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Biofilm formation of bacteria isolated from a food processing environment

Diploma thesis

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submitted by

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1. Introduction

1.1. Introduction to biofilms

Since the discovery of microorganisms in the 17th century traditional research on bacteria focused on their living form as single, planktonic cells (Gest 2004, Donlan 2002). However, over the last decades the prevailing method of studying bacteria focuses on their surface-related, cooperative form - the biofilm mode of growth. As the biofilm mode of growth varies widely from the life of free-living single bacteria considerable research was and is required to understand microbial biofilms in their entirety; such an activity is essential to not let our conventional understanding of bacteria lead us to biased views of the bacterial lifestyle (Costerton et al. 1995, Parsek and Fuqua 2004).

Bacterial biofilms are mergers of single and/or mixed species bacteria, embedded in a self-produced matrix of extracellular polymeric substances (EPS). Through the close cohabitation as a community, the bacterial cells benefit from a great number of advantages, so called emerging properties making them distinct from their free-living counterparts. Bacterial biofilm formation is presumably the smallest, oldest and most successful form of habitat formation existing worldwide. Natural occurring bacterial biofilms mostly consist of mixed species which share heterogeneous environments. Single species biofilms emerge almost exclusively under laboratory conditions and are therefore the most researched (Flemming et al., 2016).

Bacterial biofilms can be found in almost every nutrient sufficient environment, especially “the solid-liquid interface between a surface and an aqueous medium (e.g., water, blood) provide an ideal environment for the attachment and growth of microorganisms” (Donlan, 2002). Consequently, in all marine and freshwater ecosystems - natural and industrial - and other related institutions (e.g. wastewater treatment plants, irrigation systems) biofilms are the favoured mode of bacterial growth; human and animal body fluids provide optimal habitats for biofilm formation (Costerton et al., 1995).

Because of their ubiquity, bacterial biofilms offer solutions as well as problems thus their impacts on life are great. Native body biofilms, e.g. human vaginal flora, protect the autochthonous flora from intruding allochthonous organisms. However, as such rich breeding grounds as the body fluids cover artificial medical surfaces like contact lenses or orthopaedic implants, they also may lead to contamination and infection (Costerton et al., 1995). Further,

dental plaque and some forms of chronically infected wounds may be initiated by bacterial biofilms (Marsh, 2004; Mendoza, Hsieh and Galiano, 2019). Biotechnology makes use of bacterial biofilms in the treatment of drinking and waste water, biodegradation and biocatalytic processes, biofuel production, etc. Further, biofilms trigger biofouling, microbiologically influenced corrosion, and the contamination of water used in production and for drinking (Flemming et al., 2016).

1.2. Structure of biofilms

A biofilm may be viewed as structured chaos consisting of cells embedded in a strong hold of self-produced, hydrated matrixes.

1.2.1. Biofilm formation process

Biofilm formation is an ongoing developmental process in which planktonic cells cluster to build microhabitats.

Watnick and Kolter (2000) compared the development and composition of biofilm with a city. The development of biofilm can be divided roughly into four stages: I) reversible attachment, II) irreversible attachment, III) maturation and IV) dispersion (Fig.1). Different stages can occur temporally and spatially redundant in one biofilm (Stoodley et al., 2002). Bacterial cells deriving from one stage are physiologically distinct from cells in other stages, as demonstrated by Sauer et al., (2002) with a bacterial isolate of *Pseudomonas aeruginosa.* The cells also showed different protein profiles.

The processes of biofilm formation have been studied primarily under laboratory conditions. Factors influencing biofilm formation are physical and chemical conditions of the surrounding environment. Hydrodynamics and the shear stress of bulk fluids influence the structure, strength and density of a biofilm. Under laboratory conditions, biofilms cultured in fast-flowing environments show filamentous forms or mats, whereas slow-flows lead to tower- and mushroom-shaped structures. Low shear forces lead to rougher biofilm surfaces and lower density (Stoodley et al., 2002). Also of great importance for the bacterial organisms and their growth patterns is nutrient availability. The amount of available nutrients correlates with the capability of EPS-matrix production and cell replication, thus is an indispensable component for biofilm formation (Costerton et al., 1995). Biofilm forming bacteria are planktonic cells from the surrounding environment or bulk fluid. Some were detached from their initial biofilm for dissemination or have been free living bacteria existing in a dormant stage due to lack of nutrients (Stoodley et al., 2002).

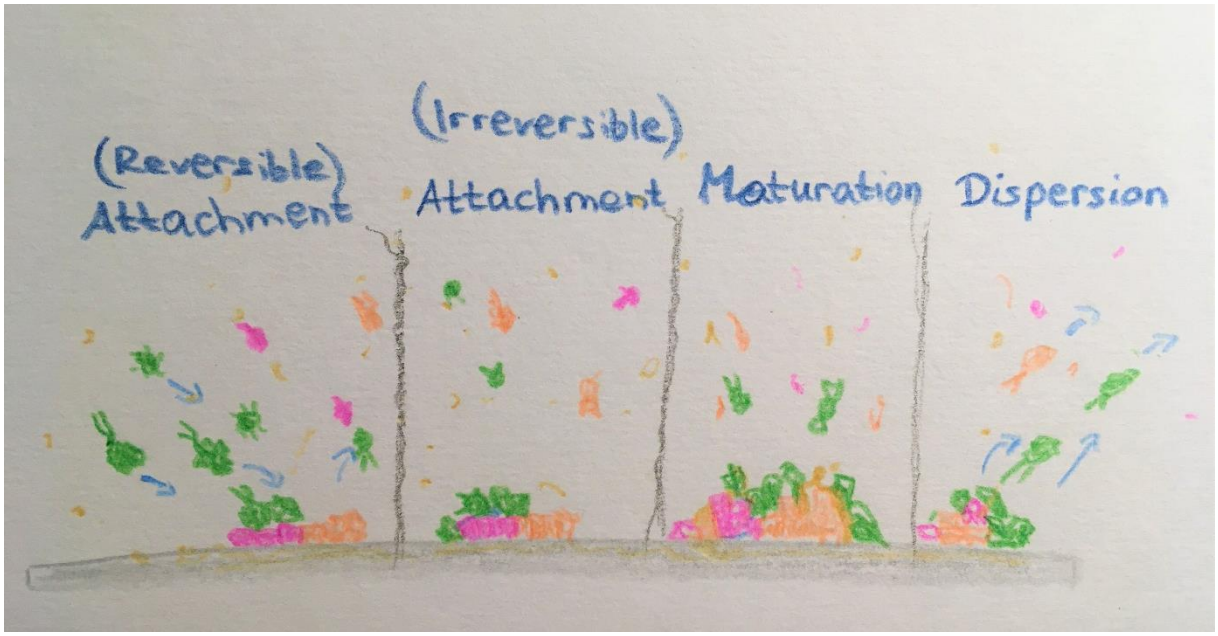

Fig. 1: Stages of biofilm development. I) reversible attachment, II) irreversible attachment, III) maturation and IV) dispersion.

1.2.1.1. Reversible attachment

Biofilm formation begins when single planktonic bacteria approach and attach to an already colonized surface. The surface provides nutrients to the cells or is itself composed of nutrients (Costerton et al., 1995). The attachment process is reversible – the cell can remove itself or change its location vis-à-vis the attached surface (Stoodley et al., 2002).

1.2.1.2. Irreversible attachment

Proximity to the attached surface leads to a change in gene expression within the bacteria. Thus, the second formation stage is initiated - irreversible attachment. Due to the alteration of their genetic profile, biofilm associated bacteria show distinct phenotypes in relation to their planktonic relatives (Costerton et al., 1995). One important phenotypical change is the stimulation of the EPS-matrix synthesis - key to biofilm formation (Costerton et al. 1995, Flemming and Wingender, 2010). The EPS-matrix is also of great importance for cell cohesion and serves as precondition for the establishment of primitive homeostasis inside the biofilm enabling the long life cycles of biofilms (Flemming and Wingender, 2010, Costerton et al., 1995).

The development of microcolonies represent another transition from reversible to irreversible biofilm attachment (Stoodley et al., 2002). Microcolonies are the basic building blocks of a physiologically cooperative bacterial community (Donlan, 2002). Depending on their physiological needs single and mixed species may benefit from a stable juxtaposition. Cooperative species with a complementary metabolism may grow together in a microenvironment that suits each bacterium (Costerton et al., 1995). To organize themselves in microcolonies some species, e.g. *Pseudomonas putida*, make use of twitching motility enabled through type IV pili (Sauer and Camper, 2001, O'Toole and Kolter, 1998).

1.2.1.3. Maturation

After the primary surface colonization and the establishment of microcolonies, the biofilm evolves into a more complex three-dimensional form. Water channels and pores emerge throughout the architecture of microcolonies. Many studies on the redistribution of bacteria indicate forms of communication between the cells, e.g. quorum sensing (see section 3.3.). Mature biofilm cells "show physiological similarity to stationary-phase bacteria" (Stoodley et al., 2002). It seems that the cell division is inhibited when a physiologically optimal size has been reached (Costerton et al., 1995).

1.2.1.4. Dispersion

Biofilms undergo constant assembly and dismantling. New bacteria attach to the biofilm while some cells or colonies disperse into the environment for dissemination (Costerton et al., 1995; Stoodley et al., 2002). In either case the process is mediated through quorum sensing, nutrient level change or physical forces. Once dispersed, the cells convert back into planktonic cells. Cells that detach due to physical force might maintain their biofilm properties (Donlan, 2002). According to several studies mentioned by Stoodley et al. (2002) differentiation must be made between active dispersal and passive sloughing mechanisms; both of which serve as forms of distribution and new habitat colonization either directly or indirectly. The studies reveal that "an increase in the concentration of an inducer molecule" is responsible for "the release of matrix polymer-degrading enzymes, [resulting] in detachment of cells from the biofilm" (Stoodley et al., 2002). Such an inducer molecule might be triggered through the lack of

nutrients; a matrix polymer-degrading enzyme might be induced by an alginate lyase (Stoodley et al., 2002, Costerton et al. 1995, Donlan 2002).

Physical forces affecting dispersion can be divided into three groups – all of which depend on the mode of cell detachment: continuous, rapid and abrasion. Continuous removal of biofilm flocks is characteristic for erosion and shearing. The amount of detached biofilm fragments increases with the biofilm thickness and the amount of fluid flowing by the interface of biofilm and bulk fluid. A rapid and massive removal of cells is called sloughing, it occurs especially in biofilms formed in nutrient-rich environments with advanced depletion of nutrients and oxygen. Abrasion occurs when larger particles from the bulk fluid collide with the biofilm (Donlan, 2002).

1.2.2. A self-produced matrix of extracellular polymeric substances

The key feature of every biofilm is its self-produced matrix of extracellular polymeric substances (EPS). The matrix is necessary for the formation, preservation and success of each biofilm; it is the cement that holds the cells together and provides the biofilm with so-called emerging properties. These additional functionalities distinguish biofilm cells from their free-living relatives i.e. planktonic cells (Flemming et al., 2016).

Depending on the environment from which the biofilm emerges, the EPS is built of different biopolymer types. Nutrient availability, bacterial species, bacterial motility, temperature, shear forces, etc. all have influence on the structural composition. Most of the EPS is produced by the organisms themselves, complemented by further compounds from the immediate environment e.g. the bulk water or the growing surface (Flemming and Wingender, 2010).

In the three-dimensional structure of the EPS-matrix, often referred to as ‘biofilm architecture’, many small microhabitats with great biodiversity are formed (Lawrence et al., 1991). The EPS matrix is the immediate habitat of the embedded cells and provides them with the profitable spatial organization and mechanical stability necessary for the survival of structured, multicellular communities (Flemming and Wingender, 2010). Furthermore the EPS-matrix passively decides upon nutrient supply and the contents of water, electric charge and hydrophobicity and cell density and further sorption processes (Flemming et al., 2007).

