentration of the disinfection product varied per farm. Table 5.2 shows the results of the microbiological load and the quantification of the chemical biofilm components of the sampled surfaces. TAC results of all samples of the five farms varied between 1.87 and 9.00 log CFU/20cm^2 while average and median values of samples taken at one sampling moment at one farm ranged from 4.27 to 7.19 log CFU/20cm^2. Average TAC for all surfaces was 6.03$\pm$1.53 log CFU/20cm^2. Enumerations for *Pseudomonas* spp. were on average 0.88 log CFU/20cm^2 lower than for TAC. Chemical analyses were performed on 43 of the 85 sampled surfaces. On 58% of the analysed surfaces, proteins were found. Carbohydrates and uronic acids were found only on 14% and 5% of the surface samples, respectively. When chemical and microbiological results were combined, 63% of the sampled surfaces during the first sampling round appeared to be contaminated with both microorganisms and at least one of the analysed chemical biofilm components. On four surfaces (9%), microorganisms, proteins and carbohydrates were found, while in two other samples (5%) microorganisms, proteins and uronic acids were found. None of the samples contained microorganisms in combination with all three of the analysed chemical biofilm substances.

Table 5.2: Presence of surface contamination in the DWS of broiler farms. The number of sampled points (n) together with the proportion of quantifiable samples for chemical analysis (%) and values for total aerobic count (TAC), *Pseudomonas* count (PSEUDO), proteins, carbohydrates and uronic acids are shown for each farm sampling. Mean and standard deviation are given for values that are normally distributed. First quartile (Q1), median (Q2, in bold) and third quartile (Q3) are given for values that did not follow this distribution. Sampling points where both TAC (≥ 2 log CFU/20cm^2) and one or several chemical components were quantified (>LOQ) are evaluated as carrying biofilm.

Broiler Farm	**Microbiological analysis**			**Chemical analysis**							**Positive points for biofilm (%)**
	n	Enumerations (log CFU/20cm^2)		n	Proteins		Carbohydrates		Uronic acids		
		TAC	PSEUDO		%[1]	Quantity (µg/20cm^2)	%	Quantity (µg/20cm^2)	%	Quantity (µg/20cm^2)	
K1	8	5.53 – **5.70** – 6.17	4.48 – **4.89** – 5.26	8	25	15.70, 22.40 *	0	/[2]	0	/	25
	15	7.19 ± 1.08	6.61 ± 1.12	0							
K2	6	6.28 – **6.35** – 7.24	5.08 – **6.29** – 7.42	6	83	14.00 – **15.85** – 16.80	17	139.44 *	0	/	83
K3	12	4.27 ± 0.64	3.36 – **3.95** – 4.38	12	75	7.10 – **16.95** – 19.05	17	59.63, 60.71 **	17	28.40, 43.20 **	83
	12	6.03 – **6.97** – 7.63	5.32 – **6.17** – 6.80	0							
K4	8	3.09 – **4.55** – 4.68	1.92 – **3.19** – 4.19	8	75	5.90 – **18.40** – 26.85	12	64.32 *	0	/	75
K5	9	4.30 – **4.71** – 6.00	4.33 ± 0.72	9	33	0.00 – **0.00** – 16.80	22	62.52, 94.04 **	0	/	44
	15	7.13 ± 1.05	5.20 – **5.67** – 6.11	0							

[1] %, proportion of quantifiable samples given in percentage; [2] /, No values were quantifiable; *, Only one value obtained; **, Only two values obtained

5.3.3 Identification of microorganisms present on surfaces on the inside of the DWS of broiler houses

5.3.3.1 Isolates from PCA

Among the few Gram-positive isolates (n=17), four families were found represented by four genera (Figure 5.1table). The genus *Microbacterium* was identified in four of the sampled broiler farms, except in farm K4 where no Gram-positive bacteria were identified among the dominant microbiota. Each of the identified Gram-positive species was found in only one of the sampled broiler farms and was present either in medium or high numbers. The identified Gram-negative bacteria (n=185) belonged to 14 families represented by 22 genera. The genera *Stenotrophomonas* and *Pseudomonas* were found in five and four of the sampled farms, respectively. The genera *Brevundimonas*, *Comamonas*, *Delftia*, *Chryseobacterium*, *Pseudoxanthomonas*, *Acinetobacter* and *Sphingomonas* were found on two of the five farms. Moreover, concerning *Stenotrophomonas* it was the same species (*Stenotrophomonas maltophilia*) that occurred at all the broiler farms. *Pseudomonas aeruginosa* and *Pseudomonas hibiscicola* were found in four broiler farms (except for K2). Overall, the most dominant genera found on PCA were *Pseudomonas* (32.2% of the identified isolates) and *Stenotrophomonas* (16.8%). However between farms there were differences in the most dominant microbiota. In broiler farm K1, besides *Pseudomonas* (36.5%), the genera *Shewanella* and *Acinetobacter* occurred with 15.4% and 13.5% of the isolates respectively. Only 14 isolates were collected in broiler farm K2, all belonging to the species *Stenotrophomonas maltophilia* (50.0%), *Microbacterium fluvii* (35.7%) or *Chryseobacterium aquaticum* (14.3%). *Pseudomonas* (64.4%), *Chryseobacterium* (11.1%) and *Stenotrophomonas* (species *maltophilia*, 11.1%) were the most identified genera at broiler farm K3, while the dominant microbiota at farm K4 mostly belonged to *Stenotrophomonas maltophilia* (40.5%) and *Sphingobium yanoikuyae* (16.7%). In broiler farm K5 the most dominant genera found were *Pseudomonas* (26.5%) and *Sphingomonas* (24.5%) and the species *Pseudoxanthomonas mexicana* (10.2%). Moreover, *Pseudomonas aeruginosa* and *Pseudoxanthomonas mexicana* isolates were found twice in time at farm K5.

Classes

- < 4 log CFU/20cm²
- 4-7 log CFU/20cm²
- > 7 log CFU/20cm²

Gram	Family	Species	K1	K2	K3	K4	K5
Gram-positive	*Microbacteriaceae*	*Microbacterium fluvii*		5			
		Microbacterium hominis			1		
		Microbacterium paraoxydans					2
		Microbacterium saccharophilum			1		
		Microbacterium testaceum	1				
	Nocardiaceae	*Gordonia amicalis***					2
		Gordonia spp. **					1
	Nocardioidaceae	*Nocardioides simplex*					2
	Promicromonosporaceae	*Cellulosimicrobium funkei*	2				
Gram-negative	*Alcaligenaceae*	*Bordetella trematum*				1	
	Brucellaceae	*Ochrobactrum* spp.	1				
	Caulobacteraceae	*Brevundimonas bullata*				2	
		Brevundimonas spp.					1
	Comamonadaceae	*Comamonas* spp.	3				2
		Delftia acidovorans			3		
		Delftia lacustris	1		1		
	Enterobacteriaceae	*Citrobacter freundii*				4	
		Klebsiella michiganensis	1				
		Leclercia adecarboxylata	2				
		Raoultella spp.	2				
	Flavobacteriaceae	*Chryseobacterium aquaticum*		2			
		Chryseobacterium hispalense			1		
		Chryseobacterium massiliae			2		
		Chryseobacterium spp.			2		
		Cloacibacterium normanense	2				
	Lysobacteraceae	*Pseudoxanthomonas mexicana****				4	5 *
		*Stenotrophomonas maltophilia****	3	7	5	17	2
	Moraxellaceae	*Acinetobacter beijerinckii*	4				
		Acinetobacter grandensis	1				
		Acinetobacter johnsonii	1				
		Acinetobacter oryzae	1				
		Acinetobacter spp.					2
	Mycobacteriaceae	*Mycobacterium saopaulense*					1

Pseudomonadaceae	*Pseudomonas aeruginosa*	6		3	3	10 *
	Pseudomonas chengduensis			1		
	Pseudomonas geniculata	9		20		
	Pseudomonas hibiscicola	1		3	1	1
	Pseudomonas monteilii					2
	Pseudomonas putida	2		2		
	Pseudomonas vranovensis	1				
Rhizobiaceae	*Rhizobium* spp.				2	
Shewanellaceae	*Shewanella xiamenensis*	8				
Sphingobacteriaceae	*Sphingobacterium daejeonense*					2
Sphingomonadaceae	*Sphingobium yanoikuyae*				7	
	Sphingomonas koreensis					2
	Sphingomonas spp.				1	
	Sphingopyxis alaskensis					8
	Sphingopyxis italica					2
	Sphingopyxis spp.					2

Figure 5.1: Family (based on http://www.bacterio.net/, verified on 25 January 2018), genus and species identity of isolates from PCA of inside surface samples of the DWS in five broiler farms (K1-K5) after disinfection. Different colors represent the magnitude of TAC enumerations of samples whereof the bacteria were isolated. * Indicates that the corresponding species was found during the first and second sampling round in the corresponding company. ** This species belongs to the family *Gordoniaceae* according to NCBI classification. *** This species belongs to the family *Xanthomonadaceae* according to NCBI classification.

5.3.3.2 Isolates from PAB

The 102 identified isolates collected from PAB were all Gram-negative bacteria, belonging to nine families represented by eleven genera (Figure 5.2). The genus *Stenotrophomonas* was found in all sampled farms, while *Pseudomonas* was found in four out of the five broiler farms (not in K2). The genus *Aeromonas* was found on two of the sampled broiler farms (K1 and K2). Overall, the most identified genera found on PAB were *Pseudomonas* (70.6% of the identified isolates) and *Stenotrophomonas* (12.8%). More specifically in farm K1, the most identified species was *Pseudomonas putida* (18.4%). In broiler farm K2, 80% of the identified isolates were *Stenotrophomonas maltophilia*. *Pseudomonas geniculata* (35%) and *Pseudomonas aeruginosa* (25%) were the most identified species at farm K3, while the dominant microbiota at broiler farm K4 belonged to the species *Stenotrophomonas maltophilia* (28.6%) and *Pseudomonas aeruginosa* (21.4%). Also in broiler farm K5, the most common species found was *Pseudomonas aeruginosa* (36.0%) and moreover this species was found twice in time.

Classes

< 4 log CFU/20cm^2

4-6 log CFU/20cm^2

> 6 log CFU/20cm^2

	Family	Genus / species	K1	K2	K3	K4	K5
Gram-negative	*Aeromonadaceae*	*Aeromonas hydrophila* subsp. *ranae*		1			
		Aeromonas spp.	1				
	Alcaligenaceae	*Achromobacter* spp.				2	
	Brucellaceae	*Ochrobactrum intermedium*					1
	Comamonadaceae	*Comamonas* spp.					4*
		Delftia tsuruhatensis	1				
	Enterobacteriaceae	*Klebsiella* spp.			1		
	Lysobacteraceae	*Stenotrophomonas maltophilia***		4	1	4	2
		*Stenotrophomonas rhizophila***	2				
	Moraxellaceae	*Acinetobacter beijerinckii*	1				
		Acinetobacter calcoaceticus	1				
		Acinetobacter guillouiae	1				
		Acinetobacter spp.	1				
		Flavimonas oryzihabitans			1		
	Pseudomonadaceae	*Pseudomonas aeruginosa*	3		5	3	9*
		Pseudomonas chengduensis			3	2	
		Pseudomonas ficuserectae	1				
		Pseudomonas geniculata	4		7	1	
		Pseudomonas hunanensis			1		
		Pseudomonas indoloxydans	2				
		Pseudomonas japonica				1	
		Pseudomonas mandelii	1				
		Pseudomonas monteilii			1		
		Pseudomonas peli	5				
		Pseudomonas putida	7				2
		Pseudomonas spp.	6			1	7*
	Shewanellaceae	*Shewanella xiamenensis*	1				

Figure 5.2: Family (based on http://www.bacterio.net/, verified on 25 January 2018), genera and species identity of isolates from PAB of inside surface samples of DWS in 5 different broiler farms (K1-K5) after disinfection. Different colors represent the magnitude of PAB enumerations of samples whereof the bacteria were isolated. * Indicates that the corresponding species was found during the first and second sampling round in the corresponding company. ** This species belongs to the family *Xanthomonadaceae* according to NCBI classification.

5.3.4 Biofilm-forming capacities of microorganisms present on surfaces on the inside of the DWS of broiler houses

According to the classification of Stepanović *et al.* (2000), 92% of all tested microorganisms (n=169) produced biofilm (Figure 5.3), ranging from 78% of the tested isolates in farm K2 to 97% in farm K3. Of all the assessed isolates, 61% were strong biofilm producers, ranging from 33% in farm K2 to 72% in farm K4. Differences were observed between isolates from farm K2 (n=9) and the other four farms in terms of a lower percentage of biofilm-forming isolates.

