

Characterisation of *Lactobacillus plantarum*

single and multi-strain
biofilms

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**Characterisation of
Lactobacillus plantarum
single and multi-strain biofilms**

Mónica D. Fernández Ramírez

Thesis

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Table of contents

Chapter 1	7
General introduction and outline of the thesis	
Chapter 2	33
Characterisation of biofilms formed by <i>Lactobacillus plantarum</i> WCFS1 and food spoilage isolates	
Chapter 3	51
Role of cell surface composition and lysis in static biofilm formation by <i>Lactobacillus plantarum</i> WCFS1	
Chapter 4	71
Quantitative assessment of viable cells of <i>Lactobacillus plantarum</i> strains in single and multi-strain biofilms	
Chapter 5	91
Strain-specific tracking of <i>Lactobacillus plantarum</i> in competitive multi-strain static and dynamic flow biofilm models	
Chapter 6	139
General discussion, conclusions and future perspectives	
Summary	161
Acknowledgements	165
About the author	167
List of publications	168
Overview of training activities	169

CHAPTER 1

General introduction and outline of the thesis

Introduction

Lactic acid bacteria (LAB) have been used for centuries as starter cultures for a large variety of fermented food products of meat, dairy and vegetable origins (Buckenhüskes, 1993; Caplice and Fitzgerald, 1999; Leroy and De Vuyst, 2004). More recently, LAB have been studied for their probiotic properties (Remus et al., 2011; Turroni et al., 2014). Besides these desired properties, LAB can cause food spoilage of a wide variety of products including beer (Sakamoto and Konings, 2003), sliced meat (Chenoll et al., 2006), salad dressings (Kurtzman et al., 1971) and ketchup (Bjorkroth and Korkeala, 1996). Food spoilage LAB typically belong to the genus *Lactobacillus*. *Lactobacillus* spp. are Gram-positive rods, generally regarded non-motile with the exception of twelve *Lactobacillus* species, including *Lactobacillus ruminis* (Neville et al., 2012) and *Lactobacillus ghanensis* (Nielsen et al., 2007). Lactobacilli mostly reported for food spoilage, include *L. brevis*, *L. buchneri*, *L. curvatus* and *L. plantarum* (Stiles and Holzapfel, 1997). *L. plantarum* has for example been found in grape must (Lonvaud-Funel, 1999) and causes spoilage of acidified food products (Sharpe and Pettipher, 1983).

L. plantarum displays large genotypic and phenotypic diversity allowing this species to occupy diverse ecological niches (Siezen and van Hylckama Vlieg, 2011; Siezen et al., 2010), thereby facilitating transmission from plant rhizosphere to foods. Its presence in food is further supported by adaptive responses to food preservation stresses such as low pH, weak organic acids and high salt concentrations (Sanders et al., 2015; van de Guchte et al., 2002). *L. plantarum* requires nutrient-rich environments (Teusink et al., 2005; Wegkamp et al., 2010) and food products offer a variety of carbohydrates, nitrogen sources, vitamins and cofactors to support its growth.

Besides the above mentioned growth supporting factors, manganese (in the oxidation state Mn(II)) is important for the defence against reactive oxygen species (ROS) in *L. plantarum*. Most microorganism use superoxide dismutase to cope with ROS; however, *L. plantarum* and several other *Lactobacillus* species (e. g. *L. fermentum* and *L. casei*) lack this enzyme but accumulate Mn(II) to over 30 mM intracellularly as an alternative mechanism to cope with ROS (Archibald and Duong, 1984; Archibald and Fridovich, 1981).

While animal derived sources contain high levels of haem-bound iron, plant derived foods can contain high concentrations of Mn(II). In food products, Mn(II) is present in concentrations ranging between 180 nM to 0.25 mM (USDA, 2015). Standard growth media for *L. plantarum*, such as MRS (de Man, Rogosa and Sharp (De Man et al., 1960)) contain 0.265 mM Mn(II).

Acquisition of Mn(II) in *L. plantarum* is mediated by several transporter systems including a Mn(II) and Cd(II) specific P-type ATPase (MntA) (Hao et al., 1999). Moreover, two Nramp-type

transporters, MntH1 and MntH2, and a Mn(II) specific ABC-transporter (MtsCBA) appear involved in uptake of this metal ion since corresponding genes are upregulated when Mn(II) concentrations in the medium are limiting (Nierop Groot et al., 2005) in agreement with responses reported for known Mn(II) transporters in *Escherichia coli*, *Salmonella typhimurium*, *Streptococcus gordonii* and *S. pneumoniae* (Jakubovics and Jenkinson, 2001). More transporters with affinity for Mn(II) may be present in *L. plantarum* since the deletion of individual or combined MtsA/MntH2 systems did not affect intracellular Mn(II) concentrations (Nierop Groot et al., 2005).