The biofilm and its matrix undergo constant dynamic formation and processes of degradation in order to align itself with the changeable environments e.g. variations in hydrodynamics, nutrition and/or water availability. Related thereto is the sloughing off of cells and cell dispersal leading to new colonisation (Flemming et al., 2016).

The EPS-matrix provides the biofilm with mechanical stability. However, it also shows viscoelastic properties. The matrix can respond to forces exerted by reversible and irreversible deformation until emerging in a viscous fluid. One concept behind this behaviour is, that "fluctuating binding points between EPS components are kept together by physiochemical interactions like van der Waals forces and electrostatic interactions" (Flemming and Wingender, 2010).

The EPS-matrix is comprised of ~97 % water. Therein distributed are soluble gel-forming organic components like polysaccharides, proteins and extracellular DNA (eDNA). Furthermore, it includes insoluble components like cellulose and amyloids. Therein embedded are bacterial cells of various species and morphologies. All together the EPS-matrix forms a non-rigid structure with different viscosities (Flemming et al., 2016).

Several studies focusing on the architecture of biofilm take a closer look at the unique basic structures of the EPS-matrix. Confocal scanning laser microscopy paired with fluorescent staining provided a non-destructive method for picturing the hidden structures of a biofilm (Costerton et al., 1995).

As mentioned above, biofilms are highly hydrated. Water channels and pores cross through the EPS and the cell aggregates like sewage canals. Some pores are not directly connected with the larger flow of the system but open to voids or cavities surrounded by bacterial colonies. The water maintains the same convective flow direction as the bulk fluid (Costerton et al., 1995). In this way the biofilm becomes permanently flooded with fresh oxygen and nutrient containing water. The substrate supply is influenced by hydrodynamic factors and can cause biofilm sloughing and erosion (Donlan, 2002). Water flow is kept up via convective flow mechanisms and diffusion (Costerton et al., 1995).

Besides water, exopolysaccharides, extracellular proteins and DNA are important building blocks of the EPS-matrix. All together they help the biofilm fulfil essential functions like adhesion and cohesion, aggregation of bacterial cells, retention of water, sorption of organic/non-organic compounds, nutrient source, exchange of genetic information, enzyme

functions etc. (Flemming et al., 2016). These main building parts will be discussed in the following subsections.

1.2.2.1. Exopolysaccharides

Exopolysaccharides can be analysed by electron microscopy, staining with fluorescently lectins and biochemical analyses. They are long, linear or branched molecules with a molecular mass of 0.5 x 10^6 Daltons to 2 x 10^6 Daltons, and look like miniature cobwebs attached to cells. Even within the same bacterial isolates exopolysaccharides can differ. There are homopolysaccharides and heteropolysaccharides with neutral and/or charged sugar residues, and with organic or non-organic components. Depending on their chemical structures their physical and biological qualities may differ. Within one biofilm cells that express polysaccharides exist next to cells that do not increasing the bacterial diversity. The most widely researched exopolysaccharide is alginate; alginate is a high molecule mass, unbranched heteropolymer consisting of 1,4-linked uronic residues of beta-D-mannuronate and alpha-L-guluronate (Flemming and Wingender, 2010).

Gacesa (1998) showed that alginate plays an important role in the irreversible attachment of mucoid *Pseudomonas aeruginosa* cells and the following maturation process. The alginate-related gene expression and thus its synthesis are upregulated shortly after the cells initial contact with the surface.

In mature biofilms alginate leads to an increased mechanical stability (Flemming and Wingender, 2010).

1.2.2.2. Extracellular proteins

Extracellular proteins in the EPS-matrix can be divided into two larger groups: enzymatic proteins and non-enzymatic proteins.

Enzymatic proteins fulfil various biochemical reactions, e.g. degradation of biopolymers and redox reactions or can even act as virulence factors in infectious biofilms. The enzymes are entrapped within the EPS-matrix by exopolysaccharides. This entrapment prevents the cells from early proteolysis enhancing their thermostability (Flemming and Wingender, 2010). This

cites an example for interaction between matrix components also referred to as ‘activated matrix’ (Flemming et al., 2007).

Non-enzymatic proteins act as structural proteins. Some of these proteins, called lectins, are used to visualize the biofilm structure under confocal scanning laser microscopy when getting fluorogenically stained. Lectins are carbohydrate-binding proteins associated with biofilm cell surfaces and support the mechanical stability and formation of biofilms by building a network between the cells and the EPS. Other proteins are amyloids, bacterial pili, fimbriae and flagella (Flemming and Wingender, 2010).

1.2.2.3. Extracellular DNA

The amount and origin of the extracellular DNA varies between bacterial species. The source of eDNA are lysed cells. Other quantities seem to be actively excreted. The function of eDNA appears to be of structural importance –the presence of nucleolytic enzymes has an negative impact on the microbial aggregation (Flemming and Wingender, 2010). eDNA can be arranged in grid-like and filamentous formations, proposing a structural and cell-trafficking purpose. Furthermore, the DNA remnants can be used for horizontal gene transfer (Flemming et al., 2007).

1.2.3. Cells of the biofilm

Single species biofilms mostly occur under laboratory conditions. The majority of naturally occurring biofilms include multiple bacterial species (Watnick and Kolter, 2000). These multi-species biofilms represent a coworking, synergistic bacterial community where cells physiologically complement each other (Flemming et al., 2016). The bacteria form aggregates, so called microcolonies, in sectors that fit their physiological and metabolic needs e.g. oxygen availability. Each species has their own preferential area or micro niche; some prefer the base of the biofilm, some prefer the liquid-biofilm interface (Costerton et al., 1995).

1.2.4. Heterogeneity

A great range of biodiversity is hidden in a variety of microhabitats (Flemming and Wingender, 2010). The arrangement of the microcolonies embedded within the EPS matrix is highly ordered (Flemming et al., 2016). However, due to constant physical and physiological changes, biofilms are spatially and temporally highly heterogenous constructions. Heterogeneity is found in biofilms from the beginning of formation and exists in both, single-species and multispecies biofilms. In the former case its onset is connected with phenotypical variations through fluctuating gene expression (Flemming et al., 2016).

The microbial cells live in close proximity to one and another because of their immobilization vis-à-vis the EPS matrix. The juxtaposition permits cellular cooperation between species with different metabolic/physiological processes and needs; e.g. the exchange of genetic material, metabolites and signalling molecules (Flemming et al., 2016). The biofilm is divided into areas of different chemical, electrochemical and physical gradients. Important gradients are oxygen levels, pH-values, nutrient levels and availability of electron donors and acceptors (Costerton et al. 1995, Flemming et al., 2016). Depending on their physiological activities different bacterial species accumulate in different areas. Concurrently their cellular metabolism affects the immediate environment, thus other bacterial species join in as second and third colonizers (Costerton et al., 1995).

Dissolved oxygen is a vital resource for many bacterial species. Aerobic bacteria are supplied with oxygen through water channels and diffusion. Interestingly, oxygen exhaustion through aerobic bacteria and limitations in the oxygen diffusion through the matrix enables anaerobic species to live in aerobic microenvironments (Costerton et al. 1995, Flemming et al. 2016).

Another example for the formation of heterogenous zones are acid-producing bacteria. Their metabolism leads to the reduction of the pH-value and thus leads to movements of charged molecules and ions within the biofilm. This can cause microbially influenced corrosion and increase antimicrobial resistance (Costerton et al., 1995).

This heterogenous structure is, among other things, one of the biofilms winning formulas. Its composition enables the biofilm to utilise a great many of environmental resources and thus form a diversified construction of microhabitats.

1.3. Emerging properties

Cells in biofilms benefit from the advantageous properties that only emerge within a biofilm. These properties do not occur in planktonic cells. The entrapment of bacteria of the same and/or different species in the EPS-matrix and their close juxtaposition promotes a lively cooperation in a favourable environment. The architecture and composition of the matrix protects them from external influences that might be detrimental. For example emerging properties are the ability of resource capture, enhanced tolerance to desiccation and antimicrobial agents, horizontal gene transfer and intercellular communication (Flemming et al., 2016).

1.3.1. The matrix as defence barrier

Many emerging properties are mediated by the matrix. The high water content of the matrix and its branched water storage protects the cells from desiccation when facing water stress. (Costerton et al., 1995; Flemming and Wingender, 2010). Another way to limit desiccation processes is skin formation of the uppermost layers of the EPS-matrix (Flemming et al., 2016). Furthermore, the matrix forms a mechanical and biochemical barrier protecting the cells from detrimental influences like oxidation, UV-radiation, the hosts immune system, protozoan grazers, metal ions and biocides (Flemming and Wingender, 2010).

1.3.2. The matrix as a resource capture and an external digestive system

Biofilms experience constant contact with the round washing bulk fluid. The EPS-matrix functions as a sponge to passively accumulate organic and inorganic particles like nutrients, molecules, gases, metal ions and humic substances. Furthermore, the matrix closely links the biofilm with the surface and nutrients situated therein. The sorption processes of the matrix are extended by ad- and absorption processes of the cells. These include active and passive cell transport with a wide range of binding sites. Debris of decayed and lysed cells remain in the biofilm and are recycled (Flemming et al., 2016).

The EPS-matrix functions as a large storehouse for nutrients and metabolic products and accumulates enzymes that are secreted by cells. These enzymes are captured in the matrix and become available in higher concentrations than in non-biofilm environments where they might

diffuse. “Thus, an activated matrix is generated that can be considered to be an external digestion system” (Flemming et al., 2016). The composition of enzymes changes depending on the components of the EPS-matrix with which they interact including the surrounding environment. Once the enzymes are bound with the matrix they “become a resource that is available to all members of the biofilm community, even when the community is a mixed-species consortium.” (Flemming et al., 2016).
These elaborate logistics enable a consortium of bacterial cells to fully process available nutrients giving them a competitive edge in contrast to their planktonic relatives.

1.3.3. Social cell interaction

The complexity of biofilm architecture suggests social interaction within the cellular community. Chemical signalling, known as quorum sensing, seems highly suitable for communication in biofilms. Because of the high number of cells and the dense structure of the EPS-matrix, quorum-sensing molecules, acyl-homoserine lactones, can accumulate with increased effectiveness (Flemming et al., 2016).
A study on the development of *Pseudomonas aeruginosa* biofilms by Davies et al. (1998) showed the necessity of quorum sensing for the establishment of a stable and complex biofilm architecture. Mutants lacking the quorum sensing inducing gene *lasI* were not able to build as thick, differentiated and complex cross-linked EPS-matrix biofilms as their wildtype counterparts (Davies et al., 1998).
Another factor enabled by quorum sensing is a “cell density-dependent gene regulation” (Stoodley et al., 2002). This enables the suppression of cell division in microcolonies once a sufficient size is reached (Costerton et al., 1995).
The activation of quorum sensing is affected by the external flow of the bulk fluid. Variations in quorum-sensing activity in different areas throughout biofilms promote the heterogeneity of the microbial community by initiating spatially and temporally differing phenotypic cell responses (Flemming et al., 2016). Additionally, signal molecules attract new inhabitants to the surface and initiate cell dispersal or cell death (Stoodley et al. 2002, Donlan 2002, Watnick and Kolter, 2000).
Another mode of cell communication is mediated over ‘nano wires’ (Flemming et al., 2016).

Electrical signals are transmitted through the biofilm using potassium ion channels (Prindle et al., 2015).

The close cohabitation of a diverse range of organisms, ensured by the covering of EPS-molecules, enables a lively communication, cooperation and exchange between the cells (Flemming et al., 2016). However, as in every social structure cooperation comes with competition. Rendueles and Ghigo (2015) reviewed the possibilities of competition like the use of bacteriocins, membrane vesicles and type VI secretion systems.