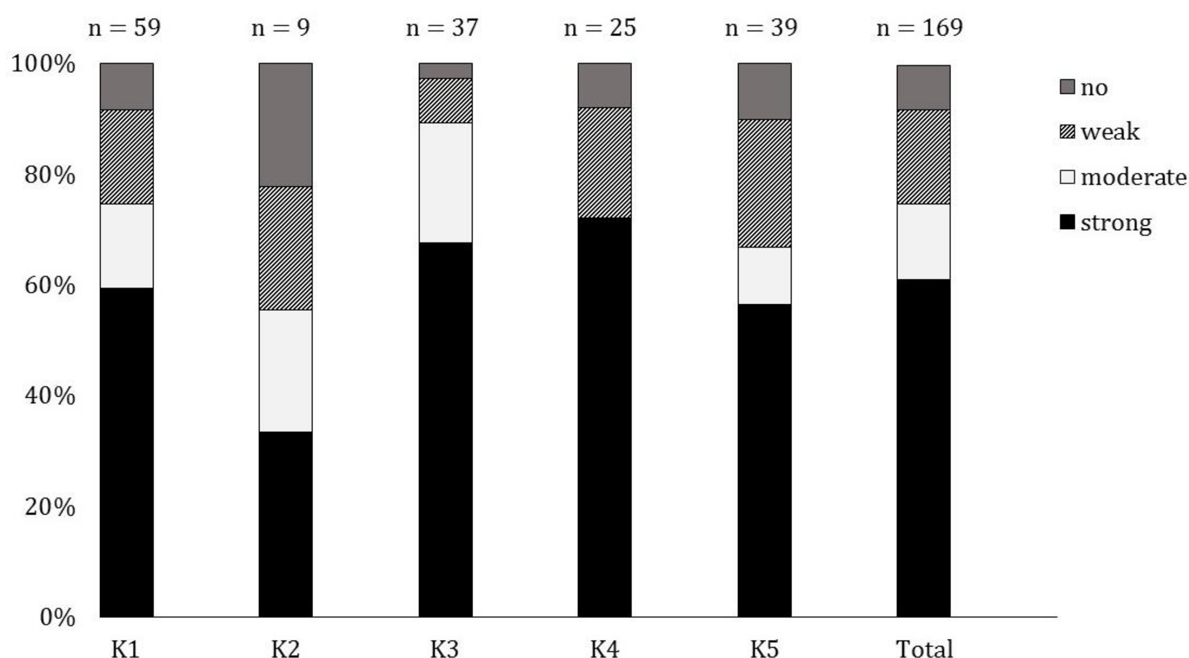

Figure 5.3: Overview of the prevalence of strong, moderate, weak and no biofilm-formers in the different broiler farms given in percentage.

The bacterial isolates per genus with strong biofilm-forming ability and their presence in the different farms (as strong biofilm producer) are summarised in Table 5.3. Several strong biofilm producers, detected on PCA, were not detected on PAB. On the other hand, only one strong biofilm producer, detected on PAB, was not detected on PCA. The strong biofilm producing bacteria mainly belonged to the genera *Pseudomonas* spp. and *Stenotrophomonas* spp. Of all the evaluated isolates of these genera, 83% (for *Pseudomonas*) and 87% (for *Stenotrophomonas*) were strong biofilm producers. Strong biofilm producers belonging to the genus *Pseudomonas* were found in every farm, except for K2. The genus *Stenotrophomonas* was present as strong biofilm producer on every farm. All isolates belonging to the three species *Pseudomonas aeruginosa*, *Pseudomonas*

geniculata and *Stenotrophomonas maltophilia* (except for one *Pseudomonas geniculata* and one *Stenotrophomonas maltophilia* strain) were evaluated as strong biofilm producers. *Pseudomonas aeruginosa* was present as a strong biofilm producer in every farm except for K2 and *Pseudomonas geniculata* was present as a strong biofilm producer in farms K1, K3 and K4. Other isolates with strong biofilm-forming capacities only occurred in one or two farms and belonged to the genera *Acinetobacter*, *Flavimonas*, *Nocardioides* and *Ochrobactrum*. The strong biofilm producers of the genus *Acinetobacter* belonged to different species, for *Ochrobactrum* to two species, and for *Flavimonas* and *Nocardioides* to one species, i.e. *Flavimonas oryzihabitans* and *Norcardioides simplex*.

Table 5.3: Bacteria with strong biofilm-forming capacities per genus and presence as strong biofilm former on the different broiler farms. The number of evaluated isolates (n) together with the proportion of strong biofilm-formers given in percentage (%) is shown per genus.

Identification	Evaluated isolates		TAC		PSEUDO		K1	K2	K3	K4	K5
	n	strong biofilm (%)	n	strong biofilm (%)	n	strong biofilm (%)					
Pseudomonas spp.	76	83	29	86	47	81	+ [1]		+	+	+
Stenotrophomonas spp.	23	87	13	92	10	80	+	+	+	+	+
Acinetobacter spp.	9	44	5	20	4	75	+				
Microbacterium spp.	8	13	8	13					+		
Delftia spp.	5	40	4	50	1	0	+		+		
Pseudoxanthomonas spp.	4	25	4	25							+
Shewanella spp.	4	75	3	67	1	100	+				
Comamonas spp.	3	67	2	50	1	100					+
Brevundimonas spp.	2	50	2	50						+	
Ochrobactrum spp.	2	100	1	100	1	100	+				
Sphingobium spp.	2	50	2	50						+	
Sphingomonas spp.	2	50	2	50							+
Flavimonas spp.	1	100			1	100			+		
Nocardioides spp.	1	100	1	100							+

[1] +, indicates the presence of the genus as strong biofilm former in the corresponding company

5.4 DISCUSSION

5.4.1 Contamination of water samples on broiler farms

Microbiological load of the incoming water samples (without disinfection product) ranged from 6 to >300 CFU/mL. This was generally lower than reported by Maharjan (2016). Participating broiler farms used water disinfection products based on oxidising agents such as chlorine or hydrogen peroxide to control microbial growth. However, the type of disinfectants and the applied concentrations are not always ideal for the disinfection of water according to the disinfectant providers.

Identified dominant microbiota in water samples were unique per farm and consisted mostly of Gram-negative bacteria. None of the species identified in the incoming water were also found as dominant flora on the inside of the DWS. On genus level on the other hand, the genus *Pseudomonas* (one of the most identified genera on the inside of the DWS) also occurred in all the water samples. Also *Chryseobacterium* spp. were found in three of the five water samples. The identified microorganisms from water samples are generally not involved in disease development in poultry (Jordan and Hampson, 2008). However, *Aeromonas media*, is reported as a putative human pathogen (Chaix *et al.*, 2017).

5.4.2 Surface contamination in DWS of broiler houses

In all the broiler farms, disinfection of the DWS was performed during vacancy with oxidising agents, containing at least hydrogen peroxide. Despite regular disinfection, most sampled surfaces on the inside of the DWS of the broiler houses showed high microbiological counts. No published results could be found concerning microbiological contamination on similar surfaces, however SE Watkins found comparable counts (6.35 and 6.83 log CFU/sponge; personal communication, December 19, 2017, University of Arkansas) to this study. It is well known that cleaning of DWS is not obvious since it consists of a mostly closed system and applying the needed mechanical force is not evident. On the other hand, not optimal action of the disinfectant due to residual dirt, biofilm formation and noncompliance of the recommended concentration can be an additional reason of the microbial contamination on the surfaces. Beside, the survival can be caused by antioxidant strategies, resistance to the disinfectant, the structure of the microbial biofilm communities (which causes reduced diffusion of the active components) and many other defensive strategies (Batté *et al.*, 2003). Due to the fact that

an insufficient reduction of bacterial numbers is obtained, it is better to speak of sanitisation of the DWS instead of disinfection.

No previous studies were found where surface samples of DWS on primary production farms were chemically analysed for biofilm EPS components. In this study, 63% of the samples where chemical analysis was performed contained at least one of the chemical components (proteins, carbohydrates or uronic acids) after the application of disinfectants. The presence of high numbers of microorganisms in combination with chemical components (possibly originating from EPS or organic pollution) sampled after sanitisation of the surface can be an indication of the presence of a biofilm (see chapter 3). This means that in this study, 63% of the analysed surfaces would be identified as carrying biofilm. This is a much higher number compared to surfaces in the food industry where the presence of biofilm was suspected in 17% of the cases (chapter 3).

5.4.3 Characterisation of isolates collected from the DWS of broiler houses

To our knowledge, no previous studies were performed describing the identity and biofilm-forming capacity of microorganisms isolated from the inside of the DWS in broiler farms. Overall, Gram-negative bacteria were identified to a higher extent compared to Gram-positive bacteria. This is possibly due to the fact that Gram-negative bacteria are generally better biofilm formers and that the niche in DWS is more favourable (Norton and LeChevallier, 2000; Smith et al., 2000). The dominant bacteria identified over the participating farms were largely similar except for K2. On this farm, except for *Stenotrophomonas maltophilia*, the dominant flora differed from the other farms. Besides a smaller sample size and consequently less collected isolates, on farm K2, water disinfectants were not applied and DWS sanitation only took place once a year. Due to the low number of isolates and the single case of the specific character of farm K2, no interpretation of the results and general discussion can be made. Bacteria originating from surfaces on the inside of the DWS (both with or without detection of chemical components) in the other four broiler farms and collected from TAC (but also identified on PAB) mainly belonged to the species *Stenotrophomonas maltophilia* (17% of the identified isolates), *Pseudomonas geniculata* (14%) and *Pseudomonas aeruginosa* (11%). Species that were also abundant but not identified on PAB were *Pseudoxanthomonas mexicana*, *Sphingopyxis alaskensis* and *Shewanella xiamenensis* (all 4% of the identified isolates from TAC). According to Anzai *et al.* (2000), *Pseudomonas*

beteli, *Pseudomonas geniculata* and *Pseudomonas hibiscicola* should not be included in the genus of *Pseudomonas* (sensu stricto) because of a higher level of homology (99.2% - 99.5%) with *Stenotrophomonas maltophilia* based on the 16S rRNA gene sequence. Although further extensive studies are required for definite taxonomic conclusion, this would shift the prevalence of *Stenotrophomonas maltophilia* to 34%. Rożej *et al.* (2015) reported the abundance of *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* in a model for drinking water distribution systems. According to these authors the abundance of these microorganisms was due to the high ability to settle and multiply on the surface of plastic pipes. *Pseudomonas aeruginosa* is a versatile Gram-negative bacterium that is one of the top three causes of opportunistic human infections (Stover *et al.*, 2000) that may become multidrug resistant (Aloush et al., 2006). Moreover, different studies reported the high mortality rate in broiler chicks due to *Pseudomonas aeruginosa* infection (Niilo 1959; Devriese *et al.* 1975; Walker *et al.* 2002). *Stenotrophomonas maltophilia* is an environmental global emerging multidrug resistant microorganism that is most commonly associated with respiratory infections in humans (Brooke, 2012). *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* are frequently co-isolated from lungs of cystic fibrosis patients and evidence suggests that *Stenotrophomonas maltophilia* modulates the virulence of *Pseudomonas aeruginosa* in a multispecies biofilm (Pompilio *et al.*, 2015). Although *Stenotrophomonas maltophilia* was detected in the caecal content of broiler chickens (Nathiya et al., 2012) and chicken eggs (Sabarinath et al., 2009), no link with water quality and with disease development in broiler chicks associated with *Stenotrophomonas maltophilia* was reported.

In this study 92% of all tested isolates had the ability to produce biofilm and 61% were even strong biofilm producers. These results support the hypothesis that nearly all bacteria have the potential to form biofilm in case they are under the right conditions. Almost all the isolates belonging to the three most abundant species (*Stenotrophomonas maltophilia*, *Pseudomonas geniculata* and *Pseudomonas aeruginosa*) were strong biofilm producers. When applying the taxonomic classification of Anzai *et al.* (2000), the percentage of strong biofilm producing bacteria would shift from 61% *Pseudomonas* and 19% *Stenotrophomonas* to 40% *Pseudomonas* and 43 % *Stenotrophomonas*.

Zoonotic pathogens mostly associated with poultry (Mor-Mur and Yuste, 2010), such as *Campylobacter* spp. and *Salmonella* spp., were not identified among the dominant microbiota of water and DWS surface samples. This is because if these pathogens are present, this would be in such low number that they would not be identified among the dominant flora on TAC. After the first sampling round, also detection methods for *Campylobacter* spp. and *Salmonella* spp. were performed on samples collected in three out of the five participating broiler farms during the second sampling round. Although no *Campylobacter* spp. or *Salmonella* spp. were detected in these samples (results not shown), the presence of biofilm-forming bacteria present in DWS could be a potential risk for the protection of these pathogens. The survival of culturable *Campylobacter jejuni* increased when cultured with a biofilm of a community sampled from a water drinker in a poultry house in a study by Hanning *et al.* (2008). The sampled community consisted mainly of *Pseudomonas* spp., *Staphylococcus* spp., *E. coli*, *Bacillus* spp. and *Flavobacterium* spp. Culotti and Packman (2015) reported not only a prolongation of the survival of *Campylobacter jejuni* when co-cultured with *Pseudomonas aeruginosa* under aerobic conditions, but also an enabling of the growth of *Campylobacter jejuni* on the surface of *Pseudomonas aeruginosa* biofilms. Comparable results were reported in other studies (Buswell *et al.*, 1998; Ica *et al.*, 2012). The presence of *Pseudomonas* spp. could also favour the growth of *Salmonella* in biofilms. Dual-species biofilms of *Salmonella* and *Pseudomonas* spp. increased the biovolume 3.2-fold compared to single-species biofilms of *Salmonella* (Habimana et al., 2010b). However, knowledge is lacking about the importance of biofilm or strong biofilm formers in the protection of zoonotic pathogens in practice. The results in this study will be the basis for more research of our group in a biofilm model system concerning the interaction between *Salmonella* spp. and the obtained field isolates from the DWS. The model will take into account the conditions that approach the situation in practice in DWS on broiler farms as good as possible.

5.5 CONCLUSIONS

Despite regular application of oxidising disinfection products, sampled surfaces on the inside of DWS in broiler houses showed rather high (average of 6.03±1.53 log CFU/20cm^2) microbiological counts. Also, 63% of the sampled surfaces contained at least one of the analysed chemical components. The presence of high numbers of microorganisms in combination with chemical components is indicative for the presence of biofilm. It is clear that the intended disinfection is not achieved and it is better to speak of sanitisation in this situation.

The most identified species over the five sampled broiler houses were *Stenotrophomonas maltophilia*, *Pseudomonas geniculata* and *Pseudomonas aeruginosa*. Moreover, these species were identified as strong biofilm formers. It is also known that some of these microorganisms can cause disease and death in humans and chickens, whereby these species are important to monitor and to eliminate.

Even without *Salmonella* spp. and *Campylobacter* spp. being detected in the present study, it was already shown in lab studies that biofilm could play a role in the maintenance of these pathogens in the drinking water system of broiler chickens. More research will be done in a biofilm model system concerning the interaction between these pathogens and microorganisms originating from DWS.