L. plantarum lacks a complete electron transport chain (ETC) and therefore was considered only capable of fermentative growth. However, it was shown that addition of haem and vitamin K2 could restore the functionality of the ETC (Brooijmans, de Vos, et al., 2009). The ETC in *L. plantarum* consists of NADH dehydrogenase, menaquinone (vitamin K2) and cytochrome oxidase, using oxygen as a final electron acceptor (Brooijmans, de Vos, et al., 2009). Respiration yields more energy per glucose molecule thereby resulting in increased biomass and less acidification as it introduces a shift from homolactic to mixed acid fermentation with production of acetate and acetoin instead of lactate compared to fermentation (Brooijmans, Smit, et al., 2009). Addition of Mn(II) to the growth medium of *L. plantarum* stimulated growth under respiratory conditions and resulted in higher cell densities (Watanabe et al., 2012). Watanabe et al. (2012) previously reported that *L. plantarum* WCFS1 cells originated from respiratory grown cultures (with added haem and vitamin K2) showed higher resistance to hydrogen peroxide in comparison to cells grown under fermentative and aerobic conditions. Nevertheless, similar activity levels of haem-dependent catalase were present in cells grown under aerobic conditions (with only haem added). The results obtained indicate that oxidative stress resistance of *L. plantarum* is affected by respiratory growth. Notably, respiratory growth has been shown to lead to the production of ROS caused by leakage of electrons from the electron transport chain intermediates by the conversion of NADH to NAD⁺ (Kohanski et al., 2007). It is conceivable that generation of ROS in respiratory grown *L. plantarum* induced oxidative stress resistance mechanisms resulting in more robust cells.

Within the *Lactobacillus* genus, *L. plantarum* WCFS1 was the first strain for which the full genome sequence was published (Kleerebezem et al., 2003). *L. plantarum* WCFS1 is used as a model strain to study the probiotic properties of LAB (Boekhorst et al., 2006; Remus et al., 2011) and fermentative properties since it is a well-studied strain that is genetically accessible. The genome-scale metabolic model (Teusink et al., 2006) and the reconstruction of the metabolic pathways (Teusink et al., 2005) of this strain supported the study of relevant biological processes.

Biofilms

A potential source of contamination of food products by lactobacilli is its presence in biofilms formed either on raw materials (Kubota et al., 2008) or during processing in food production environments (Lee Wong, 1998; Somers et al., 2001).

Biofilms are composed of microorganisms attached to a surface and are embedded in a matrix of extracellular polymeric substances (EPS) (Watnick and Kolter, 2000). The first report of a biofilm was by Antonie van Leeuwenhoek in 1684, who observed accumulation of bacteria on tooth surfaces as described in a review on microbial biofilms by Donlan (2002). Later, Heukelekian and Heller (1940) and Zobell (1943) described the distribution of marine microorganisms on glass surfaces and sand. Biofilm formation was identified in the fossil records in the South African Kornberg formation, a hydrothermal environment, dating from 3.3-3.4 billion years ago. In this fossil, coccoid and rod-shaped bacteria were embedded in granular or smooth surfaces which were interpreted as biofilms (Westall et al., 2001).

Two general models for biofilm formation have been proposed previously in literature (Lemon et al., 2008), one for motile and one for non-motile microorganisms. Figure 1.1 shows the general model of biofilm formation for motile and non-motile microorganisms (Lemon et al., 2008). The initial attachment to surfaces is for most motile bacteria dependent on flagella or type IV pili for Gram-negative bacteria that allow active movement to a surface. Following the initial attachment, bacteria lose motility (Figure 1.1A). For non-motile microorganisms, initial surface attachment depends on a passive process, driven by sedimentation, electrostatic interactions and cell surface proteins which favour the interaction among cells and between the cells and the abiotic surface (Figure 1.1B) (Lemon et al., 2008). Adherence to a surface is reversible at the initial attachment phase.