Despite all competitive mechanisms run by bacteria, Flemming et al. (2016) take the view that "for most biofilms, the majority of social interactions may indeed be cooperation".

An important form of cooperation is horizontal gene transfer enabled by the numerous cell-to-cell-contacts and the high density of genetic molecules. Examples of the gene transfer mechanism in biofilms are plasmid conjugation, type VI secretion systems and eDNA uptake (Flemming et al., 2016). Horizontal gene transfer plays a major part in the emergence and transfer of antimicrobial resistances and provide the cells with a large genetic kit for survival properties (Donlan, 2002).

1.3.4. Biofilms and antimicrobial agents

Antimicrobial resistance and the mechanisms behind it are of growing importance. The medical field faces enormous problems with resistant biofilms growing on medical devices, e.g. orthopaedic implants, catheters and contact lenses further leading to delayed healing (Mah and O'Toole, 2001). Furthermore, many infectious diseases and disturbances in tissue repair go hand in hand with biofilm formation (Potera, 1999; Mendoza et al., 2019). Therefore, much research focuses on the investigation of biocide resistance.

Cells embedded in biofilms show an increased resistance to antimicrobial agents (AG), compared with their planktonic relatives. Studies show that multiple mechanisms within the biofilm community are applied to withstand antimicrobial agents. Essential for the development of resistances is the composition of the biofilm and the antimicrobial compounds used for cleaning and disinfection. Mechanisms, which alternate the amount of intake and the cell interaction with the exposed substance, include the composition of the EPS-matrix, growth rate,

intercellular communication and the expression of special biofilm-phenotypes (Mah and O'Toole, 2001).

It appears that the exopolysaccharide matrix can act as a physical "initial barrier that can delay penetration of the antimicrobial agent" (Mah and O'Toole, 2001). However, the EPS-matrix is not impenetrable, agents can move through to the attaching surface and reach the targeted cells. The EPS-molecules react with and/or absorbs compounds of the AG, thus decreasing the amount of its transport within the biofilm and to the imbedded cells (Mah and O'Toole, 2001).

Studies on the cellular reactions to environmental stress show that a general stress response can be triggered by two major forces: nutrient limitation and high cell density. Sooner or later biofilm cells will go through both due to the heterogenous construction of biofilms. In response to stress factors the cells undergo physiological changes. Such changes prevent the cells from further damage provoked by environmental stressors like heat, cold, desiccation, pH changes and antimicrobial agents (Mah and O'Toole, 2001).

Another way to avoid detrimental effects of the AG is by slowing down growth; such an effect occurs when bacterial biofilm cells become starved and enter the stationary phase. A decrease of bacterial growth leads to an increase of resistance to antimicrobial agents (Mah and O'Toole, 2001). Or the other way round, if cells divide rapidly, they experience more downsizing by certain antibiotics (Costerton et al., 1995).

Furthermore, the induction of a specific biofilm phenotype, induced by the stressors mentioned above, can lead to increased resistance. Phenotypes alter their gene profiles by activating or repressing genes and thereby activating pumps and changes in the membrane-protein composition to help the cell conquer the AGs (Mah and O'Toole, 2001).

In conclusion there are many mechanisms working together to prevent the cells from detrimental effects of antimicrobial agents.

1.3.5. Starvation survival

Efficient bacterial growth requires favourable nutrient content. If a biofilm faces oligotrophic environmental circumstances of natural or industrial origin, a subset of cells undergoes phenotypical changes to survive. The cells become small and the metabolisms decrease. These dormant ultramicrobacteria can survive years in unfavourable environments and are

resuscitated under nutrient sufficient conditions. In this way biofilms can survive and disseminate under the most difficult conditions (Costerton et al., 1995).

1.4. Biofilms in the food industry

1.4.1. Biofilms - natural phenomena of utmost importance

Food contamination is of great concern to the food industry. Source of the introduction of microorganisms to the processing environment may be raw materials of the primary production, air, water, product and process surfaces and personnel (Holah and Kearney, 1992). Wet surface and organic residues are excellent preconditions for biofilm formation (Srey et al., 2013; Bridier et al., 2015). Once formed, biofilms are difficult to remove and present serious hygienic problems in the food industry (Kumar and Anand 1998). Sectors of concern among others are brewing, dairy processing, fresh produce, poultry processing and red meat processing (Simões et al., 2010).

Cross-contamination and post-processing contamination are hygienic problems of concern leading to lowered shelf-life, food spoilage, economic losses and, at worst, food-borne illnesses (Kumar and Anand, 1998).

Cross contamination arises when products come in contact with food-contact surfaces harbouring biofilms, or if fragments of biofilms detach from non-food-contact surfaces and relocate to food contact materials (Wagner et al. 2020). Biofilms can be relocated from their initial place of formation via aerosol arising during cleaning (Kumar and Anand 1998). Besides food contact surfaces, common hotspots for biofilms are environmental surfaces like floors, walls, water pipes and rubber seals (Kumar and Anand 1998). Another source of biofilms may be water hoses. Wagner et al. (2020) isolated biofilms from water hoses that were used for rinsing food contact materials. Hence, disinfected tools and surfaces may be recontaminated by the use of hoses.

Food-borne illnesses may occur if pathogens like *Listeria monocytogenes, Yersinia enterocolitica, Campylobacter jejuni, Salmonella spp., Staphylococcus ssp.* and *Escherichia coli O157:H7* are part of the biofilm forming bacterial flora (Simões et al., 2010). Stress factors within the food processing environment like disinfection, cooling temperatures, acidity and salinity may harm bacteria. Pathogens, and spoilage causing bacteria, e.g. *Pseudomonas ssp., Acinetobacter ssp., Lactobacillus ssp*. seem to be protected of these stresses within a biofilm (Bourdichon and Rouzeau, 2012; Giaouris et al., 2014).

Additional, biofilms are also the cause of several technical malfunctions e.g. filter clogs and metal surfaces corrosion, heat exchangers failure and cooling tower and chain lubrication system malfunction (Kumar and Anand 1998, Meyer 2003).

According to Lindsay and Holy (2006) beside a great extent of available nutrients and water, further factors promoting biofilm formation in the food industry are mass production systems, lengthy production cycles and vast surface areas.

As previously discussed, biofilm formation is a recurrent event in the food processing inter alia in the meat processing environment with highly detrimental effects (Simões et al., 2010). Although there are a great number of studies on the variety of biofilms, a large share of the present knowledge comes from studies focusing on oligotrophic environments; these environments vary greatly from those found in the food industry (Kumar and Anand 1998, Chmielewski and Frank 2003). Furthermore, most of the studies examining formation and structure of biofilms mostly took place under laboratory conditions, focusing on the characteristics of single species biofilms, whereas the majority of biofilms unite microorganisms of multiple species (Elias and Banin 2012).

Many studies focused on the bacterial attachment to food contact material and food; in the meat processing sector mainly the attachment to poultry and meat surfaces (Kumar and Anand 1998). Very few studies have focused on the actual detection of biofilms within the food environment – more precisely proofing the presence of cells and matrix components (Wagner et al., 2020).

Exemplary studies that provide evidence of the existence of biofilms in the food processing environment performed Maes et al. (2017 and 2019) and Wagner et al. (2020). They defined the existence of biofilms as the presence of cells and, depend on the study of at least one or two matrix components (carbohydrates, protein, eDNA/uronic acids).

Maes et al. (2017) revealed that 17 % of their investigated sites in eight different food processing plants harboured a biofilm (cells and one matrix component). Wagner et al. (2020) took 108 samples in a meat processing plant, of which ten sites (9.3 %) hosted biofilms (cells and two matrix components). All of these biofilms found by Wagner et al. (2020) were of multispecies composition. These findings, together with the different detected matrix components, draw a colourful picture of the diversity of biofilms found in the meat processing environment.

1.4.1. Biofilm formation in the food processing environment

In the food industry favourable conditions, e.g. good nutrient supply of bacteria, generally allow biofilm formation on any surface. Variables influencing surface colonization include physicochemical properties of the environment, the contact surfaces and the bacterial cell surface (Srey et al., 2013). The variables include pH-value, temperature, texture, hydrophobicity, surface charge, nutrient composition, osmolarity, O_2 levels, the presence of other bacteria and pre-existing EPS (Srey et al. 2013, Giaouris et al. 2014, Flemming and Schaule 1988).

Of particular importance to the formation of biofilms is the development of a so called 'conditioning film'. Accumulation of organic molecules and charged ions preconditions the surface at the "solid-liquid interface on food-contact surfaces" (Chmielewski and Frank 2003, Kumar and Anand 1998). In the food industry, these organic molecules comprise proteins from milk, meat and old EPS (Shi and Zhu 2009). The conditioning film provides the surface with a high amount of nutrients and alters its physicochemical properties, both of which favour bacterial attachment (Kumar and Anand 1998). Despite the advantages bacteria gained of the preconditioning, some proteins like bovine serum albumin, gelatine, fibrinogen and pepsin are able to inhibit bacterial attachment (Chmielewski and Frank 2003).

Studies, e.g. Bos et al. (2000), have shown a connection between free surface energy and bacterial attachment. Therefore, surfaces with high free energy (e.g. glass, stainless-steel) tend to be more hydrophilic and adsorptive for organic molecules, favouring the bacterial attachment process (Chmielewski and Frank 2003).

Next to the conditioned surface, the bacterial cell surface expresses physicochemical notable properties for attachment. These properties are modifiable through factors such as growth rate, medium compositions and culture conditions (Chmielewski and Frank 2003).

One property, the net negative charge of the bacterial surface, is unfavourable for the bacterial adhesion. The resulting electrostatic repulsive forces keep the cells away from the surface. To overcome the electrostatic repulsion and get in contact with the surface, the microorganism express cell appendices (fimbriae, pili, flagella), lipopolysaccharides and produce fibrils of EPS (Chmielewski and Frank 2003, Kumar and Anand 1998).

Lipopolysaccharides are part of the outer membrane composition and provide the cell with hydrophilic properties, enabling attachment to hydrophilic materials (Shi and Zhu 2009).

Flagella consist of “fine threads of the protein flagellin with a helical structure extending out from the cytoplasm through the cell wall”, and provide the bacteria with motility (Simões et al., 2010). Thus, the cell can overcome the repulsive barrier, and attach itself to the surface. Pili, or fimbriae, are straight, thin, filamentous protein appendices, that make the cell more adhesive (Simões et al. 2010).

Appendices possess amino acid residues with hydrophobic properties (Shi and Zhu 2009). Hydrophobic interactions along with van der Walls attraction forces, electrostatic forces, dipole-dipole interactions, hydrogen, ionic and covalent bonding are part of the reversible and irreversible attachment process of biofilm formation (Kumar and Anand 1998).

Furthermore, the design and microtopography of the food contact material play an important role in the adhesion of microorganisms and biofilm formation (Kumar and Anand 1998).

1.4.2. Food contact material

Frequently used food contact materials are stainless steel, glass, rubber, polyurethane, teflon, rubber and aluminium (Kumar and Anand 1998, Srey et al. 2013). However, biofilm formation can take place on nearly any material (Meyer, 2003). Stainless steel seems to be the most practical for food processing due to its robustness (Simões et al. 2010). One disadvantage of stainless steel is its vulnerability to corrosion (Srey et al., 2013).

Of great value is the cleanability of the equipment design (Kumar and Anand, 1998). Pipe bends, corners and dead ends promote soil build up and are hardly accessible via mechanical cleaning (Kumar and Anand 1998, Simões et al. 2010). In addition, even proper usage and cleaning methods lead to normal wear and tear of surfaces, further leading to the development of cracks and crevices which provide the bacteria with shelter from shear force and cleaning (Kumar and Anand 1998).