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CHAPTER 6: POTENTIAL OF *PSEUDOMONAS PUTIDA* AS A BIOCONTROL AGENT AGAINST *SALMONELLA* JAVA IN THE DRINKING WATER SYSTEM OF BROILER HOUSES

Biofilm experiments were performed at ILVO, Technology and Food Science Unit

6 POTENTIAL OF *PSEUDOMONAS PUTIDA* AS BIOCONTROL AGENT AGAINST *SALMONELLA* JAVA IN THE DRINKING WATER SYSTEM OF BROILER HOUSES

ABSTRACT

Biofilms can provide attachment and protection of other microorganisms (among which pathogens) but can also prevent them from attaching and forming biofilm. This latter situation, wherein living organisms control the presence of other living microbes, is called biocontrol. This potential for both positive and negative interactions between microbes raises the need for in depth characterization of the sociobiology of candidate biocontrol agents (BCAs). Especially the inside of the drinking water system (DWS) is eligible to study interactions since this niche plays an important role in the contamination of broiler chickens and consequently humans with pathogens. More specifically, *Pseudomonas putida*, which is part of the natural microbiota in the DWS of broiler houses, was in this chapter evaluated as BCA against the pathogen *Salmonella* Java, which shows an increasing prevalence in the broiler production chain over recent years.

To study the interaction between these species, an *in vitro* model to simulate biofilm formation on the inside of the DWS of broiler chickens that approached practical conditions as well as possible was developed and validated. Mono- and dual-species biofilms were grown in 6-well microtiter plates and quantified based on bacterial counts and biomass. Interactions in dual-species biofilms were determined based on cooperation criterion and biodiversity effect.

The developed model was found to be repeatable and reproducible based on enumerations while quantification based on biomass needed to be further optimized. Significant differences in biofilm formation between different strains and between different applied inoculum densities were observed, whereby *Salmonella* Java was evaluated as the best biofilm former among the tested strains and biofilm formation increased with increasing inoculum density. Biofilm formation by *Pseudomonas putida* and *Salmonella* Java was always characterized by competitive interaction, independent of the *Pseudomonas putida* strain, *Salmonella* Java inoculum density and application order. However, the degree of *Salmonella* reduction did vary dependent on these factors and the strongest reduction was observed in dual-species biofilms where *Pseudomonas putida*

strain P3 in an inoculum density of 6 log CFU/mL and *Salmonella* Java in an inoculum density of 1 log CFU/mL were sequentially applied.

This study provided the first results indicating the potential of *Pseudomonas putida* as a BCA against *Salmonella* Java in the broiler environment. These results could be used in further research concerning alternative methods to eliminate pathogens in primary animal production environments and to prevent the animals and humans from being infected.

6.1 INTRODUCTION

Infections with *Salmonella* occur frequently in broiler chickens leading to animal disease, animal death and large economic losses (Rose et al., 2003; Gast et al., 2008). In Belgium, an increasing trend in the prevalence of *Salmonella* Java on broiler flocks was observed over recent years (Pierré, 2018). There are indications that this strain is mostly introduced at farm level (van Asselt *et al*., 2009) and that it spreads and persists easily (Van Immerseel *et al*., 2004). This is of main concern because consumption of poultry meat is an important source of human infections with *Salmonella* (Rose et al., 2000; Gast et al., 2008; Zhang, 2008; Antunes et al., 2016). Broiler chickens are mainly infected through environmental sources, feed and drinking water (Waage *et al*., 1999). Drinking water quality and the drinking water system (DWS) therefore play an important role in the general health and performance of broiler chickens (Maharjan *et al*., 2016) and consequently also in human health (Sparks, 2009). The number of microorganisms on the inside of the DWS can increase when conditions are favourable or when they join to form a biofilm. *Aeromonas* spp., *E. coli, Pseudomonas* spp. and *Sphingomonas* spp. were previously described as biofilm-forming organisms in water systems of bovine and humans (Elhariry et al., 2012; Van Eenige et al., 2013; Liu et al., 2014; Mulamattathil et al., 2014; van der Wielen and Lut, 2016), but also *Salmonella* spp. are capable to form biofilms in the DWS of poultry (Zhao *et al*., 2011).

Biofilms do not *per se* contain pathogens, but they can provide a niche for their attachment and protection (Buswell et al., 1998; Trachoo et al., 2002). On the other hand, the presence of specific commensal microorganisms on surfaces might also prevent pathogens from attaching and forming a biofilm which would reduce their persistence. The use of living microorganisms to control other living microbes is called biocontrol. This method could be an alternative for the usually performed chemical disinfection which is not environmentally friendly and poses a risk for resistance development (Fraise, 2002). Biocontrol agents (BCAs) to reduce unwanted organisms such as pathogens and pests have already been evaluated in several environments such as pig nursery units, floor drains in food industry and on plants (Zhao *et al*., 2006; Luyckx *et al*., 2016b; Bosmans *et al*., 2017).

Biocontrol is based on the naturally occurring competitive interactions exerted by BCAs on the pathogen. Especially when microbial species use the same nutrient sources or

when they colonize the same area of surface, competitive interactions are expected to be dominant (Ghoul and Mitri, 2016). However, microbes can also engage in other types of social interactions (Mitri and Foster, 2013). In a more limited number of cases, microbial species were found to cooperate, enhancing one another's fitness (Rakoff-Nahoum et al., 2016). This potential for both positive and negative interactions between microbes raises the need for in depth characterization of the sociobiology of candidate BCAs. With this respect, the *cooperation criterion* can be used to distinguish between cooperative and competitive interactions (Mitri and Foster, 2013), whereas the *biodiversity effect* (consisting of a selection effect and a complementarity effect) can be calculated to further quantify the level of competition (Loreau and Hector, 2001; Parijs and Steenackers, 2018). Since biofilm-growth capacities of microorganisms strongly depend on several factors, including growth conditions, contact surface and taxonomy (Chia et al., 2009; Elhariry et al., 2012; Schonewille et al., 2012; Wang et al., 2013; Lianou and Koutsoumanis, 2013), assays to evaluate social interactions should be performed under conditions that mimic the real situation as much as possible.

In chapter 5, we identified *Pseudomonas putida* as part of the natural microbiota on several locations on the inside of the DWS in broiler houses, but it is not known as a common contaminant on chickens. Several *Pseudomonas* spp. have been shown to suppress plant pathogens by antibiotic production and more specifically *Pseudomonas putida* is suggested as a BCA against plant diseases (Sun et al., 2017; Bernal et al., 2017). Moreover, several isolates from this species with different biofilm-forming capacity (evaluated in chapter 5) were collected. Because of its presence in the niche of interest, its variation in biofilm-forming capacity, its harmless character and its previously indicated potential as a BCA, *Pseudomonas putida* was chosen in the current study to investigate its potential as BCA against the pathogen *Salmonella* Java on the inside of the DWS in broiler houses. The interaction between these species was investigated based on the cooperation criterion and biodiversity effect to assess if *Pseudomonas putida* biofilms promote or impede the attachment and biofilm formation by *Salmonella* Java. Hereto, an *in vitro* model to simulate biofilm formation on the inside of the DWS of broiler chickens that approaches practical conditions as well as possible, was developed and validated. Several field strains were evaluated for their biofilm-forming capacity and also the influence of the pathogen density and sequential application of the strains on the attachment and biofilm formation by *Salmonella* Java were investigated.

6.2 MATERIALS AND METHODS

6.2.1 Strain selection and preparation

To study the potential of *Pseudomonas putida* as BCA against *Salmonella* Java, several field strains from broiler houses were used: *Salmonella enterica* subsp. *enterica* serotype Paratyphi B variant Java (later referred to as *Salmonella* Java or S1, collected from drinking water of broiler chickens on a broiler farm in Belgium, KS243), *Salmonella enterica* subsp. *enterica* serotype Mbandaka strain (later referred to as *Salmonella* Mbandaka or S2, collected from broiler feed, KS7) and three *Pseudomonas putida* strains (later referred to as P1, P2 and P3, collected from inside surfaces of the drinking water system in broiler houses). The *Pseudomonas putida* strains were, in the study discussed in chapter 5, classified as weak (P1), moderate (P2) and strong (P3) biofilm formers in 96-well microtiter plates (MTPs).

For the preparation of the bacterial suspension for inoculation in the *in vitro* biofilm model, strains were streaked on Plate Count Agar (PCA, Oxoid, CM0325, Basingstoke, Hampshire, England) from their glycerol stocks at -80°C and incubated for one day at 37°C for *Salmonella* strains and two days at 30°C for *Pseudomonas* strains. Subsequently, one colony from PCA was transferred to a test tube containing 10mL of Tryptone Soya Broth (TSB, Oxoid, CM0129). An overnight culture was obtained by incubating the broth for 18h at 30°C or 37°C for *Pseudomonas* (8 log CFU/mL) or *Salmonella* (9 log CFU/mL) strains, respectively. Quantification of the overnight culture was done by plating on PCA and incubation for three days at 30 or 37 °C depending on the strain. Finally, overnight cultures were diluted in sterile ¼ Ringer's solution (Biokar, BR00108, Beauvais, France) to approach the desired density (1, 2, 3 or 6 log CFU/mL depending on the corresponding biofilm set-up). However, actual inoculum densities were calculated based on enumerations of the used overnight cultures and are called the inoculum suspensions.

6.2.2 Model preparation

Coupons with a dimension of 35mm to 10mm and a thickness of 2mm were cut out of new plastic drinking water lines commonly used in broiler houses (Swii'Flo, Roxell, Maldegem, Belgium). Before use, these coupons were decontaminated in 70% ethanol for 10min and dried in a laminar flow cabinet. The decontaminated coupons were vertically placed in each well of a 6-wells MTP (Novolab, SPL30006, Geraardsbergen, Belgium) using

sterilized tweezers in a way that only the 2mm sides of the coupons touch the wells. For each experiment, three 6-well plates were required.

6.2.3 Mono- and dual-species biofilm formation

6.2.3.1 Attachment of the bacterial strains

Per biofilm experiment, two out of three 6-well MTPs were used for biofilm formation. In these plates, 11mL of inoculum suspension was added per well to completely submerge the coupons. The third 6-well MTP was used as blank. In this plate, 11mL of diluted TSB (equally diluted as the inoculum suspension) was applied in each well. Well plates were incubated for four hours at 25°C and very slightly shaken at 50 rpm making attachment of the bacteria to the coupons possible. After four hours of incubation, coupons were removed from the 6-well MTPs and transferred to 15mL falcon tubes (Sigma-Aldrich, Z720461-50EA, Overijse, Belgium) using sterilized tweezers. To remove non-attached bacteria, coupons were rinsed once by submerging them in 10mL sterile ¼ Ringer's solution in the falcon tubes. Afterwards, the ¼ Ringer's suspension was poured away and coupons with attached bacteria were placed vertically in new 6-well MTPs.

6.2.3.2 Biofilm formation by the attached bacterial strains

The three new MTP's with coupons were filled with 11mL of sterile 1/20 diluted TSB per well and subsequently incubated for 18h at 25°C and 50rpm to allow the attached bacteria to form biofilm. After incubation, coupons were removed from the 6-well MTPs and transferred to 15mL falcon tubes using sterilized tweezers. To remove non-attached bacteria, coupons were rinsed three times by consecutively adding 10mL of sterile ¼ Ringer's solution in the falcon tubes and pouring away the suspension. Finally, coupons were transferred to new sterile 15mL falcon tubes.

6.2.4 Quantification of biofilm formation based on bacterial counts

Coupons originating from the first MTP per biofilm experiment were used for quantification of biofilm formation by conventional microbiology. Three blank coupons from the third MTP were also used to ensure no contamination did occur during analysis. First, 10mL of sterile ¼ Ringer's solution was added to the falcon tubes containing the coupons. Then, three consecutive rounds of sonication for 30s at 42kHz in a ultrasonic water bath (Branson, 2510, Eemnes, the Netherlands) and vortexing for 30s were performed to harvest the biofilm.

The liquid suspension containing the detached biofilm cells was plated on Tryptone Soya Agar (TSA, Oxoid, CM0131) for enumerations of total aerobic count (TAC) and a second, more selective, medium. This selective medium was Xylose Lysine Desoxycholate Agar (XLD, Oxoid, CM0469) for *Salmonella* biofilms and Pseudomonas Agar Base (PAB; Oxoid, CM0559) with Pseudomonas CFC Selective Agar Supplement (Oxoid, SR0103) for *Pseudomonas* biofilms. For dual-species biofilms with a *Salmonella* and *Pseudomonas* strain, quantification was done using TSA, XLD and PAB. Appropriate 10-fold dilutions were made in sterile 0,1% w/v Peptone Water with 0,85% w/v Salt (BioTrading, K110B009AA, Mijdrecht, the Netherlands) and pour plated. TSA plates were incubated for three days at 30°C or 37°C for *Pseudomonas* or *Salmonella* biofilms, respectively. XLD plates were incubated for three days at 37°C and PAB plates were incubated for three days at 30°C. The lowest microbiological enumeration limit was 1,16 log CFU/cm^2.

6.2.5 Quantification of biofilm formation based on biomass

Coupons originating from the second MTP per biofilm experiment together with the remaining three blank coupons of the third MTP were used for quantification of biofilm formation based on biomass. 10mL of a 0.1% crystal violet solution (containing 0.1g/100mL crystal violet (Merck, 101418, Darmstadt, Germany) dissolved in one part of methanol (Biosolve, 13687802, CE Valkenswaard, the Netherlands), one part of isopropanol (Merck, 1.09634) and 18 parts of Phosphate Buffered Saline (Oxoid, BR0014G)) was added to each of the falcon tubes for 20min at 350rpm for the staining of the total biomass of the biofilm on the coupons. The excess stain was removed by placing the tubes under gently running tap water. Retained crystal violet was dissolved by adding 10mL of 33% acetic acid (Merck, 1.00063) for 15min at 350rmp. The absorbance was measured at 590nm using a spectrophotometer (Jasco, V-660, Pfungstadt, Germany). OD-measurements of the blank coupons were subtracted from the OD-measurements of the biofilm coupons.