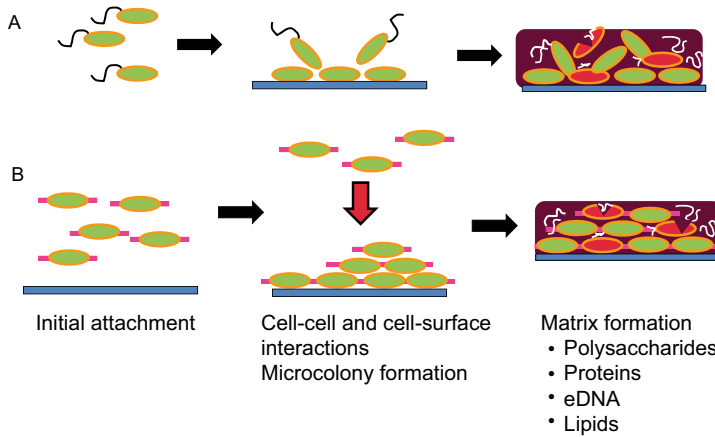


Figure 1.1. Biofilm formation model for (A) motile and (B) non-motile microorganisms. Adapted from Lemon et al. (2008).

As most of the research has been performed with motile bacteria, the role of flagella in the initial attachment to surfaces has been extensively reviewed for *Salmonella*, *Listeria*, *Bacillus* and *Pseudomonas* (Davey and O'Toole, 2000; Simões et al., 2010; van Houdt and Michiels, 2010). For some non-motile Gram positive bacteria, pili or fimbriae were involved in attachment including the pathogenic species *Corynebacterium diphtheriae* (Ton-That and Schneewind, 2003), *Enterococcus faecium* (Hendrickx et al., 2008), *Enterococcus faecalis* (Nallapareddy et al., 2006) as well as several *Streptococcus* species such as *S. pneumoniae* (Barocchi et al., 2006), *S. pyogenes* (Nakata et al., 2009) and *S. suis* (Fittipaldi et al., 2010). Pili producing LAB include *L. rhamnosus* GG (Reunanen et al., 2012) and several *Bifidobacterium* species, namely *B. longum* subsp. *longum*, *B. dentium*, *B. adolescentis* and *B. animalis* subsp. *lactis* (Foroni et al., 2011). The relevance of pili in biofilm formation was demonstrated for *L. lactis* by overexpression of the *pil* operon (*yhgD*, *yhgE*, and *yhhB*) in strain *L. lactis* IL1403 (Oxaran et al., 2012). Overexpression of pili encoding genes induced auto-aggregation of cells in liquid cultures and resulted in high biofilm formation compared to the WT. Other *L. lactis* strains were screened for the presence of pili and in four out of eleven strains tested the presence of pili was detected. However, the biofilm forming capacity of these strains was not determined.

Notably, biofilm maturation requires cell-cell interaction and coordinated cellular responses. Cell-cell communication is possible via quorum sensing systems present in bacteria, in which signal molecules called autoinducers are produced, released and detected, inducing a response in the receiver cells. Quorum sensing systems respond to cell density and autoinducer concentrations and it enables bacteria to regulate gene expression required for bioluminescence, biofilm formation, sporulation, virulence and other cellular responses (Bassler, 2002; Parsek and Greenberg, 2005).

The biofilm matrix is composed of EPS released to ensure several functions, namely 1) to support the integrity of the biofilm, 2) to enhance cell-cell interactions and 3) to protect cells embedded in the biofilm against environmental stresses. The biofilm matrix is often composed of lipids, proteins, polysaccharides and extracellular DNA (eDNA). The exact composition of the matrix varies among species (Flemming and Wingender, 2010). In addition, membrane vesicles were found to be part of the biofilm matrix of Gram negative microorganism such as *Pseudomonas aeruginosa* (Schooling and Beveridge, 2006).

Proteins, including enzymes, secreted proteins, adhesins, carbohydrate binding proteins, biofilm associated proteins, lectins and pili/flagella have been described previously as relevant matrix components (Branda et al., 2005; Flemming and Wingender, 2010). Several studies point to a role of biofilm associated proteins for cell-surface and cell-cell interactions in *Staphylococcus*, *Enterococcus*, *Salmonella* and *Pseudomonas* (Cucarella et al., 2001; Espinosa-Urgel et al., 2000; Latasa et al., 2005; Tendolkar et al., 2004; Toledo-Arana et al., 2001). The role of cell surface proteins in host-microbe interaction has been analysed using model systems following treatment of microbial cells with proteases or lithium chloride (Lorca et al., 2002; Tallon et al., 2007). The treatment with lithium chloride or proteases is used to extract cell surface associated proteins by 'shaving' the cell surface. For *Lactobacillus* species this treatment reduced the attachment capacity to fibrinonectin, mucus and Caco-2 cells thereby suggesting a role of surface proteins in attachment in biotic surfaces (Lorca et al., 2002; Tallon et al., 2007; Tuomola et al., 2000). Nevertheless, no further work has been performed to determine the relevance of cell surface associated proteins in attachment to abiotic surfaces.