1.4.3. The importance of sanitation programmes

Hygiene, cleaning and disinfection are key measures in the food processing environment for preventing bacterial contamination. Hence, the implementation of thorough sanitation programs is essential for effective biofilm control. Despite hygienic precautions, the "removal of biofilms is a very difficult and demanding task" (Kumar and Anand 1998). This is due to the protective functions of the EPS matrix and the lively exchange of genes promoting resistance development (Flemming et al., 2016). Therefore, the breaking of the EPS matrix is the most important aspect of the cleaning process so that the antimicrobial reagents can gain access to the cells (Kumar and Anand 1998, Chmielewski and Frank 2003). Furthermore, the purpose of the surface (food contact surface or environmental surface like a floor or wall) and the characteristics of the residues must be considered when choosing the frequency and detergent for cleaning and disinfection (Simões et al., 2010).

Cleaning agents, high temperatures and mechanical forces (e.g. brushing, scrubbing) form the first step of the cleaning process and help to remove the accumulated residues. This is of utmost importance for allowing disinfection success as disinfectants can only be fully effective if they have direct contact with the viable cells. Therefore, existing matrix components must be removed in order to be killed by the disinfectants (Simões et al., 2010). Care should be taken when using high pressure cleaning as the aerosolization of biofilm parts can lead to the unintentional spreading of microorganisms (Meyer, 2003). The cleaning agents most often used in the food industry are alkaline cleaners and acid cleaners for persistent residues (Chmielewski and Frank, 2003; Simões et al., 2010).

After thorough cleaning, disinfectant agents must be applied to kill the microorganisms. If not, detached cells can relocate and form biofilms elsewhere (Simões et al., 2010). Commonly used disinfectants are halogens, peroxygens, acids and quaternary ammonium compounds (Chmielewski and Frank, 2003).

Attention should be paid to the fact that there is no 'one' biofilm control strategy, as there is no "unique model which is representative for all biofilms" (Meyer, 2003; Simões et al., 2010).

As referred to earlier (section 3.4.), biofilms show strong resistance to antimicrobial agents. The EPS matrix impedes the penetration of antimicrobial detergents and a wide range of environmental conditions faced in food processing reduces its efficacy (Mah and O'Toole 2001, Kumar and Anand 1998). Together with gene alterations of the cell during the attachment progress, referred to by Meyer (2003) as "phenotypic adaption" and the rather fast attachment (just a few hours) it becomes hard to control the biofilm with conventional cleaning and disinfection methods (Kumar and Anand, 1998). Thus, additional ways for effective removal of biofilms are required (Simões et al., 2010).

Examples in the literature include the use of enzymes and bacteriophages, hyper-swimming tunnelling bacteria, quorum-sensing inhibiting molecules and physical approaches like ultrasonication and electricity used for biofilm degradation (Meyer, 2003; Simões et al., 2010; Bridier et al., 2015). This variety of methods appears to have the best impact when applied in combination or with conventional chemical detergents (Srey et al., 2013).

The best control strategy in the case of biofilms is prevention. This goal is almost impossible to achieve due to the sophistry of biofilms. Still, new approaches are developed to minimize initial cell attachment including attempts for biofilm detectors and changing the physicochemical properties of the food contact material through adding antimicrobial additives (e.g. components of essential oils or surfactants (Simões et al. 2010; Philip-Chandy et al. 2000; Pereira et al. 2009; Giaouris et al. 2014; Srey et al. 2013).

1.5. The aim of this study

As shown, many aspects of biofilm formation especially in the food and meat processing environment need further research. The aim of this study was to examine the ability of biofilm formation of three bacteria species, namely *Microbacterium sp.*, *Acinetobacter harbinensis* and *Lactococcus piscium*., sampled in a meat processing facility. The results of the study provide new knowledge required for the research with multispecies biofilm-models, paving the way for greater understanding of biofilms.

The study examined the growing process of the three afore mentioned bacterial isolates in a static mono-species biofilm-model. The model used stainless steel slides bathed in a nutrient rich cultivation medium representatively for the meat processing environment. After a seven-day cultivation, the material was examined on the presence of bacteria (quantitative analysis of the bacterial load on agar plates) and the three main EPS matrix components: carbohydrates, protein and eDNA. Subsequently the ability of motility was tested using agar plates.

2. Materials and Methods

2.1 Mono-species biofilm assays

Mono-species biofilm assays were used to test the biofilm-forming ability of *Microbacterium sp.*, *Acinetobacter harbinensis* and *Lactococcus piscium*. The bacterial isolates were isolated from a biofilm from a meat processing environment (Wagner et al., 2020). To mimic the food processing environment stainless-steel slides were used and the incubation temperature was set on 10 °C; both settings are frequently found in the food-production environment. The slides were put into glass flasks filled with culture medium in order to imitate a humid, nutrient rich environment.

The duration of each biofilm assay was seven days. On day zero, the biofilm model was prepared and inoculated. The biofilm was incubated for seven days at 10 °C on a shaker. Subsequently, on days one, three and six a medium change and visual evaluation of biofilm growth was performed. On day seven the biofilm was harvested, the bacterial load was evaluated, and the matrix was isolated. In addition, matrix analyses were carried out to determine the presence of proteins, eDNA and carbohydrates.

The technical devices and materials used can be seen in Table 1.

Table 1: **Materials and methods used in the biofilm model**

Equipment	**Manufacturer**	**Characteristics**
Eppendorf Centrifuge 5424	Eppendorf AG	serial number: 5424YM526100
Eppendorf Centrifuge 5810 R	Eppendorf AG	serial number: 5811XG839982
Eppendorf Thermomixer compact	Eppendorf AG	shaker
Eppendorf Safe-Lock tubes	Eppendorf AG	0.5 ml 1.0 ml 1.5 ml 2.0 ml
Eppendorf Research Plus Pipette	Eppendorf AG	0.5 – 5.0 ml 100 – 1000 µl 10 – 100 µl 0.5 – 10 µl
Fisher Scientific accumet AE150	Fisher Scientific	pH-meter
Heidolph Unimax 1010	Heidolph Instruments	shaker
Shimadzu UV-Spectrophotometer UV-1800	Shimadzu Corporation	
micro scales		
Kern PCB scale	KERN & SOHN GmbH	Precision balance PCB scale
Powerpette Pro Pipet Filler	VWR	pipette boy
Greiner Bio - One Pipette Tips	Greiner Bio - One	0.5 – 5 ml 100 – 1000 µl 10 – 100 µl 0.5 – 10 µl
Cellstar tubes	Greiner Bio – one	50 ml conical bottom 50 ml + support skirt
Laboratory glass bottles	DURAN	50 ml 100 ml

		250 ml 500 ml 1000 ml
Sarstedt Tube	Sarstedt AG & Co. KG	15.0 ml
Sarstedt serological pipette	Sarstedt AG & Co. KG	25 ml 10 ml 1 ml
Inoculation Loops	Sarstedt AG & Co. KG	1 µl 1 µl
Filter for Syringe	Sarstedt AG & Co. KG	Filtropur S 0,2 REF: 83.1826.001
Vortex Genius 3	IKA	Model V3 S000
Friocell 222 cooled incubator	MMM Group	
Ringer solution	B. Braun Melsungen AG	1000 ml
Syringe Inject Luer Solo		20 ml REF: 4606205
Yeast extract	Bioklar diagnostics	REF: A1202 HA
CASO – Broth	Carl Roth	1000 g Art.number: X938.2
Dulbecco's Phosphate Buffered Saline (DPBS 1X)	gibco	500 ml REF: 14190-094
Dowex Marathon C Sodium form Na^+ - form, strongly acid, 20-50 mesh	Sigma Aldrich	REF: 91973-1KG-F

2.1.1 Model set up, preparation and use of liquid medium

The biofilm model design consisted of a stainless-steel inserted into 61 ml of culture medium suspended with bacteria within a 100 ml flask.

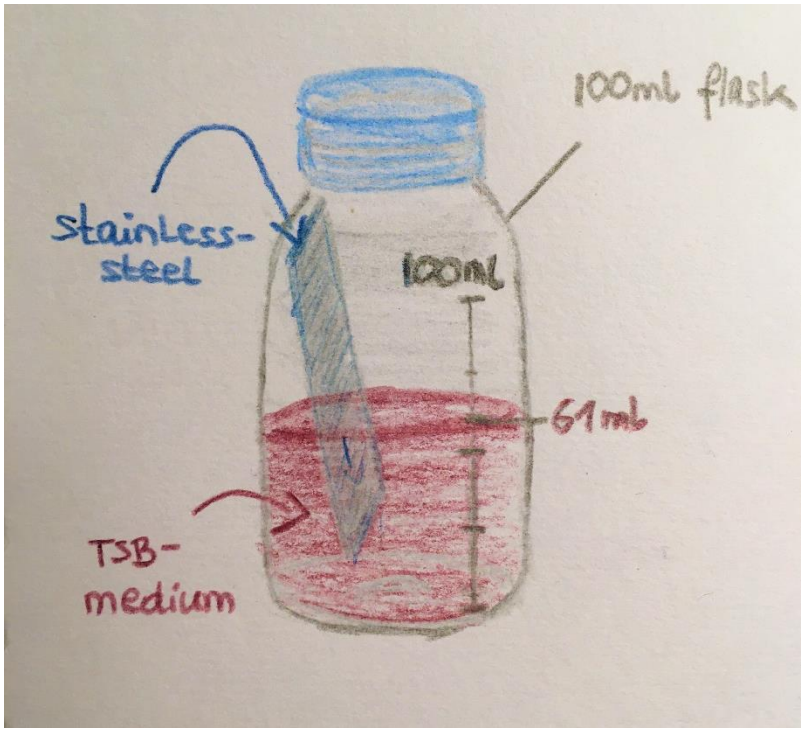

Fig. 2: Static mono-species biofilm model. The biofilm-model consisted of a stainless-steel inserted into 61 ml of medium-bacteria-suspension within a 100 ml flask.

In the course of the experiment tryptic soy broth medium (TSB) was used as a growth medium. 1000 ml of the initial TSB contained 30 g of trypticasein soy broth (CASO – Broth) and 6 g of yeast extract. The ingredients were mixed with water and then autoclaved. A 1:2-dilution was used for the cultivation of the biological replicates; the biological replicates were grown in overnight cultures. 1:10-dilution was used for the biofilm model and, subsequently, for the media-changes. The TSB was prepared the day before to allow for the opportunity to check for TSB contamination in preparation.

Cleaned and disinfected stainless-steel slides were put into clean 100 ml glass flasks and autoclaved for 15 minutes at 121 °C. After sterilisation the flasks were filled with 51 ml of 1:10 TSB-medium.

2.1.2 Overnight cultures

The desired number of overnight cultures (ONC), depended on the number of biological replicates used in the corresponding biofilm model. In general, three cultures per biological replicate were prepared - one in the morning and two in the afternoon.

Therefore, 100 ml flasks were filled using an inoculation loop with 80 ml of 1:2 TSB and inoculated with one bacterial colony per culture. The bacterial cultures were incubated overnight at room temperature with shaking.

2.1.3 Starting the biofilm-model assay

The bacterial concentration of the overnight cultures was determined in a spectrophotometer by measuring the Optical Density (OD) at a wavelength of 600 nm (OD_{600}). 1 ml of each culture was pipetted into a semi-micro cuvette and put into the spectrophotometer. The next assay steps were performed with the ONC showing the highest OD_{600}; preferable the ONC set up in the afternoon.