6.2.6 Study of interactions between bacterial strains in dual-species biofilms

In this study, the cooperation criterion and the biodiversity effect were calculated to determine social interactions between *Salmonella* Java and *Pseudomonas putida* and to consequently assess the potential of *Pseudomonas putida* as BCA. The cooperation criterion requires that the inoculation density in co-culture equals the sum of inoculation densities of the monocultures whereas the biodiversity effect imposes that the inoculation

density of each species in co-culture should be its inoculation density in monoculture divided by the number of species in co-culture (Parijs and Steenackers, 2018). A preliminary experiment was conducted growing biofilms in both set-ups but no differences in final biofilm growth were observed.

Concerning the cooperation criterion, counts for TAC of the dual culture were compared with the sum of the counts for TAC of the two monocultures of *Salmonella* and *Pseudomonas*. Also, counts for *Salmonella* spp. on XLD and *Pseudomonas* spp. on PAB were compared between mono and dual cultures.

The biodiversity effect can be calculated as follows (Loreau and Hector, 2001):

$$\Delta Y = Y_O - Y_E = N\overline{\Delta RY}\,\overline{M} + Ncov(\Delta RY, M)$$

with M_i meaning biofilm growth of species i in monoculture, $Y_{O,i}$ observed biofilm growth of species i in dual culture, $Y_O = \sum_i Y_{O,i}$ total observed biofilm growth in dual culture, $RY_{E,i}$ expected relative biofilm growth of species i in dual culture based on the inoculation proportion, $RY_{O,i} = Y_{o,i}/M_i$ observed relative biofilm growth of species i in dual culture, $Y_{E,i} = RY_{E,i}M_i$ expected biofilm growth of species i in dual culture, $Y_E = \sum_i Y_{E,i}$ total expected biofilm growth in dual culture, $\Delta Y = Y_O - Y_E$ difference between total observed and total expected biofilm growth in dual culture (=biodiversity effect), $\Delta RY_i = RY_{O,i} - RY_{E,i}$ difference between observed relative growth and expected relative growth of species i in dual culture and N the number of species which is two in dual-species biofilms. $N\overline{\Delta RY}\,\overline{M}$ represents the complementarity effect and $Ncov(\Delta RY, M)$ the selection effect. The intention was to apply strains in dual-species biofilms in a certain ratio (e.g. 1:1 or 1:0.001) depending on the experiment. However, due to differences in growth of the overnight culture per isolate, the ratio based on the actual inoculum densities was used in the study of the interactions between bacterial strains in dual-species biofilms.

6.2.7 Statistical analysis

Statistical analyses on the obtained microbiological and biomass results were carried out using Statistical Analysis System software (SAS®, version 9.4, SAS Institute Inc., Cary, NC, USA). First, normal distribution of the OD measurements and of the log transformed enumerations per microbiological parameter per biofilm experiment were evaluated based on the histogram and QQ plot. For the evaluation of the reproducibility of the model system, a Kruskal Wallis test was used to compare results for OD measurement and

enumerations between the three experiments per strain. For the comparison of mono-species biofilm formation of different bacterial strains, enumerations of total aerobic counts were evaluated per experiment using ANOVA. Post-hoc pairwise comparisons were made using Scheffe test. For the comparison of mono-species biofilm formation with different inoculum densities, enumerations of total aerobic counts were evaluated per experiment using a Kruskal Wallis test. Post-hoc pairwise comparisons were made using Dunn test. For the comparison of the quantification of different dual-species biofilms (with different strains, different inoculum densities or different application order) again a Kruskal Wallis test was performed on enumerations of TAC, *Pseudomonas* spp. and *Salmonella* spp. followed by a post-hoc pairwise comparison using Dunn test to indicate possible differences. P-values ≤ 0.05 were considered significant.

6.3 RESULTS

6.3.1 Validation of the *in vitro* biofilm model

First, an *in vitro* biofilm model was developed and validated based on mono-species biofilm formation. Practical conditions were taken into account as much as possible, in contrast to most biofilm research performed in 96-well MTPs (Coenye and Nelis, 2010), to guarantee the relevance of the obtained results and insights. Practical conditions were simulated by growing biofilms on coupons made out of plastic drinking water lines for broilers. Low flow conditions in the DWS were simulated by shaking at a low shaking speed (50rpm) and an incubation temperature of 25°C was chosen to mimic the average stable environmental temperature. Nutrient conditions were approached by applying a poor growth medium during biofilm formation. Finally, as specified in Materials and methods: Strain selection and preparation, field strains (2 *Salmonella* strains; 3 *Pseudomonas putida* strains) previously collected from water, broiler feed and from inside surfaces of the drinking water system for broilers were used to simulate biofilm formation at this specific niche.

Before interactions between *Pseudomonas putida* and *Salmonella* were investigated, mono-species biofilms were evaluated for the validation and implementation of the newly developed *in vitro* biofilm model. Biofilms were grown using a 6 log CFU/mL inoculum suspension of *Salmonella* Java strain S1 and a 6 log CFU/mL inoculum suspension of *Pseudomonas putida* P2. To evaluate the repeatability of the results, standard deviations between replicates (six for S1 and five for P2) performed in the same experiment (i.e. on

the same day) were evaluated. Concerning reproducibility, results obtained in three different experiments per strain (i.e. on different days, using diffent overnight cultures performed by different laboratory technicians) were statistically analysed and compared. Only small standard deviations between replicates and no significant differences between independent experiments were observed for enumerations of biofilm experiments using the same strain (p=0.0600 for S1 and p=0.1738 for P2). Yet, OD measurements provided large standard deviations between replicates and significant differences over time for both strains (p=0.0009 for S1 and p=0.0087 for P2). These results demonstrate the repeatability and reproducibility of the model for biofilm quantification based on enumerations while quantification based on biomass needs to be further optimized.

6.3.2 Influence of strain and inoculum density on mono-species biofilm formation

The mono-species biofilm set-up was then used to study differences in biofilm-forming capacity of different *Salmonella* and *Pseudomonas putida* strains isolated from broiler DWS and feed and to evaluate the influence of inoculum density on biofilm formation.

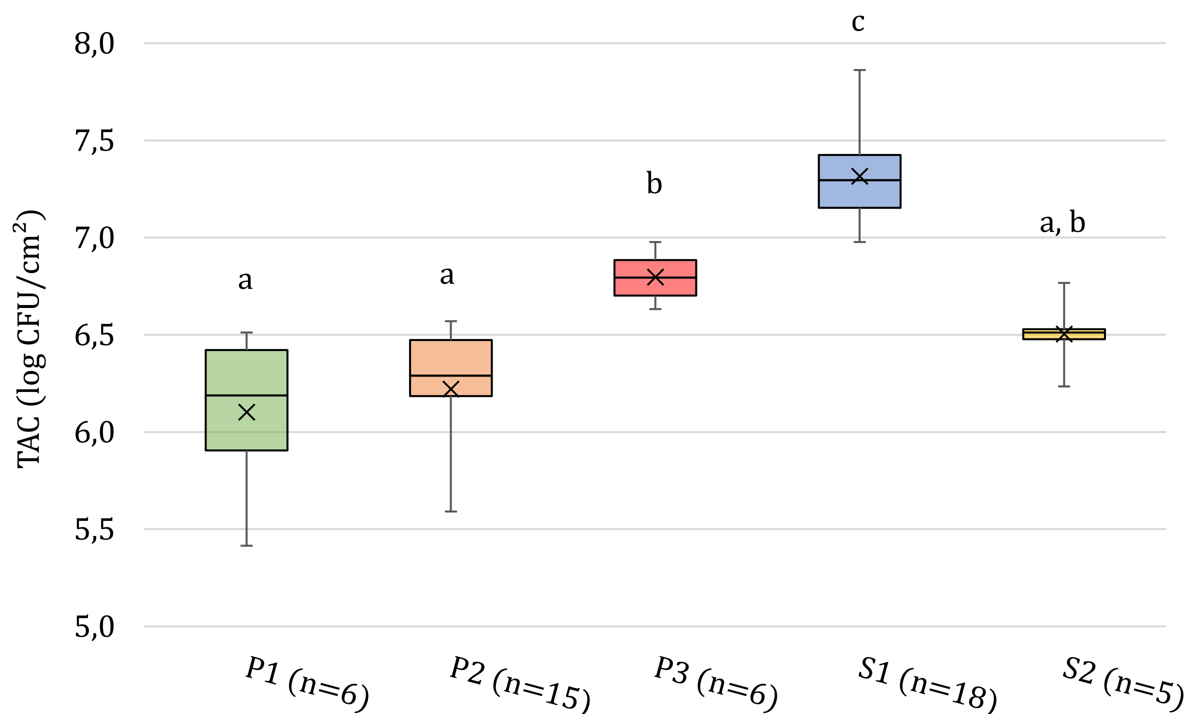

Figure 6.1: Mono-species biofilm formation by different field strains. The same inoculum density (6 log CFU/mL) was used for attachment of the different strains (P1: *Pseudomonas putida* weak biofilm former, P2: *Pseudomonas putida* moderate biofilm former, P3: *Pseudomonas putida* strong biofilm former, S1: *Salmonella* Java, S2: *Salmonella* Mbandaka) followed by biofilm quantification by enumerations of total aerobic counts (TAC, log CFU/cm^2). Strains that did not provide significantly different biofilm quantities are indicated with the same alphabetical character. P-values of ≤ 0.05 were considered as significant.

This revealed significant differences in biofilm formation between different strains applied with the same inoculum density (Figure 6.1) and between different inoculum densities of the same strain (Figure 6.2). *Salmonella* Java S1 applied with an inoculum density of 6 log CFU/mL provided the highest amount of biofilm of all strains based on microbial enumerations. The different *Pseudomonas putida* strains showed a similar trend in number of biofilms cells as obtained by crystal violet biomass staining in chapter 5, with P1 providing the lowest amount of biofilm and P3 the highest. Decreasing the inoculum density was found to lead to decreased biofilm formation. Because of its higher biofilm forming potential and its higher prevalence in practice as compared to *Salmonella* Mbandaka, *Salmonella* Java S1 was selected to study the interactions with *Pseudomonas putida*.

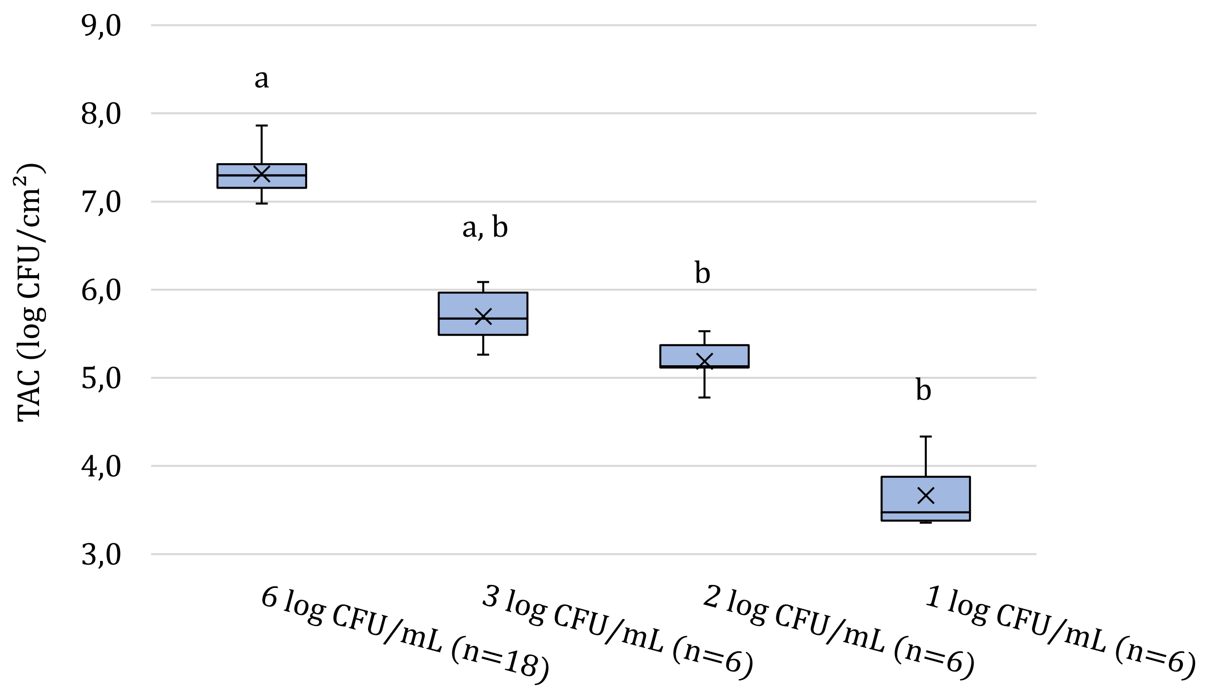

Figure 6.2: Mono-species biofilm formation by *Salmonella* Java strain S1. Four different inoculum densities (6, 3, 2 and 1 log CFU/mL) were used for attachment followed by biofilm quantification by enumerations of total aerobic counts (TAC, log CFU/cm^2). Inoculum suspensions that did not provide significantly different biofilm quantities are indicated with the same alphabetical character. P-values of ≤ 0.05 were considered as significant.

6.3.3 Influence of strain on interaction and biocontrol effect

To study the interaction between the *Pseudomonas putida* strains and *Salmonella* Java, dual-species biofilms were set up by simultaneously inoculating both species in a ratio that approached 1:1. The cooperation criterion states that cooperation occurs if both strains increase with respect to their final cell number in monoculture and the total number of cells in dual-culture is thus higher than the sum of the monocultures; otherwise competitive, neutral or accidental effects occur (Mitri and Foster, 2013). Figure 6.3 shows that all three *Pseudomonas putida* strains reduced the cell number of *Salmonella* Java S1 cells (reduction of 39% with P1, 74% with P2 and 37% with P3) in the dual-species compared to mono-species biofilms, indicating competitive interactions between both species. *Pseudomonas putida* strain P2 mediated the largest reduction of *Salmonella* Java S1. All strain combinations are characterized by mutual inhibition, with a decrease in cell numbers of both strains in the dual- compared to mono-species biofilms.