The relevance of polysaccharides in biofilm formation and as a component of the matrix has been extensively studied in biofilms growing in static or dynamic flow conditions, in pellicles and in colony biofilms (Branda et al., 2005; Sutherland, 2001). Polysaccharides produced by bacteria can be either located extracellularly, where they are loosely attached to the cells or released in the medium, or covalently attached as so-called capsular polysaccharides (Branda et al., 2005; Flemming and Wingender, 2010). The best studied extracellular polysaccharides are those formed by *P. aeruginosa* that support surface attachment and subsequent biofilm maturation under dynamic flow conditions (Ryder et al., 2007). Extracellular polysaccharides have also been studied in *L. rhamnosus* GG in the context of its probiotic properties. *L. rhamnosus* GG produces a galactose containing polysaccharide (Francius et al., 2008; Landersjö et al., 2002) and the presence of this polysaccharide decreased attachment to polystyrene surfaces and subsequent biofilm forming capacity, conceivably due to shielding of other cell surface structures like pili (Lebeer et al., 2009). The role of capsular polysaccharides in biofilm formation on (a)biotic surfaces has been extensively studied for various species including the human pathogen *S. pneumoniae*. The loss of capsular polysaccharides by this species increased the total

biofilm formed on abiotic polystyrene surfaces (Moscoso et al., 2006; Muñoz-Elías et al., 2008). Both enhancing and inhibiting properties have been reported for polysaccharides depending on the species and stages of biofilm formation studied (Starkey et al., 2004).

Earlier studies have noted the importance of eDNA for some species in the irreversible attachment and structure stability of biofilms in conjunction with other biofilm matrix components (Flemming and Wingender, 2010). eDNA is a component of the biofilm matrix of several species including *Listeria monocytogenes*, *E. faecium*, *L. plantarum* LM3-2 and *Staphylococcus aureus* (Hall-Stoodley et al., 2008; Harmsen et al., 2010; Jakubovics et al., 2013; Muscariello et al., 2013; Paganelli et al., 2013; Rice et al., 2007) and can be relevant for the initial attachment of microorganisms to surfaces (Paganelli et al., 2013; Qin et al., 2007). DNA can be released into the biofilm matrix by cell lysis or via another mechanism that involves 'active' DNA release via membrane vesicles or direct DNA secretion (Jakubovics et al., 2013; Okshevsky and Meyer, 2015). Cell lysis is the most common mechanism of DNA release for different species including enterococci (Guiton et al., 2009) and staphylococci (Qin et al., 2007; Rice et al., 2007). Furthermore, it is conceivable that autolysins play a role in cell wall degradation leading to high levels of eDNA and thereby supporting biofilm formation (Bayles, 2007; Frese et al., 2013).

Mixed species biofilms in a food context

In nature, biofilms are assumed to be composed of more than one species and/or strains (Elias and Banin, 2012; Yang et al., 2011). In mixed species biofilms, a competition for space and nutrients takes place and positive, synergistic or negative effects between the different species may occur (Elias and Banin, 2012; Rendueles and Ghigo, 2012).

Interactions of pathogens and non-pathogens have been studied for example in biofilms composed of *L. monocytogenes* – *L. plantarum* (van der Veen and Abee, 2011b) and *L. monocytogenes* – *Pseudomonas* (Saá Ibusquiza et al., 2012). *L. plantarum* can share habitats with *L. monocytogenes*, for example both species have been found in salami (Campanini et al., 1993) and green table olives (Caggia et al., 2004). Moreover, they are able to form mixed species biofilms which resulted in increased resistance of *L. monocytogenes* to disinfectants (van der Veen and Abee, 2011b), likely due to diffusion limitation. *L. lactis* is frequently used in dairy fermentations and the ability of this microorganism to form a mixed biofilm with *L. monocytogenes* has also been studied. *L. lactis* outcompetes *L. monocytogenes* by reducing the attachment and growth of *L. monocytogenes* (Habimana et al., 2011).

Complex biofilms can be found in the oral cavity containing over 700 bacterial species, including the phyla *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, and from this set over 100 different species are present in an individual's mouth as dental plaque (Paster et al., 2006). It has been shown that initial biofilm formation depends on specific strains serving either as an anchor or serving a role in preconditioning of the surface (Leung et al.; Yamada et al., 2005). For biofilms on teeth, *Lactobacillus* spp. are among the first colonizers, along with *Actinomyces* spp., *Streptococcus* sp., and *Candida* sp. (Zijne et al., 2010) subsequently paving the way for full maturation of the oral biofilm.

Tools to study biofilm formation

In the food industry, biofilms can be formed under static conditions if equipment design is not optimal leaving dead ends, or in vats and containers or under dynamic conditions in pipelines and drains (Boulané - Petermann, 1996).