After measurements were conducted, the desired volume of the overnight sample was used to adjust a bacterial density of 0.1 in the final biofilm samples. The necessary volume was calculated using the following formula:

$$\mathbf{C_1 \times V_1 = C_2 \times V_2}$$

$$\rightarrow OD_{ONC} \times V_1 = 0.1 \times 61 \text{ ml}$$

$$\rightarrow \mathbf{V_1 = \frac{0.1 * 61\ ml}{OD\ ONC}}$$

C_1 …. OD_{600} of ONC-culture
V_1 …. required volume of ONC-culture
C_2 …. desired end concentration (= OD 0.1)
V_2 …. fluid end volume in biofilm models (=61 ml)

Then, the calculated volume of the ONC culture was pipetted into a 50 ml tube and centrifuged for five minutes at 4000 G.
After centrifugation a bacterial pellet was formed on the base of the tube. The supernatant was discarded in order to transfer the bacteria into the biofilm model. The pellet was then resuspended with 10 ml of 1:10 TSB, using a 10 ml pipette, and filled into the previously sterilized biofilm model flasks. With this last step the models were filled with the necessary amount of liquid, 61 ml. Simultaneously, a uniform bacterial density of OD 0.1 was achieved within the biofilm model flasks.
The biofilm-model was then placed in the incubator for 24 hours on a shaker at 10 °C until the first medium change.

2.1.4 Medium changes and evaluation of biofilm growth

A change of medium was carried out on day one, three and six. Hereby, the bacteria were provided with fresh nutrients. The stainless-steel slides were transferred into flasks filled with fresh medium; operations were carried out using a sterile tweezer near the flame.

2.1.5 Harvest of biofilm

After seven days inoculation the biofilm model was harvested. The aim of the harvest was to separate the grown bacteria from its matrix, so that both subjects were available for further examination.

2.1.5.1 Cation-exchange resin

The cation-exchange resin (CER) was hydrated using a 1:10 phosphate-buffered saline (PBS) solution, before its usage. For each g CER 10 ml PBS were used. After washing the CER twice for 15 minutes on a magnetic stirrer, the CER was separated from the surplus washing fluid and filled into 2 ml Eppendorf safe lock tubes (2 g CER per tube).

2.1.5.2 Harvesting of biofilm

On day seven, the biofilm was harvested. First the stainless-steel slide was put into a 50 ml tube filled with 15 g sterile glass beads and ringer solution; this was performed using a sterile tweezer near the flame.

The tube was then vortexed for three minutes in order to detach fixed biofilm particles off the plates. Then, with the help of a pipette the stainless-steel slide was carefully rinsed to remove any remaining particles. The sample solution was then transferred into a new, empty 50 ml tube with a 10 ml pipette. 2 g CER were added into the tube and put on the shaker for 20 minutes at 500 rpm. Hereafter 50 µl of the sample were taken and transferred into the 1.5 ml Eppendorf tube for the following dilution series. The remaining sample was centrifuged for another 20 minutes in order to clear the fluid of CER and bacteria accumulated on the base of the tube.

The supernatant was carefully transferred into a 15 ml tube using a 10.0 ml pipette. The last drops were pipetted with a 1.0ml pipette to avoid recontamination with the bacterial pellet and CER. To ensure no bacteria got into the matrix sample, the liquid was pressed through a 0.20 µm filter with a 20 ml syringe. Afterwards the matrix sample was stored at -20 °C.

2.1.6 Analysis of harvested biofilm samples (bacterial load and matrix)

A biofilm contains bacteria and matrix components; protein (P), eDNA and carbohydrates (CH) (Flemming and Wingender 2010). In order to determine biofilm formation, the matrix sample was analysed for the presence of these constituents. In the current study cultivated bacteria were also plated and quantitatively analysed.

2.1.6.1 Bacterial load - quantitative analysis of colony forming units

The amount of bacteria present in the biofilm, bacterial load, was checked by performing an agar plate-based cultivation of the harvested sample in order to determine the colony forming units (CFU). The sample was diluted in a 1:10 dilution series. Several dilution steps (e.g. 10^{-4}, 10^{-5}, 10^{-6}) were plated for each sample in triplicate. The agar plates were then incubated for two days and the colonies were subsequently counted.

2.1.6.2 Matrix analysis

The matrix analyses were performed with aliquots of the harvested matrix solution. The protein and eDNA assays were performed in duplicate.

The matrix was aliquoted into Eppendorf tubes. 500 µl/2 ml tube for eDNA-, 1000µl/2 ml tube for protein analysation and 1000 µl/1.5 ml tube for carbohydrate analysation.

The 1.5 ml-tubes for the carbohydrate assays were weighed on a fine scale before filling.

2.1.6.2.1 Protein analysis

Precipitation

Controls were performed during the analysis - one negative (1 ml dH_2O) and two positive (1 ml 0.5 g/l bovine serum albumin and 1 ml 0.1 g/l bovine serum albumin).

For precipitation the samples were put on ice. First 100 µl of 1 g/l TCA/Acetone was added, followed by 11 µl of 2 % Deoxycholate. Afterwards the solution was mixed thoroughly with a pipette and put on 4 °C overnight.

The next day the samples were centrifuged for 30 minutes at 14000 rpm at a temperature of 4 °C. Next, the supernatant was carefully removed using a 1000 µl- and a 100 µl-pipette. Afterwards, the tube with the pellet was air-dried for approximately five minutes. Shortly thereafter, the pellet was dissolved in 30 µl of a 0.5 M Tris-HCl solution.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) separated proteins by size; using staining methods for proteins, the presence of protein after separation becomes evident through emerging colour lines on the gel. The SDS-PAGE was used for a qualitative detection of proteins in the biofilm matrix.

The gel consisted of a separating and a stacking gel. The gel was made in two steps. First, the separating gel was mixed, poured into the mould and dried for half an hour. 60 ml of 15 % separating gel contained 13.8 ml H_2O, 30 ml acrylamide, 15 ml 1.5 M TRIS buffer (pH 8.8), 600 µl SDS, 600 µl APS (10 %) and 48 µl TEMED.

After the separating gel was dry, the stacking gel was mixed and poured on the dry separating gel. A sample comb was put into the liquid stacking gel. 20 ml of 30 % stacking gel contained

14 ml H_2O, 3.3 ml Acrylamide, 2.5 ml 1 M TRIS buffer (pH 6.8), 200 µl SDS, 200 µl APS (10 %) and 20 µl TEMED.

The gel was filled vertically in a mould. The mould consisted of two opposing glass plates separated by two spacers on the left and right side to leave room for the gel. The plates were held together with clasps on either side and fixed in an upright position. After 30 minutes drying the sample comb was taken out. Lopsided pockets were adjusted to an upright position with water.

Meanwhile the precipitated samples were mixed with 10 µl laemmli sample buffer and the proteins were denatured at 95 °C for 5 minutes.

After these preparations the gel was put into the electrophoresis device. The first gel pocket was filled with a protein ladder (PageRulerTM Prestained Protein Ladder, Thermo Fisher), in the following gel pockets the prepared samples were pipetted.

The electrophoresis was performed for one hour and a half using 60 mA and 200 V.

Staining

After electrophoresis the gel was stained in seven steps. The gel was placed in a small glass tank and put on a shaking board to make sure the gel was covered equally with the fluids.

Table 2: **Silver Staining Protocol**

Staining step	**Solution composition per litre**	**Incubation time**
Fixation	30% ethanol 10% acetic acid 60% UHQ-water	60 minutes minimum up to several weeks maximum
Sensitizing	2 g sodium thiosulfate $5H_2O$ 68 g sodium acetate $3H_2O$ in 700 ml UHQ-water 30% ethanol	60 minutes minimum up to overnight maximum
Washing	UHQ water	3 x 20 minutes
Silver Staining	2 g/l silver nitrate	1 x 30 minutes
Developing	25 g sodium carbonate in UHQ-water 100 µl formaldehyde (37%)	1 x 1 minute, followed by 3 x 10 minutes
Stopping	10 g/l glycine	1 x 20 minutes
Washing	UHQ water	2 x 15 minutes

Scanning

The stained gel was scanned on a commercial scanning device (HP Scanjet G4050).

2.1.6.2.2 eDNA analysis

EtOH precipitation of DNA

Standards were included in the precipitation and analysis to control precipitation outcome. Therefore, we used one negative control with H_2O and two positive controls (50 ng/µl and 10 ng/µl DNA standard).

On ice, 50 µl of 3 M Na-Acetate (pH = 5.2), 50 µl of 0.1 M $MgCl_2$ were added. For the next 24 hours the samples were stored at -20 °C to let the DNA precipitate.

After 24 hours the samples were centrifuged at 10000 g for 15 minutes. Afterwards the supernatant was carefully removed with a 1000 µl pipette. Subsequently the pellet was resuspended with 500 µl of 70 % ethanol. Next, the sample was centrifuged at 14000 rpm for 15 minutes. After the second centrifugation the supernatant was carefully removed - first with a 1000 µl pipette and then, to get as much supernatant liquid out as possible, with a 100 µl pipette.

The pellet was then air dried. The last step included the resuspension of the dried pellet with 30 µl distilled water.

Agarose-gel electrophoresis - qualitative detection of eDNA

The electrophoresis was carried out using a 1 %-agarose gel. 1 g of agarose powder was dissolved in 100 ml of TRIS-Borat-EDTA buffer (TBE) and heated for three minutes. Before filling the liquid gel into the plate and adding the combs to form the 20 pockets, 2 µl of DNA/RNA-color (peq-green) was added.

For all assays two duplicates of the matrix samples were pooled. 5 µl of each duplicate sample were placed and mixed on parafilm. Then 3.3 µl of sample loading buffer was added. These 13.3 µl were then pipetted into the intended pocket of the agarose gel.

One pocket per row was filled with 3 µl of kilobase ladder. The electrophoresis was conducted for 40 minutes with a constant voltage of 100 V.
After the electrophoresis run was complete, a picture of the gel was made using the GelDoc2000.

DeNovix measurements (quantitative detection of eDNA)
Additionally, the eDNA concentration was measured using DeNovix. 1.2 µl of each eDNA sample were dropped on the measuring surface. Each matrix-sample duplicate was measured twice.

2.1.6.2.3 Carbohydrate analysis

The samples were weighed on a fine scale. Next the samples were opened and put on the preheated thermoblock and incubated at 95 °C for one hour.
Meanwhile, the standards for the assay were prepared. The standard curve consisted of following concentrations: 5399, 4319, 3239, 2159, 1080, 540, 270, 0 ng/50 µL glucose.
After one hour on the thermoblock the samples were weighed again. The presence of carbohydrates was determined according to Masuko et al. (2005).
The assay was carried out with a 96-well plate. Each sample and the standard was pipetted in triplicate.
Each well was filled with 50 µl of sample or standard. In the next step 150 µl of concentrated sulfuric acid was added, followed by 30 µl of 5 % phenol in water solution. After these two components were added, the contents of the wells were mixed with the help of the multi-tip pipette.
Subsequently, the 96-well-plate was covered with an adhesive foil and incubated for five minutes over a 90 °C-water-bath. When the plate returned to room temperature, the foil was removed.
Using TECAN, the absorbance at 490 nm was measured. Using the standard curve, the respective amount of glucose equivalents within the samples was calculated.

2.2 Motility assay

To explore further characteristics of the bacterial isolates used in the biofilm assay, motility assays were carried out.

The analysed bacterial isolates were the same as in the biofilm model, namely *Microbacterium sp.*, *Acinetobacter harbinensis* and *Lactococcus piscium*. Additionally, *Pseudomanoas simiae* was used as a positive control.

The bacteria were assessed on their ability to swim, swarm and twist. The assay was carried out in duplicate and repeated three times. After inoculation the agar plates were incubated at 10° C for one week. Evaluation of the motility was done after 24, 48 and 72 hours and again after seven days. The average diameter was measured with a ruler in mm.

2.2.1 Swimming agar

1 l of agar contained 10 g tryptone, 5 g NaCl and 3 g agar. After its preparation the agar was autoclaved at 121° C for 20 minutes. The agar plates were filled with approximately 20 ml agar/plate.