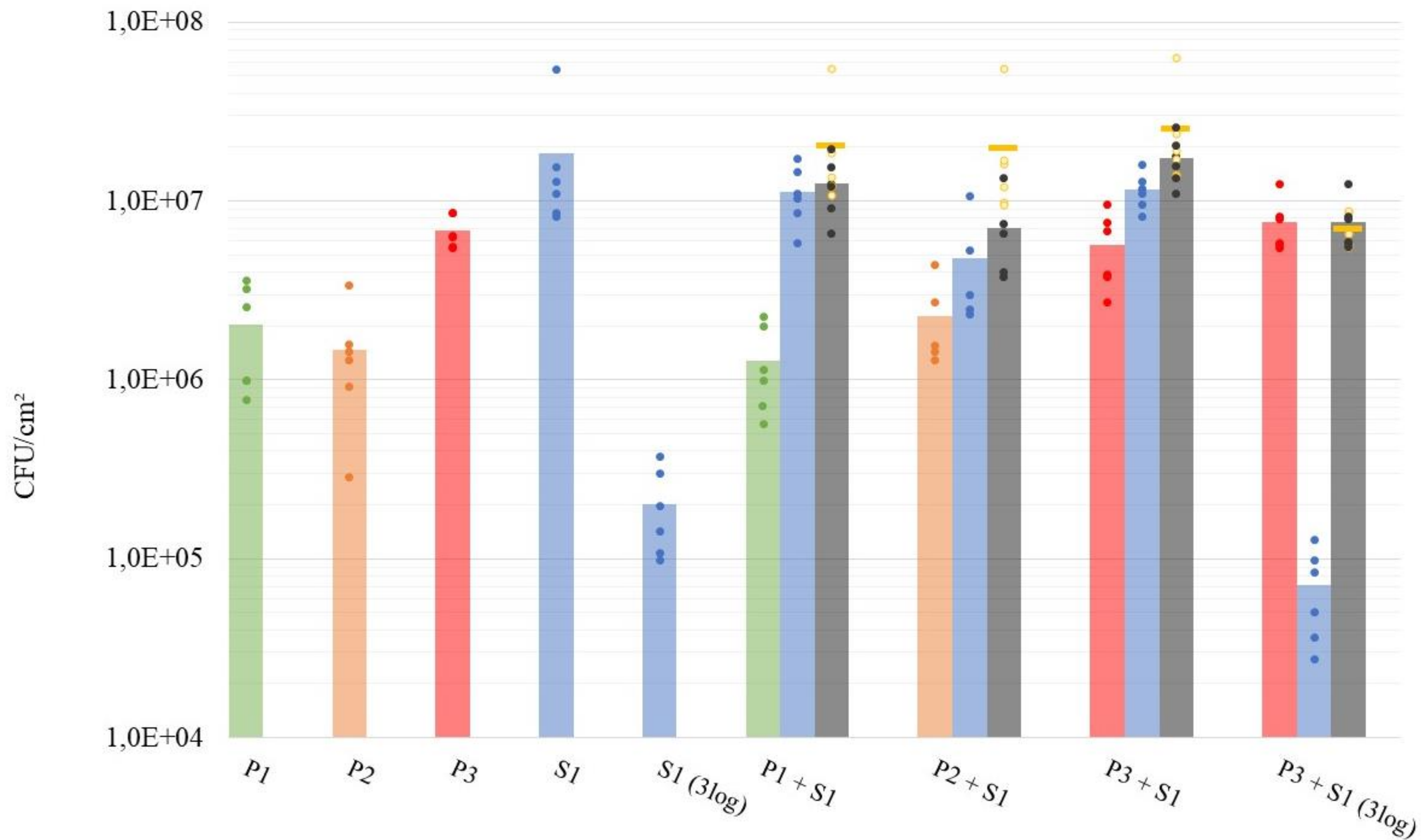

Figure 6.3: Influence of strain and pathogen density on the interaction and biocontrol effect. Cell number of each strain (P1: *Pseudomonas putida* weak biofilm former, P2: *Pseudomonas putida* moderate biofilm former, P3: *Pseudomonas putida* strong biofilm former and S1: *Salmonella* Java) in mono-species biofilms and in dual-species biofilms are indicated. Also the influence of S1 density on its cell numbers in dual-species biofilms was examined. The total amount of cells expected for cooperation in dual-species biofilms is indicated with yellow dots. Six biological replicates (dots) and their average (bars) are shown per strain and in total (grey) for the dual-species biofilms.

To further characterize the level and nature of competition we calculated the biodiversity effect (Loreau and Hector, 2001; Parijs and Steenackers, 2018). In case inter-species competition is equal to intra-species competition, the observed biofilm formation in dual-species is expected to be equal to the average of the mono-species biofilm formation, weighed by the inoculum densities (expected biofilm formation). The biodiversity effect is defined as the difference between the observed and the expected dual-species biofilm formation and is therefore a measure for the extent to which inter-species interactions differ from intra-species interactions. Dual-species biofilms between S1 and P1 showed lower bacterial counts than expected, whereas the combination of S1 and P2 or P3 produced higher cell counts than expected. This is respectively indicated by a negative and positive biodiversity effect in Figure 6.4.

The negative biodiversity effect between S1 and P1 can be caused by selection of the worse mono-species biofilm former or by strong interference competition (Loreau and Hector, 2001; Parijs and Steenackers, 2018). To distinguish between both cases we analysed the two components of the biodiversity effect, i.e. the complementarity effect and the selection effect. The dual-species biofilms containing P1/S1 showed a positive complementarity effect, indicating that the *Salmonella* Java and *Pseudomonas putida* strains do not show a complete overlap in use of nutrients and space. This, however, cannot exclude that interference competition took place. The selection effect was negative which suggests a dominance of the worse mono-species biofilm former (i.e. P1 and P2) in the dual-species biofilm. This negative selection effect compensates for the degree of positive complementarity, ultimately leading to a negative biodiversity effect. The positive biodiversity effect between S1 and P2 or P3 on the other hand can be caused by niche separation and/or selection of the best biofilm former. These combinations of strains were also found to be characterised by a positive complementarity effect and thus niche separation, which was in this case associated with a positive selection effect (selection of the best mono-species biofilm former, i.e S1), summing up to a positive biodiversity effect.

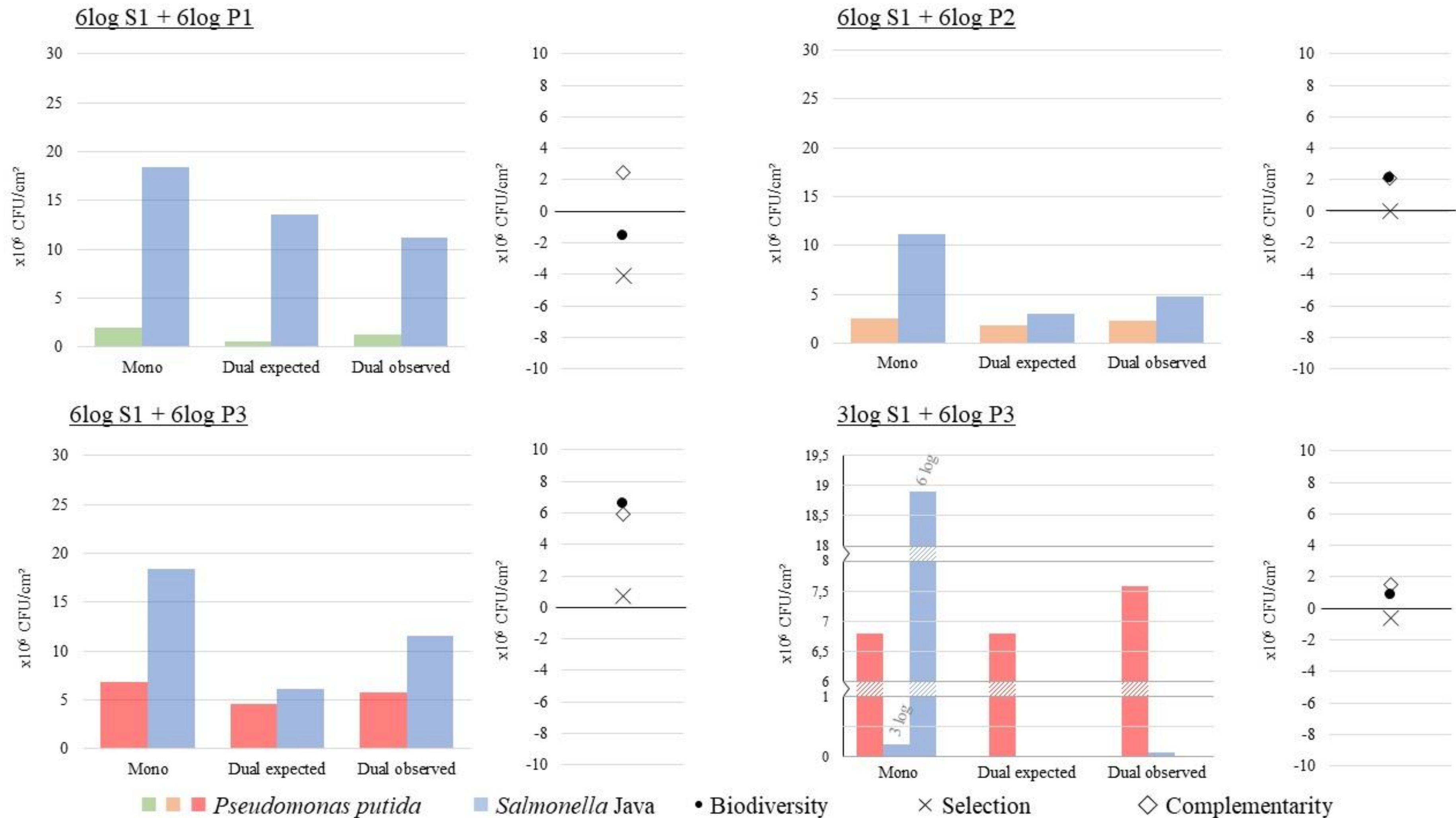

Figure 6.4: Mono-species growth (Mono), expected dual-species growth (Dual expected) and observed dual-species growth (Dual observed) of strains (P1: *Pseudomonas putida* weak biofilm former, P2: *Pseudomonas putida* moderate biofilm former, P3: *Pseudomonas putida* strong biofilm former and S1: *Salmonella* Java), together with biodiversity, selection and complementarity effect of four dual-species biofilm experiments.

6.3.4 Influence of pathogen inoculum density on interaction and biocontrol effect

We next studied the influence of the pathogen's inoculum density on the interaction with *Pseudomonas putida* by lowering the inoculum density of S1 from 6 CFU/mL to a more realistic 3 log CFU/mL. Because of its high mono- and dual-culture level of biofilm formation and associated expected persistence in practice (Borucki et al., 2003), *Pseudomonas putida* P3 was selected for this study. In case of this lower inoculum, *Salmonella* S1 was repressed by P3 to a higher proportional extent (65% decrease in dual- vs mono-species biofilm) as observed for the higher inoculum (37% decrease) (Figure 6.3). Interestingly, lowering the inoculum density of S1, changed the mutually competitive interaction (at 6 log CFU/mL) into an exploitative interaction (at 3 log CFU/mL), with slightly increased cell number of *Pseudomonas putida* in dual-species compared to mono-species biofilms. The lower inoculum size lowered the magnitude of the overall positive biodiversity effect and also its components changed in sign and/or magnitude (Figure 6.4). Indeed, although still positive, the complementarity effect did decrease when lowering the S1 inoculum, whereas the selection effect turned negative, pointing at a selection of the worse mono-species biofilm former, being P3. The negative selection effect could not compensate for the degree of positive complementary, leading to an overall positive biodiversity effect.

6.3.5 Influence of sequential application of BCA and pathogen on interaction and biocontrol effect

Finally, the effect of sequential application of *Pseudomonas putida* P3 and *Salmonella* Java S1was studied to evaluate the potential of *Pseudomonas putida* as a preventive BCA against *Salmonella*. To enhance realism we included even lower S1 inoculum densities. The potential BCA strain P3 was first allowed to attach for 4h and proliferate for 18h, providing biofilms of 6.83±0.09 log CFU/cm^2. Then, different inoculum densities of S1 (3, 2 and 1 log CFU/mL) were applied for attachment (4h) and biofilm formation (18h) on the pre-existing P3 biofilm. Significant differences between observed cell numbers in dual-species (after 44h of incubation) and cell number expected for cooperation for all applied pathogen densities were determined ($p=0.0039$, $p=0.0039$ and $p=0.0090$ for 3, 2 and 1 log CFU/mL, respectively) and absolute cell numbers were lower in dual-species compared to mono-species biofilms for both strains (Figure 6.5). This indicates that competitive interactions are also present between P3 and S1 in the sequential set-up. *Salmonella* Java biofilm formation on a pre-existing *Pseudomonas putida* biofilm was

significantly lower than on a clean surfaces for all the evaluated inoculum densities. For 3, 2 or 1 log CFU/mL of S1, a decrease of 83%, 93% and 94% was respectively observed when applied a on pre-existing P3 biofilm compared to mono-species biofilms using the same inoculum densities. Also, the presence of a pre-existing biofilm of P3 could reduce the cell number of S1 (applied at 3 log CFU/mL) to a higher extent (83%) than when the strains were applied simultaneously (65%). Remarkably, despite the low inoculation densities of S1, P3 was in this case not able to exploit S1, in contrast to the simultaneous inoculation. Also unlike the simultaneous inoculation, the biodiversity effect (only considered for the last 22h of incubation i.e. when both strains were present) was negative in this case. This was mainly due to a negative complementarity effect, which is indicative of strong chemical or physical interference. This effect became more pronounced with decreasing amounts of *Salmonella* Java S1. The slightly positive selection effect indicates selection of the best mono-species biofilm former (i.e. S1).