A static biofilm model is often used for research in the laboratory in microtiter multi well plates as it allows high throughput analysis of factors affecting biofilm formation. Using a static model, different variables for biofilm formation such as different media compositions, temperature and mutants can be tested in parallel. Additionally, different materials can be used as coupons from different materials can be inserted when 12 or 24 wells are used (Merritt et al., 2005).

Dynamic flow conditions (Figure 1.2) are achieved by growing the biofilm in a flow cell providing a continuous flow of nutrients (Sternberg and Tolker-Nielsen, 2006). Flow cells are commercially available and provided by IBI Scientific (Iowa, USA) or BioSurface Technologies (Montana, USA). In contrast to the IBI Scientific system that allows only polystyrene surfaces to be analysed, different surfaces can be analysed with the BioSurface system; however, the latter is not a high throughput system.

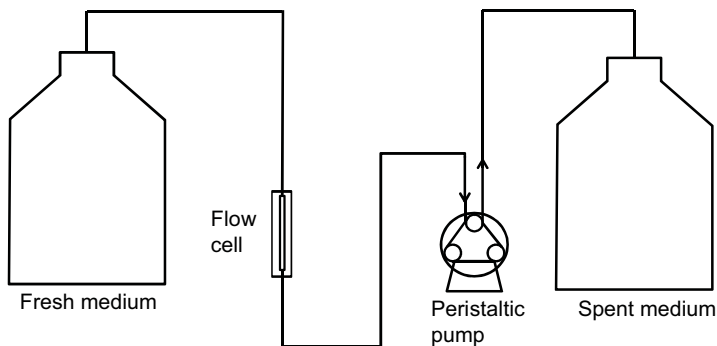


Figure 1.2. Schematic overview of a dynamic flow cell circuit to study biofilms.

The biofilms resulting from dynamic or static growth can be different. As an example, *L. monocytogenes* biofilms display a “network of knitted chains” under dynamic conditions whereas grown under static conditions unstructured biofilms are formed (Rieu et al., 2008). Additionally, it is conceivable that under dynamic conditions oxidative stress is increased because of continuous exposure to fresh oxygenated medium. Moreover, other stress mechanisms could affect biofilm formation and consequently the physiological status of cells within the biofilm. For *L. monocytogenes* it was shown that the generation of genetic variants in biofilms grown under dynamic conditions is considerably faster in comparison to the generation rate under static biofilm formation (van der Veen and Abee, 2011a) stressing the impact of oxidative stress on heterogeneity and diversity in the cell population.

Biofilm quantification and visualisation

Biofilm formation can be analysed by several methods. Table 1.1 provides an overview of the tools used for biofilm quantification and visualisation describing the main advantages and disadvantages of each method.

A widely used method is the crystal violet (CV) assay. In this assay, CV binds to all biofilm components including the matrix and the dead and living cells. The CV assay is a good technique for a general and high throughput evaluation of biofilm formation capacity. This method has been applied for a wide range of microorganisms (Merritt et al., 2005; Peeters et al., 2008). This assay destroys the original biofilm structure due to the washing steps involved, but enables quantification of biofilms that are firmly attached in a range of conditions. Notably, this assay does not reveal information about the physiological state and culturability of the cells in the biofilm. For *Bacillus cereus*, a linear relation between CV assay and the number of culturable cells in the biofilm was observed (Hayrapetyan et al., 2015), but this is not the case for microorganisms such as *L. monocytogenes* (Kadam et al., 2013). In *L. monocytogenes* it was found that the CV assay did not show a linear relation with the number of culturable cells because of the high amount of dead cells present as well as eDNA (Kadam et al., 2013).

The number of cells in the biofilm can be assessed in different ways. Plate count enumeration is one option; however, only culturable cells can be determined by this method and not the total number of viable cells (Postollec et al., 2011). For these purposes, quantitative PCR can be performed taking into account some further considerations. For instance, the number of cells can be overestimated when eDNA is present. However, a DNA intercalating agent such as propidium monoazide can be used to avoid over-quantification (Nocker et al., 2006) and to assure that only cells without a compromised membrane (referred to as viable cells) are detected. The primers have to be specific and optimised for quantification. Quantitative PCR therefore is very precise and provides useful information on relative abundance of individual strains in mixed species biofilms and to evaluate the contamination or recontamination potential of the viable cells in the biofilm.