To inoculate the plates a single colony was picked up with a sterile toothpick and placed in the middle of the agar in the centre of the plate.

2.2.2 Swarming agar

1 l of agar contained 10 g tryptone, 5 g glucose and 5 g agar. After its preparation the agar was autoclaved at 121° C for 20 minutes. The agar plates were filled with approximately 20 ml agar/plate.

A single colony was picked up with a sterile toothpick and placed on top of the agar in the centre of the plate.

2.2.3 Twitching agar

1 l of agar contained 10 g of tryptone, 5 g yeast extract, 10 g NaCl and 10 g agar. After its preparation the agar was autoclaved at 121° C for 20 minutes. The agar plates were filled with approximately 20 ml agar/plate.

A single colony was picked up at the top of a sterile toothpick. The toothpick was then stabbed through to the bottom of the agar. The colony was placed in the centre of the plate.

2.3. Statistics

Statistical analysis was performed using IBM SPSS.20 (SPSS Inc. Chicago, USA). The mean values and standard deviations (SD) were calculated. Brown Forsythe and Welch tests were applied to confirm variance homogeneity. Games-Howell posthoc test (variance heterogeneity) was used to determine significant differences between the bacterial loads, carbohydrates and eDNA and motility parameters strains. p-values < 0.05 were considered to be statistically significant.

3. Results

3.1 Mono-species biofilm model-assay

A static mono-species biofilm model was used to test the ability of biofilm formation of three bacterial isolates, namely *Lactococcus piscium, Microbacterium ssp.*and *Acinetobacter harbinensis.* The bacteria were incubated separately for 7 days at 10 °C mimicking food processing environments. After the course of seven days the biofilm was harvested.

3.1.1 Bacterial load

To determine the harvested biofilm's bacterial load an agar plate-based cultivation was carried out. The bacterial load was determined successfully.

All isolates showed high bacterial load on the ss-plate. *Microbacterium sp.* showed the highest bacterial load (~ 5.1 x 10^8 CFU/cm^2), followed by *Lactococcus piscium* (~ 9.2 x 10^6 CFU/cm^2) and then *Acinetobacter harbinensis* (~ 2.7 x 10^6 CFU/cm^2).

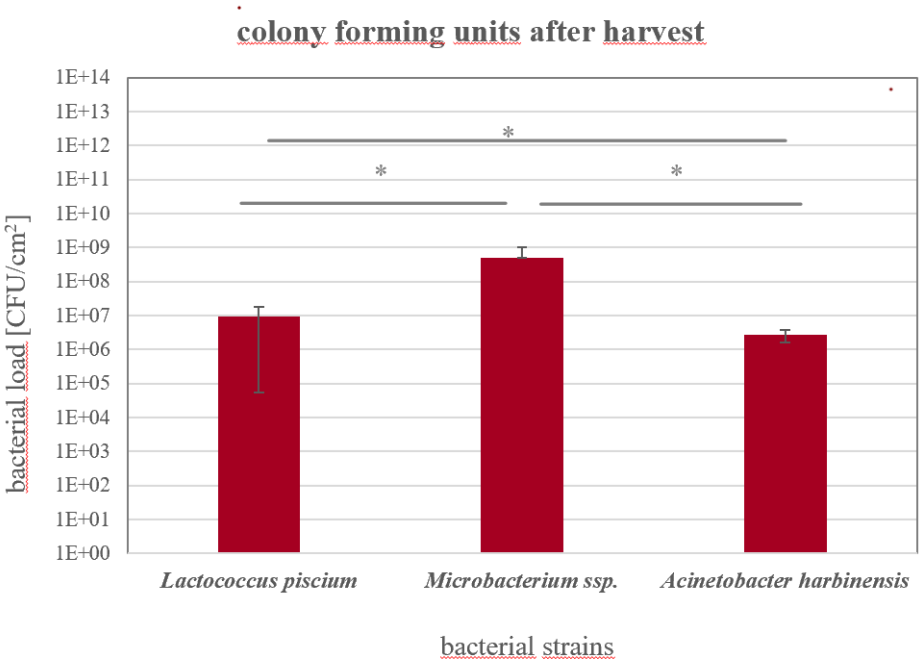

Fig. 3: Bacterial load of biofilm samples. Using agar plate-based cultivation the colony forming units of the samples were determined. *Significant differences between the isolates, $p < 0.05$.

3.1.2 Presence of matrix components

3.1.2.1 Proteins

Using SDS-PAGE and subsequent silver staining we detected proteins in 23 out of 36 samples. The positive samples belonged to *Microbacterium sp.* and *Lactococcus piscium*. All twelve samples of *Microbacterium sp.* showed positive results. Out of the twelve samples of *L. piscium* only one sample was negative. In the samples of *Acinetobacter harbinenis* no protein presence could be detected in the matrix.

3.1.2.2 eDNA

Precipitated matrix samples underwent qualitative and quantitative analysis. Qualitative detection of eDNA was performed using a 1 %-agarose-gel electrophoresis. Each of the twelve samples of *Microbacterium sp.* and *Lactococcus piscium* showed positive eDNA results using this approach, whereas all twelve samples of *Acinetobacter harbinensis* showed no detectable eDNA (data not shown).

The quantitative detection, using spectrophotometric measurements, showed that eDNA was present in all 36 samples. A significant difference in the matrix samples was seen between *Microbacterium sp.* – which showed the highest amount of eDNA with 600 ng/cm^2 – and *Lactococcus piscium,* which showed the lowest amount of eDNA at 278 ng/cm^2. The amount of *Acinetobacter harbinensis* was 563 ng/cm^2. Lightly lower amount than in the samples of *Microbacterium ssp.*. However, the amount of eDNA varied between the experiments.

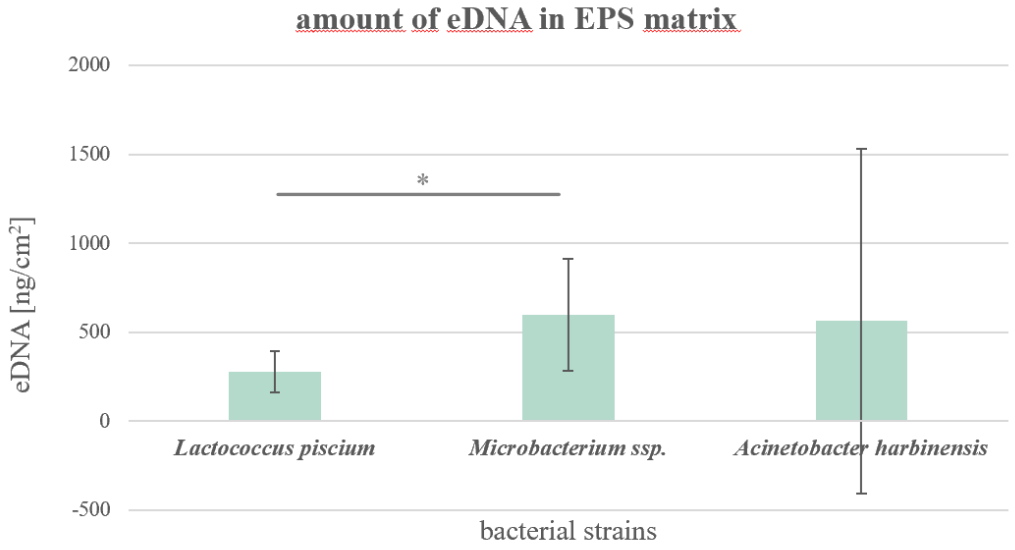

Fig. 4: Presence of eDNA in biofilm matrix samples. Using spectrophotometric measurements, the precipitated matrix samples were examined on their eDNA content. *Significant differences between the isolates, $p < 0.05$.

3.1.2.3 Carbohydrates

The presence of carbohydrates in the harvested matrix samples was determined using the colorimetric phenol-sulfuric analysis method, and subsequently measuring absorbance at 490 nm using TECAN.

Carbohydrates could be detected in all 36 matrix samples. The highest amount of CH was found in the samples of *Acinetobacter harbinensis* (3102 ng/cm^2), the lowest amount in the samples of *Lactococcus piscium* (1396 ng/cm^2). All three isolates showed significant differences between one another in the amount of CH present.

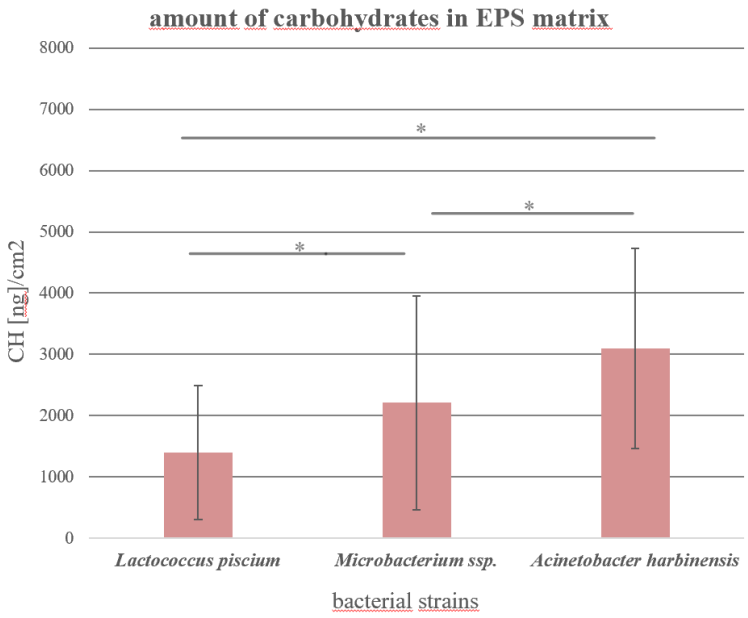

Fig. 5: Presence of carbohydrates in biofilm matrix sample. Using the colorimetric phenol-sulfuric analysis method with subsequent measuring of the absorbance at 490 nm the amount of carbohydrates in the sample was determined. *Significant differences between the isolates, $p < 0.05$.

3.1.2.4 Summary of the results

Cultivable bacteria were determined in all three bacterial isolates. The presence of all three matrix components were determined in two isolates, namely *Microbacterium sp.* and *Lactococcus piscium.* The samples of *Acinetbacter harbinensis* were positively tested for eDNA and CH.

Bacterial isolate	**Bacterial load**	**Protein**	**eDNA**	**Carbohydrates**
Microbacterium ssp.	+	+	+	+
Acinetobacter harbinensis	+	-	+	+
Lactococcus piscium	+	+	+	+

Fig. 6: Summary of the results (bacterial load, proteins, eDNA and carbohydrates). Results of bacterial load and matrix analysis. Green/+ = positive result, red/- = negative result.

3.2 Motility assays

The motility was determined using plating trials. The bacterial isolates (*Microbacterium sp., Acinetobacter harbinensis, Lactococcus piscium*) were plated on different agars forcing different motility patterns. The bacterial locomotion was measured at 24 h, 48 h and then after seven days.

3.2.1 Swimming motility

Swimming motility was detected in all three bacterial isolates. *Microbacterium sp.* and *Acinetobacter harbinensis* showed a steady increase in diameter between the 24-hour mark and seven days. After seven days the diameter amounted to over 3 mm with *Microbacterium sp.* and 4 mm with *Acinetobacter h.*

Generally, *Acinetobacter harbinensis* showed the fastest/highest swimming motility (24 h – 2 mm, 48 h – 2.83 mm, 7 d – 4 mm). On the contrary, *Lactococcus piscium* showed the slowest and least motility (24 h – 1.33 mm, 48 h – 1.75 mm, 7 d – 1.5 mm).

The swimming motility of *Lactococcus piscium* differed from the other isolates as it was diminishing between 48 h and 7 d.