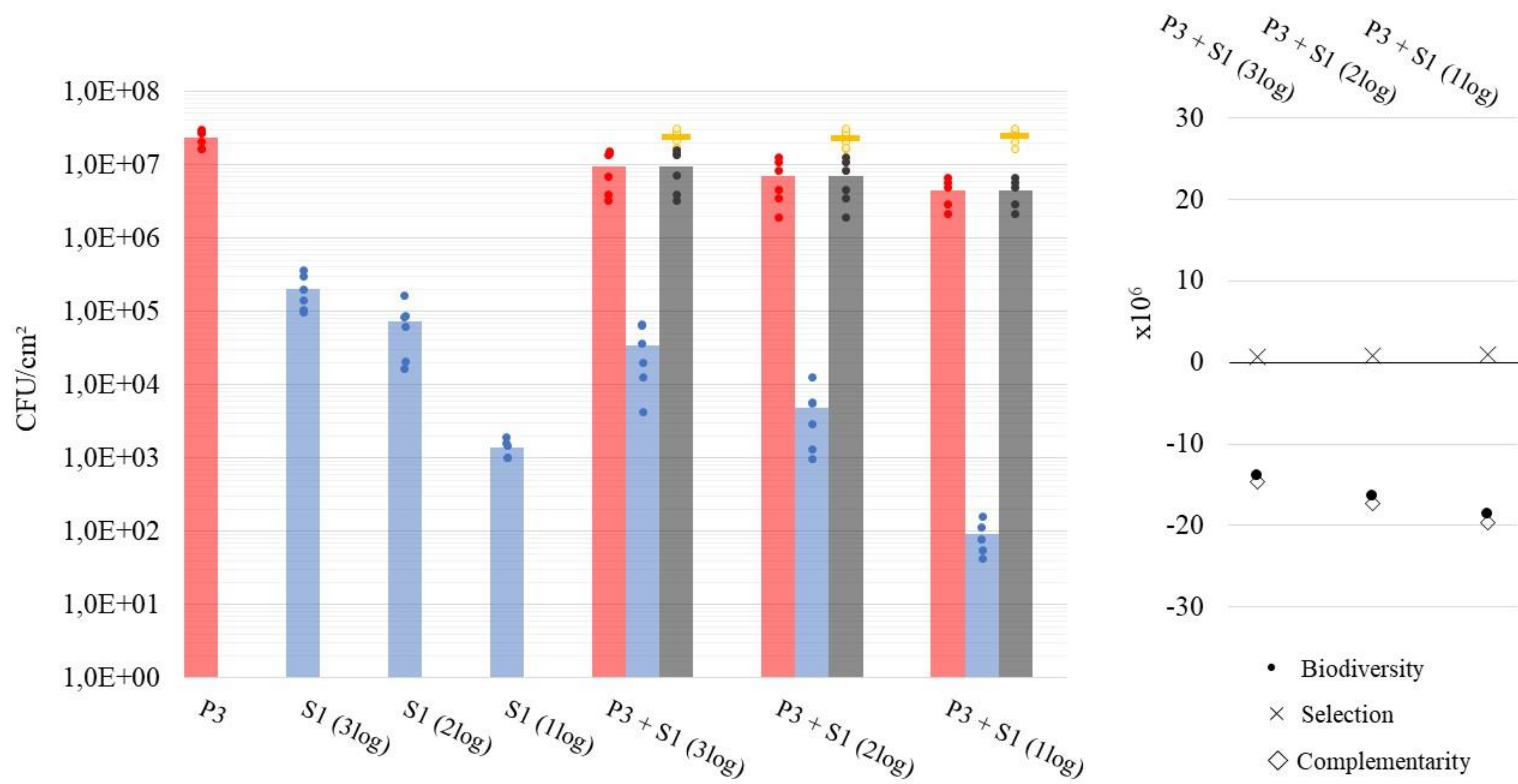

Figure 6.5: Influence of sequential application of biocontrol agent and pathogen on interaction and biocontrol effect. Cell number of each strain (P3 and S1) in each inoculum density (3, 2 and 1 log CFU/mL) in mono-species biofilms and in dual-species biofilms are indicated. The total amount of cells expected for cooperation in dual-species biofilms is indicated with yellow dots. Six biological replicates (dots) and their average (bars) are shown per strain and in total (grey) for the dual-species biofilms after 44h of incubation. Also biodiversity, selection and complementarity effect of three dual-species biofilm experiments are shown but were calculated only for the last 22h of incubation i.e. when both strains were present.

6.4 DISCUSSION

Over recent years, biocontrol as an alternative for chemical disinfection has gained strong interest. Studies have already been performed in primary animal and plant production, food industry and even in hospitals (Zhao et al., 2006; Vandini et al., 2014; Luyckx et al., 2016b; Bosmans et al., 2017; Hossain et al., 2017; Zheng et al., 2018). However, the possibility to use biocontrol agents in the fight against persistent pathogenic strains in the broiler environment has only been studied briefly (Alves *et al.*, 2015). In this research, a realistic *in vitro* model for biofilm formation on the inside of the DWS was developed and validated to study interactions between *Salmonella* Java and *Pseudomonas putida* and to consequently evaluate the potential of *Pseudomonas putida* as BCA in this niche.

Very few literature data were found concerning biofilm-forming capacity of *Salmonella* Java. Agarwal *et al.* (2011) screened a multitude of *Salmonella* serotypes, among which one *Salmonella* Java strain, for biofilm formation in 96-well MTPs: the *Salmonella* Java strain was evaluated as a weak biofilm former based on OD measurements. In the current study, where biofilm formation was evaluated under more realistic conditions, the used *Salmonella* Java field strain was evaluated as the best biofilm former based on bacterial counts compared to the other strains included (among which another *Salmonella* serotype i.e. *Salmonella* Mbandaka). The difference in biofilm-forming capacity between the three *Pseudomonas putida* strains confirmed the previous observations concerning biofilm formation by these strains in 96-well MTPs (chapter 5). Differences in biofilm-forming capacity between strains of the same species were previously described and can be due to mutations in biofilm regulating genes (Arevalo-Ferro et al., 2005; Chia et al., 2009; Agarwal et al., 2011; Lianou and Koutsoumanis, 2013; López-Sánchez et al., 2016). Even at low inoculum densities, which are more realistic for the investigated niche, *Salmonella* Java was capable to form a significant amount of biofilm. It was already demonstrated for *Listeria monocytogenes* that persistent strains showed increased biofilm formation relative to non-persistent strains (Borucki *et al.*, 2003). The strong biofilm forming capacity of the *Salmonella* Java strain in this study could therefore be an explanation for the persistent character of this *Salmonella* variant in broiler houses.

Besides the difference in biofilm-forming capacity, the three *Pseudomonas putida* strains also showed different inhibitory effect on *Salmonella* Java. All the tested *Pseudomonas putida* strains provided a reduction of the pathogen when applied in equal numbers. The

reduction of *Salmonella* Java was the result of competitive interactions with the *Pseudomonas putida* strains. Strain P2 was best capable to inhibit *Salmonella* Java.

Despite the fact that *Salmonella* Java biofilm formation was reduced in the presence of *Pseudomonas putida*, *Salmonella* was observed as the dominant species as counts for *Salmonella* spp. were always higher than counts for *Pseudomonas* spp. when applied in equal inoculum densities. The dominance of *Salmonella* Typhimurium relative to *Pseudomonas aeruginosa* in dual-species biofilms was also described by Pang *et al.* (2017), but in the same study *Salmonella* Enteritidis was equally distributed to *Pseudomonas*. In contrast, dual-species biofilms containing *Salmonella* Enteritidis and *Pseudomonas putida* revealed the latter as the dominant species (Chorianopoulos et al., 2008). Also, in contrast to our study, coexistence between *Pseudomonas* and *Salmonella* enhanced biofilm formation by *Salmonella* in the study of Habimana *et al.* (2010). The above mentioned studies and ours indicate that behaviour of *Salmonella* in dual-species biofilm with *Pseudomonas* is strongly dependent on respectively serotype and strain. However, differences in biofilm growth conditions (flow, incubation time, incubation temperature, surface type, etc.) could also lead to different interactions between the strains (Dai et al., 2017).

When *Pseudomonas putida* and *Salmonella* Java were brought together at the same time, no complete niche overlap was observed (which is indicated by a positive complementarity effect), indicating an attenuation of the competitive interactions. However, when *Pseudomonas putida* is first allowed to form biofilm on the surface of drinking water lines, the spatial niche is already occupied, leading to increased competition when *Salmonella* Java is added (negative complementarity effect). It is also possible that in this situation, the competing species directly fight each other through antagonistic factors such as the production of antimicrobials, enzymes that prevent cell communication by quorum sensing or contact dependent inhibition (Stubbendieck and Straight, 2016; Parijs and Steenackers, 2018). The increased competition when *Pseudomonas putida* was applied prior to *Salmonella* Java could also be observed in the stronger decreased cell number of *Salmonella* (%) compared to the decrease when applied at the same time.

It is more and more recognised that competitive interactions dominate between genotypically different microbial species (Mitri and Foster, 2013; Ghoul and Mitri, 2016; Parijs and Steenackers, 2018) and this was also confirmed in this study. More specifically, interactions between *Pseudomonas aeruginosa* and *Salmonella* (Enteritidis and Typhimurium), between *Pseudomonas fluorescens* and *Salmonella* Typhimurium and between *Pseudomonas putida* and *Salmonella enterica* were also identified as competitive (Leriche and Carpentier, 1995; Chorianopoulos et al., 2008; Pang et al., 2017; Pang and Yuk, 2018). In this study, all evaluated *Pseudomonas putida* strains were able to reduce the attachment and biofilm formation by the pathogenic strain *Salmonella* Java. This supports the potential of *Pseudomonas putida* as a BCA against *Salmonella* Java in the DWS of broiler houses. The ability of *Pseudomonas* strains to inhibit the growth of pathogenic bacteria (among which *Salmonella*) was previously reported and could be a consequence of siderophore production for iron captation and production of the toxic pigment pyocyanin (Oblinger and Kraft, 1970; Gram, 1993; Cheng et al., 1995; Das and Das, 2015). To profit from its biocontrol potential, it could be interesting to preserve *Pseudomonas putida* biofilms on the inside of the DWS in broiler houses and not to remove them by chemical disinfection.

However, despite the fact that *Salmonella* Java was reduced, this was probably not to such an extent that would be sufficient to prevent the animals from being infected. Also, biofilms on the inside of the DWS in broiler houses are composed by a very diverse range of microorganisms (as described in chapter 5). Social interactions will take place between all the present strains to finally reach an equilibrium. Therefore, it is not known whether the biocontrol characteristics of *Pseudomonas putida* will achieve the same result in practice as in the current model using only two strains. Possibly, other *Pseudomonas putida* strains could be able to reduce *Salmonella* Java even more, since strain variety was already observed in this study. Therefore, it would be interesting to evaluate the biocontrol potential of other *Pseudomonas putida* strains and also the combination with other *Salmonella*-reducing species.

Despite the fact that co-culturing *Salmonella* and *Pseudomonas* can lead to less biofilm formation by *Salmonella*, different studies reported an increased *Salmonella* resistance to disinfectants in dual-species biofilms (Leriche and Carpentier, 1995; Pang and Yuk, 2018). Parijs and Steenackers (2018) reported this increased tolerance as a consequence of

reduced competitive interactions between the strains in the biofilm. Also, the negative downside of biofilms present on the inside of the DWS in broiler houses (independent on the strain composition) remains among others in the form of clogging of the pipes and capture of medicine particles, leading to under dosing of the animals and increasing the risk for animal health and the development of resistant strains (Roberts et al., 2008; Høiby et al., 2010a).

6.5 CONCLUSIONS

In conclusion, the potential of *Pseudomonas putida* as a biocontrol agent against *Salmonella* Java was indicated in the present study, although strain differences were observed. These findings were derived from competitive interaction, observed between both genera in a newly developed and validated *in vitro* model that simulates biofilm formation on the inside of the DWS in broiler houses under realistic conditions. In this biofilm model, differences in biofilm formation between different strains and different inoculum densities could be observed. It was also demonstrated that *Salmonella* Java is a strong biofilm former, which could be a possible explanation for its persistent nature. In the future, it would be interesting to observe interactions between multiple strains, representing the community in the DWS niche. The biocontrol potential of *Pseudomonas putida* could be investigated for other *Salmonella* strains and even for other zoonotic pathogens frequently occurring in the broiler industry such as *Campylobacter* spp. On the other hand, currently applied disinfection products could be applied in the model to investigate the influence on interactions and on the equilibrium between strains in the DWS in broiler houses.

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CHAPTER 7: GENERAL DISCUSSION

7 GENERAL DISCUSSION

In this study, research was conducted in food industry and in primary animal production concerning surface contamination after (cleaning and) disinfection. In food industry, the study consisted of an evaluation of surface sampling methods, the monitoring and analysis of surface contamination after C&D in several food companies and also the identification and evaluation of the spoilage potential of the dominant residual bacteria. In primary animal production, monitoring and analysis of contamination after disinfection of inside surfaces of the DWS in broiler houses was performed together with identification and evaluation of *in vitro* biofilm-forming capacity of the dominant residual bacteria. Finally, an *in vitro* model for biofilm formation on the inside of the DWS in broiler houses was developed and validated to study the interaction between *Salmonella* Java and *Pseudomonas putida* and to evaluate the potential of *Pseudomonas putida* as a biocontrol agent (BCA). In this way, more insights in the presence and characteristics of residual bacterial contamination and biofilm and their importance in the food industry and in primary animal production (i.e. the DWS in broiler houses) were obtained.