Fluorescence microscopy is a convenient method to determine cell viability by the use of fluorescent dyes such as Syto9 and propidium iodide. Moreover, this method can give an indication of the presence of certain matrix components such as eDNA by the use of fluorescent DNA binding dyes. The main disadvantage is that it provides no insight on the biofilm structure or the cell distribution in a biofilm.

Table 1.1. Tools for biofilm quantification and visualisation.

Technique	Advantages	Limitations
Crystal violet assay	High throughput method when multiple wells are used	No information of physiological state or composition of the biofilm
	Inexpensive	No information about structure
	Total biomass quantification	
Cell enumeration by plate counting	Quantification of culturable cells in the biofilm	Only culturable cells are quantified
		Underestimation can occur when clumping or chaining cells are present
Quantitative PCR	Quantification of intact cells (without a compromised membrane) when DNA intercalators are used.	Exclusive primers can only be developed when the genome sequence is available
	Without intercalators eDNA can also be quantified	Good quality DNA is needed
		Depending on the amount of samples it can be laborious
Next generation sequencing	Quantification and identification of individual strains in complex biofilms.	
	Based on variations of 16S rRNA genes or other conserved regions containing SNPs	Strains from the same species cannot be discriminated when 16S rRNA gene is analysed
	Genome information is not required if 16S rRNA gene is used	
	The price is still high but decreasing rapidly	
Fluorescence microscopy	Qualitative information of dead and living cells.	No structures can be observed Fluorescent reporters, dyes or auto fluorescence needed
Confocal Laser Scanning Microscopy	High throughput for static biofilms	
	Three-dimensional analysis of intact biofilms.	Fluorescent reporters, dyes or auto fluorescence needed
	Macromolecules and cells are localized when fluorescent probes are used.	Limitation in the depth of laser penetration for image acquisition
Scanning Electron Microscopy	Biofilms can be analysed on any surface	Sample needs to be dehydrated
	3D impression of the surface	Loss of structure Artefacts in the image due to dehydration
Magnetic Resonance Imaging	Intact biofilms are analysed	Limited resolution
	Information about chemical composition	Not magnetic surfaces can be used to grow biofilms
Scanning Transmission X-ray	Analysis of inorganic and organic compounds	Synchrotron and beam time necessary
	Quantitative mapping of chemical compounds	Maximum sample thickness 300 nm
	Analysis of intact biofilms	Destructive method

Confocal laser scanning microscopy (CLSM) is not only a way to visualise the 3D structure of the biofilm but it can also be used for quantification of living or dead cells and matrix components such as eDNA if combined with fluorescent labelling (Stoodley et al., 1999). Fluorescent labelling of cells can be achieved by stains such as propidium iodide and Syto9 or by fluorescent reporters. The cells can then be quantified in an indirect way as biovolume which reflects calculation of a cell's volume in three-dimensional space with fluorescent signal. The quantification can be done by the use of image-processing platforms such as COMSTAT (Heydorn et al., 2000) and Icy (de Chaumont et al., 2012), among others. With this technique, the 3D structure of the biofilms can be visualised as the biofilm is not disturbed and it can be adapted for high throughput imaging (Bridier et al., 2010).

Another way to visualise biofilms is by scanning electron microscopy (SEM). This technique offers a higher magnification and therefore the surface of the biofilm and single cells can be visualised. The disadvantage of this technique is that the sample is dehydrated and the structure of the biofilm is disturbed as a major component is water. Dehydration can create artefacts and it is important to combine with another technique to visualise the biofilm 3D structure (Alhede et al., 2012).

Additional methods include magnetic resonance imaging (MRI) and scanning transmission X-ray. MRI can be used for measuring the diffusion of molecules and transport characteristics within microbial biofilms (Neu et al., 2010). The scanning transmission X-ray method can be used to map biomolecules and metals (Neu et al., 2010). These two techniques are not widely used because they are still very specialised and expensive equipment as well as expert operators are needed (Neu et al., 2010).

For the ideal visualisation, characterisation and quantification of relevant biofilm parameters, multiple complementary techniques are combined (Alhede et al., 2012; Bridier et al., 2013).

Genomic tools

Linking diversity in biofilm formation to genomic content of biofilm forming species can aid in understanding factors involved in biofilm formation. In the past, genomic diversity within species was determined using genome hybridization on microarrays (Malloff et al., 2001). This technique was previously applied to determine the genomic diversity of 42 *L. plantarum* strains (Molenaar et al., 2005; Siezen et al., 2010). However, nowadays the costs of genome sequencing have decreased dramatically and whole genome information is in reach to provide insight in species diversity.

Whole genome sequencing and annotation make it possible to link genotypes to phenotypes. The number of genomes available is increasing rapidly as a result of next generation sequencing activities. Next generation sequencing allows not only massive DNA parallel sequencing but it is also used to gain information on gene expression by sequencing total RNA (Todd et al., 2016).