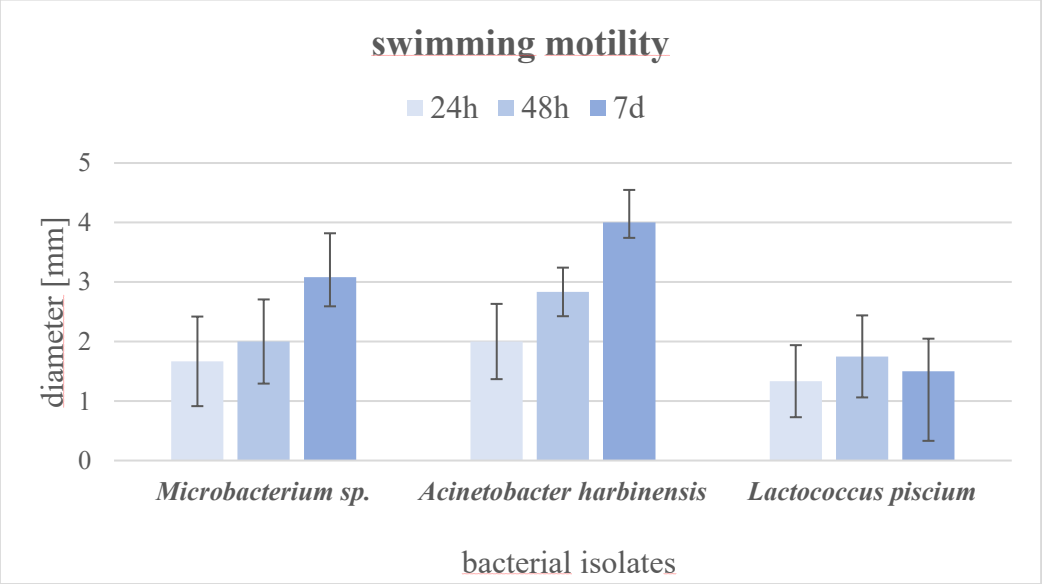

Fig 7.: Swimming motility of the tested isolates. Using plating trials, the swimming motility of the respective isolates was determined after 24 h (light blue), 48 h (middle blue) and 7 d (dark blue). Measurements are provided in diameter [mm].

3.2.2 Swarming motility

Swarming motility was determined in all three bacterial isolates. *Acinetobacter harbinensis* and *Microbacterium sp.* showed a similar increase in diameter over the course of seven days, although the motility of the former slightly topped the motility of the latter. *Lactococcus piscium* showed the smallest increase of all three isolates and a decrease in diameter of ~ 0.26 mm between 48 h and seven days.

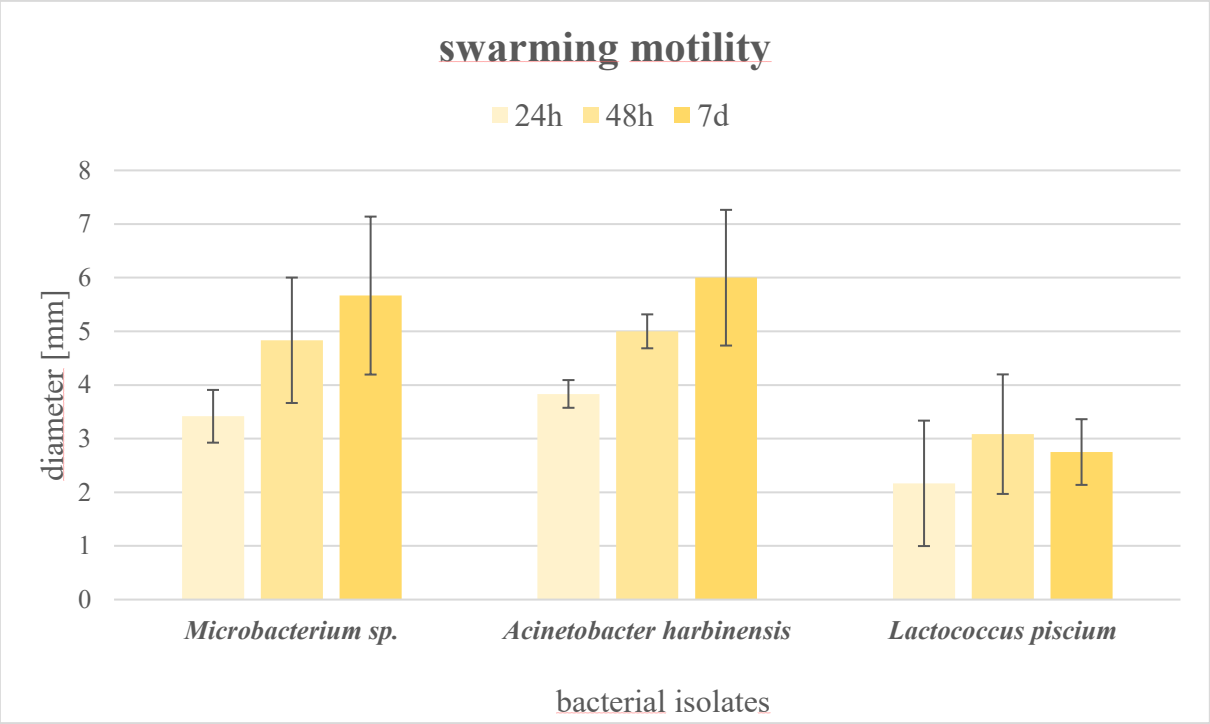

Fig 8.: Swarming motility of the tested isolates. Using plating trials, the swarming motility of the respective isolates was determined after 24 h (light yellow), 48 h (middle yellow) and 7 d (dark yellow). Measurements are provided in diameter [mm].

3.2.3 Twitching motility

Twitching motility could be detected in all three isolates. All three isolates showed a steady increase in diameter over seven days.

A. harbinensis presented the fastest and greatest increase of all three isolates. And already after 24 h its diameter measured 3.33 mm, a length which *Microbacterium sp.* reached only after the 48-hour mark and not reached at all with *Lc. piscium*. Six days later *A. harbinensis* showed a diameter of 6 mm and *Microbacterium sp.* a diameter of 4.25 mm.

Lc. piscium showed a diameter of 2.66 mm after seven days, thereby presenting the lowest form of twitching motility out of the three isolates.

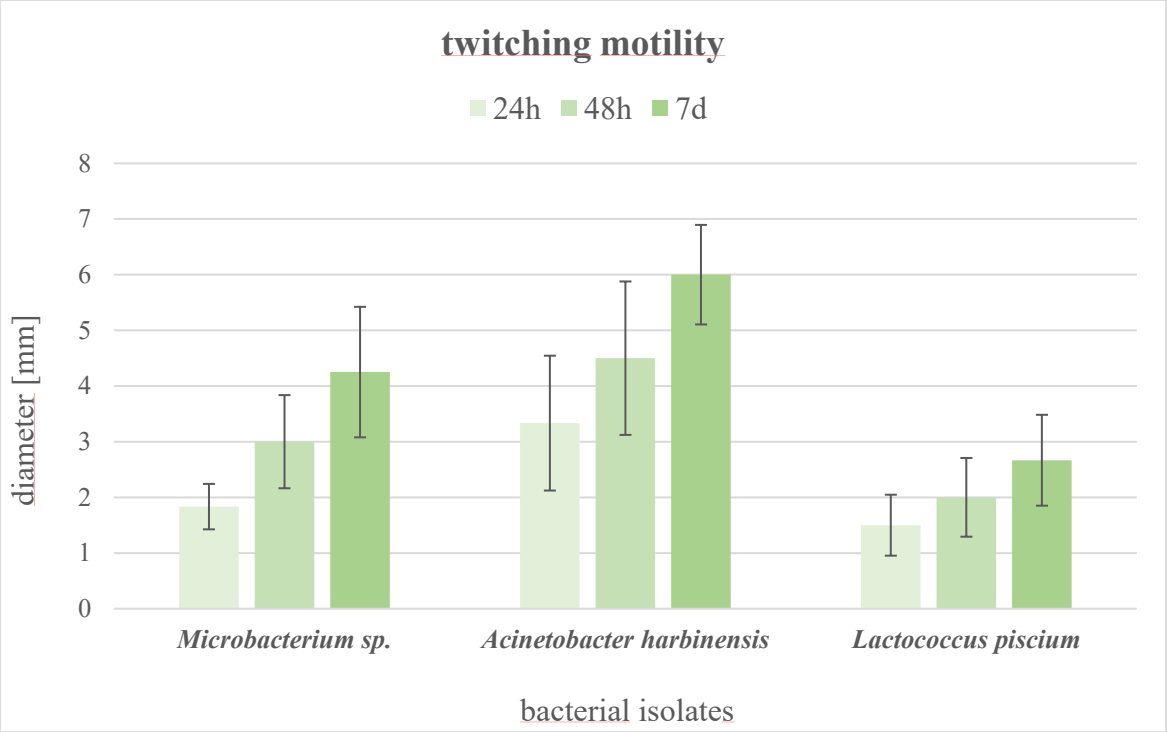

Fig 9.: Twitching motility of the tested isolates. Using plating trials, the twisting motility of the respective isolates was determined after 24 h (light green), 48 h (middle green) and 7 d (dark green). Measurements are provided in diameter [mm].

4. Discussion

The aim of this study was to examine three bacterial isolates, namely *Microbacterium sp.*, *Acinetobacter harbinensis* and *Lactococcus piscium;* particularly to examine their ability to form biofilms under conditions mirroring the food processing environment.

In order to distinguish between biofilm-forming and non-biofilm-forming bacterial isolates we link each specimen to certain criteria related to forming. In this study, the decisive criteria are the presence of cultivable bacteria and at least two matrix components in the sample.

The consequent definition of positive biofilm samples follows Wagner et al. whose 2020 study examined a meat processing environment for the presence of biofilm hotspots. Such similar criteria are to be found in Maes et al.'s 2017 study investigating biofilm positive sites in food processing facilities. However, unlike Wagner et al. Maes and her team took into account those samples including only one matrix component. The latter approach disregards the fact that in a food processing environment many matrix-related products are innately present, e.g. food residues, and can lead to false positive results (Wagner et al., 2020). Thus, in an effort to cultivate a more robust definition, the presence of two matrix components was considered to be biofilm positive, both in Wagner et al. and the current study. However, the current study included no food residues, thus no interaction within the experiments was expected.

The present study identified bacterial growth as well as the presence of at least two matrix components in all three isolates. Therefore, *Microbacterium ssp.*, *Acinetobacter harbinensis* and *Lactococcus piscium* present the ability to form biofilms. Thus, this study provides further insight to the way in which the three bacterial isolates frequently associated with the food industry grow.

The occurrence of biofilms goes hand in hand with the food industry and presents a long list of hygienic risks (Kumar and Anand, 1998). The issues become more significant when the bacterial isolates become associated with food spoilage. According to Møretrø and Langsrud (2017) all three isolates, especially *Acinetobacter ssp.* and *Lactococcus ssp.* (a representative for lactic acid bacteria), are commonly found in the food processing environment, and are all involved in food spoilage. The great abundance of these microbes within the food-processing environment could result in biofilm formation, which is determined within the present study.

Maes et al. (2019) examined samples of dominant bacteria remaining on food-contact surfaces after being cleaned as well as exploring disinfection methods in several food companies. *Microbacterium ssp.* was found on the surfaces of an oven food plant, a meat plant and an egg-processing plant. *Acinetobacter ssp.* were also identified at a meat and a sauce plant respectively. A isolate of *Lactococcus*, namely *Lactococcus lactis subsp. cremoris* was found in the oven food plant (Maes, Heyndrickx, et al., 2019). As previously discussed in my introduction (chapter 1.3.1 and 1.3.2), bacteria in biofilms benefit from the sheltering EPS-matrix; biofilm associated bacteria with their emerging properties may survive cleaning and disinfection better than planktonic bacteria (Flemming et al. 2016). Hence, the detection of bacteria capable of biofilm formation are responsible for increased risk of bacterial contamination in food-processing environments.