7.1 FOOD INDUSTRY: SURFACE CONTAMINATION AFTER C&D

In food industry, C&D is regularly performed to reduce the contamination level to acceptable levels. The main goal is to eliminate soil and remove pathogenic and spoilage organisms, to prevent them from contaminating the produced goods and eventually avoid early food spoilage and foodborne illness. Most research focusses on the detection of pathogens in the food processing environment since these microorganisms have a direct impact on human health. However, the majority of the microorganisms present on food contact surfaces is non-pathogenic (Møretrø and Langsrud, 2017). There is not much information available for food companies about where surface contamination after C&D is still present, what the microbial and chemical composition of this contamination is and what impact it has on the produced food products and human health. Therefore, the focus in the first part of this thesis was to investigate residual bacterial contamination on surfaces after C&D in different food sectors.

7.1.1 Monitoring and sampling methods

In the food industry, there is a need for quick methods to evaluate hygiene and C&D procedures. Online monitoring would be the ideal method to accurately follow up the

deposition and removal of surface contamination. However, it is not possible to apply online monitoring on open surfaces which are abundantly present in the food companies included in this study. The detection of chemical compounds on surfaces by means of commercial kits can be a measure of C&D efficiency, it can be an indication of a possible nutrient source for microorganisms in the environment or it can indicate the presence of biofilm since most chemical components can be part of the biofilm's EPS matrix. Unfortunately, no definite answer can be given about the source of this chemical contamination. On the other hand, detection of microorganisms is often used for hygiene evaluation using ACPs or swabs followed by microbial enumerations. For the detection of biofilms, these two aspects (chemical compounds and microorganisms) have to be monitored in parallel.

In this study two monitoring/sampling methods (called scraper/flocked swab method and sponge stick method) were evaluated for their suitability to detect and quantify microorganisms and chemical compounds (proteins, carbohydrates and uronic acids) on food contact surfaces after C&D. Both sampling methods were equally sensitive for the capture and cultivation of microorganisms but only the scraper/flocked swab method allowed for the analysis of chemical compounds. Consequently, the scraper/flocked swab method was the best suitable method for monitoring/sampling of biofilms as it is able to determine its two main parts. Concerning its application in practice (e.g. to include the method in routine control of hygiene in food companies) there are still some shortcomings. The scraper/flocked swab method might be too labour intensive as it includes several steps. The sponge stick method is a good alternative that is less labour intensive but only provides information about the microbiological load on surfaces due to its chemical interference. However, the detection of residual bacterial contamination after C&D can already provide interesting information for food companies as an indication of biofilm or tough contamination possibly posing a risk to contaminate the food products. The scraper/flocked swab method also requires further processing in a laboratory in terms of enumerations and spectrophotometric quantification of chemical compounds, for which not all food companies have the necessary devices. This sampling method only allows for the investigation of a restricted number of locations but this is actually a major drawback of almost all detection methods for surface contamination. Finally, the method might take too much time before food companies get the results and can take measures to act against it. However, results will indicate if locations should be checked more or less

frequently and can provide interesting information for the food companies about the type of contamination. Also, this sampling method was satisfying in this research as it made it possible to achieve the research goals concerning biofilms and residual surface contamination.

7.1.2 Occurrence and analysis of surface contamination after C&D in different types of food companies

Using the scraper/flocked swab method, surface contamination after C&D in different types of food companies and food sectors was sampled and investigated with regard to microbiological and chemical composition. Chemical compounds were detected on relatively few locations after C&D and in addition, it concerned low quantities (micro to milligrams per $100cm^2$). Due to regular C&D, it is possible that biofilms do not get the chance to go into the maturation phase where EPS production is increased. This could explain the low levels of chemical compounds on the surfaces after C&D. Even less locations were evaluated as carrying biofilm when microbiological results were also taken into account. It may be questioned if it actually concerns biofilms since chemical compounds were present in such low quantities. However, the fact that locations posing a risk for the contamination of the produced foods are indicated is for food companies more important than putting a name on the type of contamination they are dealing with. This also indicates that a definition for biofilm in a food processing environment is more difficult to determine than a theoretical definition or a definition applicable for model biofilms. Also in clinical and natural environments, where there is less disruption of biofilm formation, a definition for biofilm is more clear. In industrial environments such as food companies, there is a lot of intervention, e.g. C&D, whereby the chance that a mature biofilm develops is small. Exceptions are locations that are not reached efficiently by C&D. On these locations a more mature biofilm with higher microbiological and chemical quantities can be expected.

Since the presence of microorganisms after C&D is the highest concern for food companies (whether or not accompanied by chemical compounds), the focus during the further research in food companies was on the characterisation of the microorganisms present. On average, 43% of the sampled food contact surfaces provided 2 log CFU/$100cm^2$ or more after C&D. However this percentage ranged from 0 to 64% between the different companies. The average bacterial load on contaminated surfaces was 3.62 ± 1.20 log

CFU/100cm^2 but also reached up to 7.23 log CFU/100cm^2. This indicates that C&D is more effective in some companies than in others. These results already provided interesting information for the food companies to improve their C&D procedures. However, corrective actions were not followed up to assess their impact on hygiene and residual surface contamination.

Identification of the dominant flora from surfaces that were still contaminated with 2 log CFU/100cm^2 or more after C&D revealed that *Pseudomonas* and to a lesser extent *Microbacterium* and *Stenotrophomonas* were the most identified genera over all the food sectors. This confirmed the findings in previous studies (Carpentier and Chassaing, 2004; Møretrø and Langsrud, 2017). However, 50 different genera were identified over all the food companies whereof 60% were company specific. This indicates that most companies have to deal with a specific 'house' flora. The results obtained in this study are presumably still an underestimation of the actual surface contamination in terms of percentage of contaminated points, bacterial load and identification diversity since damaged cells could be present (possibly as a result of C&D and sampling) together with viable but non culturable (VBNC) cells (Cappitelli *et al.*, 2014). An alternative method for the quantification of the bacterial load after C&D could be flow cytometry. Advantages of this technique are a shorter analysis time (minutes to hours compared to days for culture-based techniques) and the possibility to quantify viable, dead and VBNC bacterial populations (Berney et al., 2007; Khan et al., 2010). This distinction can be made by the use of multiple dyes with different membrane permeability and target affinity. However, cell numbers of approximately 10^4/mL are required for good counting results (Khan *et al.*, 2010), which is not the case in a significant proportion of the surface samples taken in the food industry after C&D. With regard to identification, the use of culture-based methods as a start for isolate selection and identification could bias the results since only culturable and morphologically different bacteria are identified. It would be interesting to also identify the VBNC cells since they could as well have an impact on the food products after resuscitation in terms of spoilage and foodborne transmission of diseases. Using metagenomics, the total bacterial microbiota in surface samples could be identified and relative abundances can be determined. However with this method, also dead bacterial cells would be identified which are of less interest to food companies since they cannot colonise other surfaces and they will not pose a risk for food spoilage since they cannot grow to sufficient numbers on the food products. This shortcoming can be addressed by

the use of fluorescent intercalating dyes (for example ethidium monoazide or propidium monoazide) that covalently bind to nucleic acids in solution or in cells with damaged membranes, followed by photoactivation (Tantikachornkiat et al., 2016). This treatment can be performed prior to 16S PCR to limit identification to intact, living bacterial cells.

The majority of the evaluated isolates (88.5%) had some kind of spoilage potential, with the ability to break down lipids as the most prevalent property. All *Microbacterium* and *Stenotrophomonas* strains, which are among the most identified genera in the study, showed some kind of spoilage potential. We speak of a potential to cause spoilage because it is not sure if these properties will come to expression in the food products. First , there is no proof that the microorganisms present on the surfaces will actually detach during production and contaminate the produced food products. Second, the growth and survival potential of the evaluated isolates on relevant food products was not evaluated. Due to the broad range of produced food products in the different food companies concerning pH, aw, chemical composition, conservation temperature, conservation atmosphere, etc., it was not possible to also test spoilage caused by the field isolates on relevant food products under realistic storage conditions. In addition, very little information was obtained from the included food companies concerning the identity of actual spoilage organisms found in the past on spoiled end products. Usually, an assessment of bacterial growth on early spoiled products is done by determination of TAC or a specific parameter (e.g. LAB on oven food products), but mostly no identification of the spoilage organisms is performed. In some rare cases where identification in end products was performed, a few links could be made with bacterial surface identifications. Examples are *Microbacterium* spp. identified on a surface in a dairy company and in the same period identified in an end product for which there was a consumer's complaint, *Paenibacillus* spp. in a sensory abnormal end product and also present on several surfaces in the egg processing company and high counts for LAB on oven food products manufactured on a production line which was shown to be contaminated with high numbers of *Lactococcus lactis* subsp. *cremoris*. More knowledge about the identity of spoilage organisms in products at the end of the shelf life would be interesting to link to the identified microorganisms on surfaces in the production environment and to consequently assess the importance of the residual bacterial contamination after C&D on food quality.

The previous sectors contained general findings from results obtained in several food companies and sectors. In the next sections, results will be more discussed by food sector. Sampling in the company producing baker's yeast did not reveal any problematic locations after C&D concerning bacterial load and chemical compounds. Moreover, no bacterial isolates could be collected and identified. Consequently, no information about the dominant microbiota in this type of company could be obtained. It is presumed that the production environment was dominated by the presence of yeast cells, competing with bacterial cells.

In the dairy industry, biofilm formation is a well-recognised problem as it can lead to poor hygiene and equipment malfunctioning (Simões et al., 2010; Marchand et al., 2012). In this study however, few locations with high bacterial load or biofilm were found. It is possible that, by sampling of a select number of locations on the production line, higher contaminated locations were missed. Possibly, areas with the highest bacterial load or the most biofilm formation are located at difficult to reach places or dead ends in the closed production system whereby C&D cannot reach the contamination enough and sampling is also not possible. Mostly Gram-positive bacteria belonging to the genera *Microbacterium* and *Streptococcus* were identified in the dairy companies. The ability to survive pasteurisation and/or to form biofilm are most likely an explanation for their presence on surfaces that undergo cleaning at high temperatures (Marchand *et al.*, 2012). The importance of *Bacillus* spp. in surface contamination and spoilage of dairy products was also indicated in this study by its presence in low to medium numbers and strong spoilage potential. Pseudomonads, which are considered to be the main cause of spoilage of ultra-heat treated dairy products (Marchand et al., 2009b; Simões et al., 2010), were barely identified in the included dairy companies. It is possible that, despite the fact that *Pseudomonas* spp. were not frequently identified on the sampled surfaces in the dairy companies, *Pseudomonas* strains were present in the raw milk product and produced heat-stable enzymes that could lead to spoilage in the heat-treated products. Independent of their identification, most of the isolates produced lipases which could lead to rancidity of the product if the strains or their enzymes end up in the product after heat treatment.

The majority of the sampled locations in the sauce producing company showed a high bacterial load and quantities of carbohydrates were higher compared to other companies. It is not clear if these carbohydrates originate from the biofilm's matrix or from product

remains since most produced sauces contain significant amounts of carbohydrates. The dominant flora found on surfaces in this company were mostly identified as Gram-negative *Pseudomonas* spp. and all had spoilage potential. Despite this spoilage potential, actual spoilage of end products does not occur frequently due to the product's intrinsic and extrinsic factors such as pH and addition of preservatives. Microorganisms that do have a potential to grow are yeasts, moulds and LAB (Vermeulen, 2008). However, these groups of organisms were not selectively enumerated and subsequently identified in this study. The search for yeasts and moulds on processing surfaces in sauce companies could be interesting in future research since they are expected to have the most impact on product spoilage and safety.

Locations where C&D procedures clearly failed in the egg processing company were indicated as conveyor belts and small (portable) equipment. The application of mechanical action during cleaning would possibly improve the efficiency of C&D on these locations. Gram-positive Staphylococci were abundantly present on processing surfaces, which presumably originate from the eggshell (De Reu *et al.*, 2008). However, all the identified microorganisms on surfaces (except for *Bacillus* spp., *Paenibacillus* spp. and *Streptococcus* spp. collected from a more specific growth medium) are generally sensitive to pasteurisation, i.e. they should be inactivated by this processing step. This would mean that only heat-tolerant bacteria together with microorganisms present on surfaces that come into contact with the already pasteurised product are of importance since they can survive in or post-contaminate the product and lead to spoilage. Of the bacteria that are eligible for these criteria, only *Acinetobacter* spp. isolates in this study show spoilage potential. Bacterial spoilage of the egg white would manifest itself through liquefaction and spoilage of whole egg or egg yolk would be visible as coagulation and discolouration (Techer et al., 2013).

In the meat industry, 39% of the sampled surfaces (mostly conveyor belts) were contaminated with 2 log CFU/100cm^2 or more after C&D. *Pseudomonas* and *Stenotrophomonas* were the dominant genera whereof most isolates showed spoilage potential. Especially *Pseudomonas* spp. are well recognized in the refrigerated food processing environments and as spoiler on food stored at low temperature such as meat (Chmielewski and Frank, 2003; Møretrø and Langsrud, 2017), but the presence of *Stenotrophomonas* spp. was not yet described. The role as spoilage organisms of

Brochotrix thermospacta on meat products was confirmed in this study. Spoilage of refrigerated meat products is generally expressed as off-odours, off-flavours and sometimes change of colour, slime and gas production (Borch *et al.*, 1996).

Many locations in the sampled oven food company were still contaminated with significant numbers of bacteria after C&D whereof most isolates were identified as *Pseudomonas* spp. or *Microbacterium* spp. LAB were also present in high numbers on several surfaces after C&D. In case these microorganisms contaminate the produced food products, they could lead to sensory abnormalities (acidification) which will not be neutralised despite proper heating before consumption. This research provided as good as the first results concerning identification and characterisation of residual bacterial contamination on food contact surfaces in the oven food industry producing ready-to-heat products.