Currently, there are several next generation sequencing platforms including Roche 454-pyrosequencing, Illumina-Solexa (MiSeq), Life Sciences Ion-Torrent and Pacific Biosciences Single-molecule real-time sequencing. A good genome annotation is crucial for genome comparison purposes in so called gene-trait matching (Dutilh et al., 2013). It is important to note that not all phenotypes are linked to presence or absence of genes but are also affected by gene expression levels. Thus, a combination of gene content comparison and transcriptomics or proteomics is also valuable.

PhenoLink is a tool which has been developed to link phenotypes to presence or absence of certain genes (Bayjanov et al., 2012). The potential power of gene-trait matching approaches was recently shown for *B. subtilis* leading to the identification of a mobile genetic element (Tn1546-like) which confers increased heat resistance to *B. subtilis* (Berendsen et al., 2016). In an effort to link expression levels to phenotypes, a transcriptome-phenotype matching fermentation platform (FermDB) was developed for *L. plantarum* (Bron et al., 2012). This platform was constructed by full genome transcriptomics of *L. plantarum* WCFS1 obtained under diverse fermentation conditions and in parallel study of physiological characteristics. FermDB helped to identify the role of conserved genes during oxidative stress and growth (Bron et al., 2012) and it was also used to identify genes responsible for GI tract persistence (van Bokhorst-van de Veen et al., 2012).

Impact of bacterial biofilms for industry

According to the Food and Agriculture Organization of the United Nations (FAO, 2011) one third of the food for human consumption is wasted or lost. These data include food loss due to physical and chemical damage and also spoilage. Food waste causes great economic losses, for example it has been reported that annual losses are in the range of US\$ 680 billion in industrialized countries and US\$ 310 billion in developing countries. The bacterial food spoilage could be partially attributed to contamination by biofilms (Olaimat and Holley, 2012). Biofilms in the food industry have proved to be very problematic as they are not only responsible for contamination and recontamination of the food products but they also cause corrosion in equipment, reduced heat transfer, modification of fluid dynamics and limit the operational time due to cleaning of equipment between

production runs (Characklis and James D. Bryers, 2009; Gilbert et al., 2003; Verran, 2002). Moreover, the annual worldwide process engineering costs related to biofouling (biofilms and organic deposits), could reach up to billions of dollars for the combined food, pharma and biotechnological sectors (Riedewald and Sexton, 2006).

Furthermore, the eradication of the biofilms can be very difficult and the microorganisms could be released from the biofilm into the production line resulting in spoilage or disease when pathogens are involved (Lee Wong, 1998; Simões et al., 2010). The microorganisms within a biofilm have a different phenotype in comparison to their planktonic counterpart. Often, the cells located inside and/or originating from biofilms are more resistant to cleaning agents, disinfectants and antibiotics (Bridier et al., 2011; van Houdt and Michiels, 2010). Different mechanisms have been described leading to increased resistance: limited penetration of antimicrobial agents caused by the biofilm matrix, reduced growth rate, phenotypic adaptation or gene transfer and/or mutation (Bridier et al., 2011; Jahid and Ha, 2012).

***Lactobacillus* biofilms**

A possible source of contamination or recontamination of food products by lactobacilli are the biofilms on raw materials (Jahid and Ha, 2012; Olaimat and Holley, 2012) or in processing equipment Somers et al. (2001). Furthermore, *Lactobacillus* spp. capable of biofilm formation have been isolated from a range of raw materials including onions and olives (Domínguez-Manzano et al., 2012; Kubota et al., 2008).

Somers et al. (2001) reported biofilm formation from nonstarter LAB on stainless steel equipment in a cheese factory. Biofilm forming capacity has been reported for some *Lactobacillus* strains including *L. rhamnosus* GG (Lebeer, Verhoeven, et al., 2007), *L. reuteri* (Tannock et al., 2005), *L. fermentum*, *L. acidophilus* (Millsap et al., 1997) and *L. plantarum* (Kubota et al., 2008; Kubota et al., 2009; Millsap et al., 1997; Sturme et al., 2005).

In addition, it was found that *L. plantarum* cells within a biofilm were more resistant to ethanol and acetic acid in contrast to their planktonic counterparts (Kubota et al., 2008); this increased resistance may result from diffusion limitation of disinfectants in the biofilm matrix. Kubota et al. (2009) found that *L. plantarum* JCM 1149 displayed higher resistance to several organic acids used in food preservation when grown as a biofilm. Organic acids such acetic and lactic acid are also found in natural fermented products. The cells originating from resuspended biofilms were also more resistant compared to the planktonic cells (Kubota et al., 2009). These results show that cells originating from a biofilm pose a risk for contamination of the food products during processing.