The findings of the present study can be associated with the results of Wagner et al. (2020), who detected ten multispecies biofilm hotspots in a meat processing facility. In seven biofilm hotspots within the facility at least one of the three species presented in the current study (*Acinetobacter ssp., Microbacterium spp. and Lactococcus ssp.*) could be identified. For this reason, *Microbacterium ssp.*, *Acinetobacter harbinensis* and *Lactococcus piscium* may be considered a part of biofilm formation under natural conditions.

Tang et al. (2013) called *Microbacterium ssp.* a “strong biofilm former”. This is in positive agreement with the biofilm forming abilities of *Microbacterium ssp.* found in the present study.

The results of the present study showed no presence of protein in the matrix samples of *Acinetobacter harbinensis*. For this reason, it must be considered that our method of protein analysis was only semiquantitative. Additionally, the protein levels may have been below our detection limits. Various studies indicate that *Acinetobacter ssp.* are able to form biofilms. *Acinetobacter ssp.,* are commonly found in the food processing environments, especially of cold and aerobically-stored foods (Møretrø and Langsrud, 2017). In 2013, Møretrø et al. observed biofilm formation of *Acinetobacter ssp.* isolated from a meat abattoir. *Acinetobacter calcoaceticus* isolated from meat processing environments show highly structured, channelled biofilms formed under static and dynamic conditions (Habimana et al., 2010).

Studies from the medical sector provide further evidence for biofilm formation by *Acinetobacter ssp*. These showed that a harmful, nosocomial germ, *Acinetobacter baumannii*, is able to form biofilms (Longo et al., 2014). Interestingly, Greene et al. (2016) showed that *A.*

baumannii readily develops biofilms on stainless steel. The present study showed biofilm formation by *Acinetobacter harbinensis* on stainless steel plates in the static biofilm models. This finding is of importance because stainless steel is a material commonly found in food processing facilities (Dewangan et al., 2015).

The biofilm forming potential of a *Lactococcus* isolate, namely *Lactococcus lactis*, is discussed in studies by Habimana et al. (2009) and Mercier et al. (2002). Studies, e.g. Saraoui et al. (2016b) and Sakala et al. (2002) outline that *Lactococcus piscium*, a psychrotrophic species, plays a major role in the spoilage of meat. Contaminated products show major changes in their sensory qualities (T. Saraoui et al., 2016b). Packed meat products in particular are affected. Sakala et al. (2002) isolated *Lactococcus piscium* from vacuum-packed refrigerated beef. Rahkila et al. (2012) showed that *Lactococcus piscium* is well adapted to live in chilled environments and within various types of Modified Atmosphere Packaged (MAP) meat products (e.g. broiler products, minced meat of beef and pork, turkey, pork). Furthermore, *Lactococcus piscium* is the most common species present at the end of storage; the inoculation of pork with the respective bacterial isolate reduced the sensory quality and shelf-life of the product, indicating that the growth of *Lactococcus piscium* leads to meat spoilage.

Pathogenic bacterial isolates are highly relevant for food safety. Since pathogens entering the processing environment are likely to encounter a pre-colonized surface, studies on their interaction with non-pathogenic, spoilage-causing isolates are a significant area of research (Bridier et al. 2015). A study by Habimana et al. (2010) showed that the presence of an *Acinetobacter ssp.* isolate isolated from meat-processing environments, namely *A. calcoaceticus*, had great impact on the surface colonisation of *E. coli O157:H7*. The presence of an *Acinetobacter*-biofilm led to a 400-fold increase in the total biovolume of *E. coli O157:H7* between 24 and 48 hours. *E. coli* cells were found embedded and covered by cell-clusters of *A.calcoaceticus* under both static and dynamic growth conditions. These findings lead to the assumption that *Acinetobacter*-biofilms provide shelter from environmental stress to pathogenic isolates like *E. coli O157:H7* (Habimana et al., 2010).

The presence of biofilms can also lead to a minimisation of pathogenic surface colonization. Already in 1999, Leriche et al. determined a bactericidal effect of *Lactococcus lactis* on *Listeria monocytogenes*. More recently, Saraoui et al. (2016a) showed the contact-dependent inhibition of *Listeria monocytogenes* by *Lactococcus piscium*.

If the presence of a biofilm can enhance or weaken the persistence of a pathogenic isolate, it highlights the importance of studying biofilm formation; these studies would include the ability to form biofilms of non-pathogenic bacterial isolates as well as spoilage causing bacterial isolates. The results of such studies, including the present one, may help to prevent and/or ease the occurrence of pathogenic isolates in the food-processing environment.

Cell-appendices play an important role in the attachment process of bacterial cells to surfaces. This initial bacterial attachment may lead to biofilm formation (Simões et al., 2010). Flagella-driven motility enables bacteria to migrate towards a more favourable environment and is regulated via intracellular chemotactic signal pathways (Nakamura and Minamino, 2019).

Flagella operate like rotating propellers, enabling the bacteria to swim in liquids and to swarm over moist solid surfaces. Another way to move over humid, solid surfaces is through twitching motility, enabled through the expression of type IV-pili (Jarrell and McBride, 2008). The connection of motility and biofilm formation of four pathogenic isolates (*Bacillus, Pseudomonas, Vibrio, and Escherichia*) has been reviewed by Guttenplan and Kearns (2013). Their study showed that if bacteria remain attached to a biofilm over a long period of time the flagella-transcription in a cell is inhibited, enabling the bacteria to stay closely packed in the biofilm.

Another study, O'Toole and Kolter (1998), outlined the importance of Flagella and type IV-pili for the biofilm formation of *Pseudomonas aeruginosa*. A study on the motility and biofilm formation of *Vibrio cholerae serotype O1* and *O139* under the influence of Polymyxin B suggested that Polymyxin B influenced the flagella-based motility. Polymyxin B-influenced motility led to a lower ability in biofilm formation (Giacomucci et al., 2019). Further studies from the medical sector focus on the motility of known biofilm formers such as *Acinetobacter baumannii*, e.g. Vijayakumar et al. (2016).

The results of the motility assays of the present study demonstrate that all three isolates are capable of locomotion. The highest movement-diameter was observed on agars supporting swarming and twitching, which suggests that the isolates move faster over solid and moist surfaces (as seen on swarming-agar) and solid, humid surfaces (as seen on twitching-agar) (Jarrell and McBride, 2008). An inconsistency was noted in the swimming and swarming motility of *Lactococcus piscium,* showing a decrease of diameter after seven days.

Currently, to the best of my knowledge, there is no published research material focusing on the

connection between the motility of food-borne pathogens or spoilage bacteria and their ability to form biofilms. Thus, our findings provide first insight into the motion patterns of three food spoilage causing bacterial isolates. Since physical parameters, like light and temperature and chemical gradients have impacted locomotion, further research is needed to gain thorough knowledge of these isolates' motility properties (Adler, 1966; Vijayakumar et al., 2016).

In conclusion, this study demonstrates the ability of three food spoilage causing bacterial isolates *Microbacterium ssp.*, *Acinetobacter harbinensis* and *Lactococcus piscium* to form biofilms under static conditions. And while it further outlines their motility ability, the present study has only investigated the isolates within a mono-species biofilm model set up. Since natural occurring biofilms comprise multiple species, further research would focus on the investigation of mixed-species biofilm formation. Nevertheless, our findings might help to gain deeper knowledge of biofilm formation in the food-processing environment leading to increased prevention of biofilm-borne food contamination.

5. Abstract

Bacterial biofilms are mergers of single and/or mixed species bacteria, embedded in a self-produced matrix of extracellular polymeric substances. Wet surfaces and nutrient-rich environments are commonly found in food-processing plants and provide good conditions for bacterial colonisation and potential biofilm formation. Bacteria in a biofilm may benefit from emerging properties, e.g. high tolerance to antimicrobial agents. Thus, biofilm formation in food-processing environments constitute a high hygienic risk; possible consequences are food contamination and spoilage. This study examined three bacterial isolates isolated in a meat-processing plant on their ability to form biofilms. For this, the bacterial isolates, namely *Microbacterium ssp.*, *Acinetobacter harbinensis* and *Lactococcus piscium* were cultivated in static, mono-species biofilm models under conditions mimicking food-processing environments. After a seven-day long cultivation the samples the bacterial load and the presence of the three major EPS-matrix components (protein, eDNA and carbohydrates) were determined. All three isolates showed a high bacterial load; eDNA and carbohydrates were determined in biofilms of all three bacterial isolates. However, proteins were only detected in biofilm samples of *Microbacterium ssp*. and *Lactococcus piscium*. Furthermore, the swarming, swimming and twitching motility was analysed. Overall *Lactococcus piscium* showed a lower motility compared to *Microbacterium ssp.* and *Acinetobacter harbinensis.*

Considering previous studies on biofilm detection, the decisive criteria in the present study for a biofilm positive sample are the presence of cultivable bacteria and at least two matrix components in the sample. Thus, the study concluded that all three isolates are capable of biofilm formation. Furthermore, the study tested and determined the isolates' swimming, swarming and twitching motility ability. Thus, the study provides further knowledge on biofilm formation in the food industry.

6. Zusammenfassung

Biofilme sind Einzel- und/oder Mischpopulationen von Mikroorganismen, die in einer selbst produzierten Matrix aus extrazellulären polymeren Substanzen eingebettet liegen. In Lebensmittelbetrieben bieten feuchte Oberflächen und nährstoffreiche Umgebungen optimale Bedingungen für bakterielle Besiedelung und mögliche Biofilmbildung. Enger, biofilmbedingter Zellkontakt und der besondere Aufbau der Matrix verschaffen den Bakterien Vorteile für ihr Überleben. Ein Beispiel dafür ist die oftmals hohe Toleranz gegenüber antimikrobiellen Wirkstoffen. Für die Lebensmittelindustrie stellen Biofilme ein großes hygienisches Risiko dar – Folgen sind bakterielle Kontamination und Verderb von Lebensmitteln. Die vorliegende Arbeit beschäftigt sich mich mit der Entstehung und Bedeutung von bakteriellen Biofilmen in der Lebensmittelindustrie. Weiters wurden drei aus einem Lebensmittelbetrieb isolierte Bakterienisolate - *Microbacterium ssp.*, *Acinetobacter harbinensis* und *Lactococcus piscium* auf deren Fortbewegungs- und Biofilmbildungsvermögen untersucht (Wagner et al. 2020). Die Bakterien wurden hierfür in statischen Einzel-Spezies Biofilmmodellen kultiviert. Nach sieben Tagen wurden die Anzahl der Bakterien im Biofilm und das Vorhandensein der drei wichtigsten EPS-Matrix Bestandteile (Proteine, eDNA und Kohlenhydrate) analysiert. Die Untersuchungen zeigten hohe Bakteriendichte in allen Biofilmen. eDNA und Kohlenhydrate konnten in Biofilmen von allen drei Bakterienisolaten nachgewiesen werden; Proteine jedoch nur in den Biofilmen von *Microbacterium ssp*. und *Lactococcus piscium*.

Die vorliegende Studie konnte folglich zeigen, dass alle drei Isolate Biofilme bilden können. Des Weiteren wurden die Bakterien in Versuchen zu ihrer Motilität (schwimmen, schwärmen und twitching) näher untersucht. Alle drei Isolate zeigten Motilität, jedoch zeigte *Lactococcus piscium* ein geringeres Fortbewegungsvermögen als *Microbacterium ssp.* und *Acinetobacter harbinensis*. Die vorliegende Arbeit trägt daher einen kleinen Beitrag zum Verständnis und Wissen über bakteriellen Biofilm bei.

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