7.2 PRIMARY ANIMAL PRODUCTION: BIOFILMS IN THE DWS OF BROILER HOUSES

In primary animal production such as broiler farms, C&D is performed as part of the internal biosecurity. This is done to reduce or even prevent infectious agents to spread and cause animal and human disease. Sanitation or disinfection of the DWS in broiler houses is not performed by default but at some farms it is carried out yearly or during vacancy. However, sampling of the inside of the DWS is not part of the regular and mandatory evaluation of hygiene measures at broiler farms. Therefore, hardly any information about presence, importance and composition of surface contamination or biofilm on the inside of the DWS in broiler houses is available and these topics were addressed in the second part of this thesis. On the other hand, there is an increasing problem with *Salmonella* Java contamination on broiler chickens in Belgium. There are indications that this strain is introduced at farm level (van Asselt *et al.*, 2009), however, very few information about specific locations where this pathogen persists is available. A final research question was if biofilms composed by the natural present microbiota on the inside of the DWS, could enhance or reduce the attachment and persistence of *Salmonella* Java. This hypothesis was in this thesis evaluated using a newly developed biofilm model.

7.2.1 Occurrence and analysis of biofilms in DWS of broilers

In the first part of this thesis, the best sampling method for biofilms in the food industry was evaluated and implemented. Despite its proven value, the scraper/flocked swab method could not be used to sample biofilms on the inside of the DWS in broiler houses

after the application of disinfectants since the diameter of the drinking water tubes and other points to access the inside of the DWS were too narrow for the scraper. Consequently, only the flocked swab was used to sample biofilms on the inside of the DWS in broiler houses and to determine its chemical and microbiological composition. It could be expected that using only the flocked swab, lower bacterial counts and lower amounts of chemical compounds are detected since less mechanical action is applied to loosen the surface contamination, which could lead to a lower yield. However, a higher percentage of the locations in the DWS of broilers were contaminated with chemical compounds after the application of disinfectant compared to surfaces in the food industry but it also concerned low quantities. The origin of these chemical compounds is again unsure since they can be part of the biofilm's matrix or they can emanate from organic dirt from the well.

A higher proportion of locations were indicated as carrying biofilm after sanitation compared to the food industry (63% in DWS of broiler houses versus 17% in food industry). Primary animal production is in general a more dirty environment compared to food production. Another explanation could be that sanitation of the DWS is only performed after each production round (approximately every six weeks) or yearly which could lead to more contaminated points. This again indicates that locations that are not often disrupted, e.g. by C&D, have a higher chance of developing a more mature biofilm with higher microbiological and chemical quantities. This is also confirmed by the higher bacterial counts that were observed in the DWS compared to food contact surfaces. All but one of the sampled surfaces provided 2 log CFU/20cm^2 or more after sanitation with an average bacterial load of 6.03 $\pm$ 1.53 log CFU/20cm^2 (ranging up to 9.00 log CFU/20cm^2). A large part of the microbiota consisted of *Pseudomonas* spp. since enumerations were on average only 0.88 log CFU/20cm^2 lower than enumerations for TAC. The genus *Pseudomonas* (and more specifically *Pseudomonas aeruginosa*) is known to be abundantly present in natural waters (Mena and Gerba, 2009; Casanovas-Massana and Blanch, 2012). There was no difference observed in bacterial counts between broiler farms that performed sanitation of the DWS after each production round or the one that performed sanitation yearly. The high bacterial counts indicate that the currently applied disinfection products (in all the sampled farms based on hydrogen peroxide) are insufficient to reduce the contamination to low levels. The application of mechanical action (e.g. flushing) before and after disinfection or the use of agents that disrupt organic

matter and EPS could enhance the efficacy of disinfection since already a part of the residual dirt and biofilm would be removed and microorganisms would be better affected. However, there is no regulation concerning disinfection of the DWS and the acceptable level of microorganisms that has to be obtained. Due to this lack of legislation, lack of time and the need for flushing devices and appropriate chemicals, an equally thorough C&D of the DWS as in the food industry is not yet performed by all broiler farmers. Up to now, it was not known which impact this community of microorganisms on the inside of the DWS in broiler houses had on the animals, the performance and on the presence of pathogens. Therefore again, the focus was on the further characterisation of the present microorganism in DWS in broiler houses after the application of disinfectants.

The bacterial load of the well water was not directly correlated to enumerations of TAC on the inside of the DWS on the same farm. Also, there was no agreement between the dominant species identified in the well water and the inside surfaces of the DWS. *Pseudomonas* spp. were identified among the dominant flora in all the investigated water samples but isolates were unique on species-level per farm. However, this part of the study was limited and more sampling should be done do draw general conclusions.

Identification of the dominant flora indicated *Pseudomonas* as the most present genus on inside surfaces of the DWS, followed by the genus *Stenotrophomonas*. The most dominant species were identified as *Stenotrophomonas maltophilia*, *Pseudomonas geniculata* (to be considered as *Stenotrophomonas maltophilia* according to Anzai *et al.* (2000)) and *Pseudomonas aeruginosa*. Twenty-six different genera were identified on all the broiler farms, whereof 62% were farm specific. This indicates that also broiler farms deal with a specific 'house' or farm flora. Infection of broiler chickens with *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* was already demonstrated in the past (Devriese et al., 1975; Nathiya et al., 2012; Shukla and Mishra, 2015), however the role of the DWS in these infections was not discussed. Infection with *Pseudomonas aeruginosa* can pose a threat for animal health by causing inflammation of the eye and respiratory disorder leading to a high mortality rate (Kebede, 2010; Shukla and Mishra, 2015) and for human health since it is an opportunistic human pathogen with often observed resistance against antibiotics and disinfectants (Stover et al., 2000; Aloush et al., 2006). *Stenotrophomonas maltophilia* is not known as a broiler pathogen, but it has emerged as an opportunistic human pathogen with high intrinsic and acquired resistance levels causing respiratory tract

infections in immunocompromised patients (Looney et al., 2009; Brooke, 2012). The simultaneous presence of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* on broiler meat could pose an even bigger threat for pneumonia or cystic fibrosis patients because co-infection with these two microorganisms implies a significantly higher mortality rate than infection with only one or none of these organisms. This synergistic effect may be due to increased biofilm formation, among others leading to higher resistance to antibiotics making the general treatment less effective, and increased virulence of *Pseudomonas aeruginosa* in co-culture (Pompilio *et al.*, 2015; Yin *et al.*, 2017).

Of all the tested isolates originating from the inside of the DWS in broiler houses, only 8% did not show any biofilm formation in 96-well MTPs. Moreover, the majority of the screened isolates (61%) were evaluated as strong biofilm formers. Of the most identified genera 83% of the *Pseudomonas* isolates and 87% of the *Stenotrophomonas* isolates were strong biofilm formers. The fact that these genera easily form biofilm can be an explanation for their abundant presence and identification in the investigated niche. Similar to previous studies (O'Toole et al., 2000; Chia et al., 2009; Agarwal et al., 2011; Silva et al., 2011), differences in biofilm-forming capacity on species and even strain level were observed.

7.2.2 Potential of *Pseudomonas putida* as BCA against *Salmonella* Java evaluated in a model system to simulate biofilm formation on the inside of the DWS of broilers

The presence of biofilm-forming bacteria in the DWS could be a potential risk for the protection of pathogens such as *Salmonella*. On the other hand, commensal bacteria can also prevent other microbes to attach and form biofilm. This principle, whereby living organisms control the presence of other living microbes, is called biocontrol. The potential of *Pseudomonas putida* (a commensal species that was found to be part of the natural microbiota on the inside of the DWS in this study and previously indicated as BCA in other niches) as BCA against *Salmonella* Java (an emerging pathogen in the broiler production chain), was not yet evaluated in a realistic set-up. Therefore, a model system to simulate biofilm formation on the inside of the DWS in broiler houses was developed and validated. Since conditions were chosen that were representative for the situation in practice, obtained results were very specific for the evaluated situation and cannot be extended to other niches. When the correct parameters are adjusted, the model can be

used to solve research questions in other sectors or niches. The developed model concerned a closed system, while an open system would represent practical conditions even better. However, open or continuous systems are mostly more labour-intensive and more complex providing less throughput data.

Several *Pseudomonas putida* and *Salmonella* field strains could be ranked according to their biofilm-forming capacity in this new model. *Salmonella* Java was evaluated as the best biofilm former which could be an explanation for its persistent character in broiler houses. On the other hand, the different *Pseudomonas putida* strains could also be characterized by differences in biofilm-forming capacity. However, results were not completely the same in the 96-well MTP set-up versus the 6-well MTP set-up. These differences can probably be explained by the difference in quantification method. In 96-well MTPs, biofilm formation was quantified based on total biomass while in the 6-well MTPs, biofilms were quantified based on bacterial counts. Also the difference in growth conditions between the models could influence the amount of biofilm formed.

The presence of *Pseudomonas putida* could reduce biofilm formation by *Salmonella* Java in the *in vitro* model due to competitive interactions. These results suggest that *Pseudomonas putida* could serve as a BCA against *Salmonella* Java in the DWS in broiler houses. However, different observations could be made in practice since there is a more complex community at this niche which will influence the interaction between biocontrol strain and pathogen. As said before, sanitation or disinfection of the DWS in broiler houses is not performed by default. The results obtained in the biofilm model suggest that this is not necesarrily wrong, since the naturally present microbiota can possibly prevent the persistence of pathogens. Of course, more strain combinations should be evaluated for their social interactions to make sure other strains do not upregulate biofilm formation by possible pathogens.

7.3 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

This study provided a suitable sampling method that allowed chemical and microbiological characterisation of biofilms or residual contamination on food contact surfaces. Using this method, the current hygienic conditions after C&D in several food companies from different food sectors could be monitored and investigated into detail. However, further research concerning a highly sensitive and quick monitoring method that detects and preferably quantifies microorganisms and a suitable chemical compound

should be undertaken. Preferably, a previous selection of potential sampling devices or methods could be first evaluated in more controlled conditions, e.g. on model biofilms. The value of quantification of EPS components stays questionable since chemical components were found on few locations in low quantities and moreover, the origin of these components cannot be determined. Therefore, further research to identify chemical compounds that are more unique for the biofilm's EPS matrix should be performed to differentiate more easily with residual organic soil.

Our main interest went out to the occurrence and characterisation of the present microorganisms after C&D since these could pose a direct risk concerning food spoilage and foodborne illnesses. Mainly *Pseudomonas* spp. *Microbacterium* spp. and *Stenotrophomonas* spp. were identified, which confirmed earlier findings about identification of bacterial surface contamination in the food industry. However, the other identified genera were mostly considered as 'house' flora, providing interesting information for the corresponding company and possibly other companies producing similar food products but less interesting for generalisation. To get a full image of the microbiota present on food contact surfaces after C&D, a culture-independent identification method, such as metagenomics, could be used in the future. It would also be interesting to look for pathogenic bacteria and to consequently investigate the interaction of these pathogens with the accompanying community on these niches. This could be done in the established biofilm model that was used to simulate biofilm formation in the DWS of broiler houses, taking into account that the surface material, temperature, nutrient conditions, shaking speed and the used strains have to be adjusted. In this way, it could be better understood how pathogens can persist in specific niches in food companies due to their own or other strain's biofilm-forming capacities. A lot of the residual microorganisms present on food contact surfaces after C&D had spoilage potential in the lab. However, this should also be investigated on food products to assess the actual impact of these microorganisms.

In the DWS of primary animal production sites (broiler houses), more and higher contaminated surfaces were encountered than in the food industry. This was the first study to provide detailed information about the presence and composition of contamination on the inside of the DWS in broiler houses. This information can be the basis for further research about biofilms and residual contamination on this specific niche

and to investigate the impact on animal and human health more into depth. To further expand this basic knowledge, sampling of inside surfaces of the DWS could be implemented in the standard and legal prescriptions concerning hygiene control in broiler environments. In addition to the determination of TAC, problem causing pathogens in the broiler industry such as pathogenic *E. coli*, *Enterococcus* spp., *Salmonella* spp. and *Campylobacter* spp. could be traced. The search for antibiotic resistant bacteria can also be interesting since biofilms are considered as hotspots for plasmid transfer. Also in this industry, *Pseudomonas* spp. and *Stenotrophomonas* spp. dominated. On the niche in this part of the research, more generalisations could be made concerning identifications of the present microorganisms, however still a significant part of the microbiota represented the 'house' flora. This study indicated the potential of the naturally present *Pseudomonas putida* in the DWS as biocontrol against *Salmonella* spp. These results can be the basis for further research about the possible application of BCAs as a replacement of the classical (chemical) disinfection in the DWS of broiler houses but also in the stable environment. It would be interesting to evaluate the influence of other strains that are part of the natural microbiota in the DWS (most preferably strains that are even more abundant on broiler farms such as *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*) against pathogens such as *Salmonella* spp. and *Campylobacter* spp. since species and strain variability were already experienced. Finally, the effect of the currently applied disinfection products could be evaluated in the model system to investigate the impact on *Salmonella* survival and on its interaction with potential biocontrol strains.

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CURRICULUM VITAE

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PERSONALIA

Sharon Maes was born on October 23, 1990 in Ghent. After her secondary education science-mathematics (6u) at Leiepoort campus Sint-Hendrik in Deinze, she obtained her Bachelor degree in Biomedical Laboratory Technology, specialisation Pharmaceutical and Biological Laboratory Technology at KaHo Sint-Lieven (now Odisee) cum laude in 2011. Afterwards, she started her Master education in Bio Science, specialisation Food Industry at Hogeschool Gent (now University of Ghent) where she graduated magna cum laude in 2013. A first working experience was acquired as a quality employee at Buurtslagers, a meat-processing company in Aalter. Then, she started her doctoral research in 2014 at Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Technology and Food Science Unit (T&V). During the first two years she cooperated with the Laboratory of Enzyme, Fermentation and Brewery Technology (University of Leuven), the Laboratory for Food Microbiology and Biotechnology (University of Ghent) and the Centre of Microbial and Plant Genetics (University of Leuven) in the Flanders' Food project "KILLFILM – Control of biofilm formation in the production environment to guarantee a longer shelf life". The next two years (financed by ILVO), further research was conducted concerning contamination in the food industry and in primary animal production and a biofilm model system was developed.

Sharon Maes is the author or co-author of scientific publications in national and international journals and she actively participated in national and international conferences by poster and oral presentations.

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