The structure and morphology of biofilms is quite diverse and species dependent. For example, the morphology of *L. plantarum* WCFS1 biofilms can be described as “flat and homogeneous” with hollow voids (Aoudia et al., 2016) whereas other microorganisms such as *P. aeruginosa* form the characteristic mushroom structures.

The biofilm matrix of *L. plantarum* LM3 has been suggested to be composed of eDNA and proteins because treatments with Proteinase K or DNase removed a significant fraction of the biofilm (Muscariello et al., 2013). However, the impact on the number of cells remaining in the biofilm was not determined and the potential for recontamination was not analysed. The elucidation of the matrix composition is important to design efficient cleaning and disinfecting strategies for the food industry.

Genes involved in *Lactobacillus* biofilm formation

By comparing the performance of the wild type (WT) strains and mutant derivatives some biofilm formation mechanisms have been identified. Most data result from *in vitro* studies that aim to analyse biofilm formation as a probiotic trait. For example, by using *Lactobacillus rhamnosus* GG (ATCC 53103), which has shown not only adherence to epithelial cells and mucus *in vitro*, but also to abiotic surfaces (Lebeer, Verhoeven, et al., 2007). In addition, the effect of deletion of *wzb*, which regulates the synthesis of extracellular polysaccharides and *dltD*, an important factor for lipoteichoic acid synthesis, were studied. For the WT and mutants, biofilm formation on polystyrene was variable and depended on the growth medium. However, the effect of the deletion of *dltD* resulted generally in increased biofilm formation measured by CV staining (Lebeer, Verhoeven, et al., 2007).

A cell surface associated protein that has been characterised is the enolase EnoA1, which was found to be involved in the attachment of *L. plantarum* LM3 to human fibronectin (Castaldo et al., 2009). The deletion of *enoA1*, which encodes for EnoA1 alfa enolase, resulted in reduced biofilm formation measured as CV compared to the WT (Vastano et al., 2016).

In addition to cell surface properties, quorum sensing can affect biofilm formation. The quorum sensing associated gene *luxS* was studied in *L. rhamnosus* and *L. reuteri* (Lebeer, De Keersmaecker, et al., 2007; Tannock et al., 2005). This gene encodes the enzyme LuxS which catalyses the conversion of S-ribosylhomocysteine, yielding autoinducer 2 (AI-2) and homocysteine (Sturme et al., 2007). In *L. reuteri* the deletion of *luxS* resulted in a thicker biofilm on a plastic surface (nature not specified) under dynamic flow conditions (Tannock et al., 2005), but the effect of the deletion of this gene on static biofilm formation has not been studied.

Deletion of *luxS* not only affected total biofilm formation (measured with CV) in *L. rhamnosus* GG but also growth. Reduced biofilm formation under static conditions on polystyrene was found; moreover, the phenotype could not be restored to that of the WT when the signalling molecule AI-2 was supplemented to the medium (Lebeer, Verhoeven, et al., 2007). Lebeer, Verhoeven, et al. (2007) found that LuxS also plays a role in bacterial growth due its role in metabolism of methionine and cysteine. This might offer an explanation for the observation that the effect *luxS* deletion varied depending on the medium used for biofilm formation (Lebeer, Verhoeven, et al., 2007). Sturme et al. (2005) identified the locus *lamBDCA* as an *agr*-like module in *L. plantarum* WCFS1 based on its homology to the *agr* quorum sensing system in staphylococci. The regulator *lamA* is involved in the attachment to glass surfaces and the genes *lamBD* are responsible for the synthesis of the autoinducing peptide precursor (LamD₅₅₈). Later, Fujii et al. (2008) studied the two component system composed of *lamK* and *lamR*, and reported that *lamR* and *lamA* are cooperative genes playing a role in adhesion of *L. plantarum* WCFS1 cells to glass surfaces.

Several stress response mechanisms also play a role in biofilm formation. The FtsH enzyme has a dual chaperon-protease function and is responsible for degrading or refolding aberrant proteins generated during stress conditions (Akiyama, 2009). Fiocco et al. (2009) identified *ftsH* as a member of the Class III stress gene Repressor (CtsR) regulon in *L. plantarum* WCFS1 and confirmed that this gene is important for stress response. The deletion of this gene resulted in lower biofilm formation compared to the WT when grown in static conditions in polystyrene wells. This phenotype was attributed to the possible role of *ftsH* in the modification of the cell envelope, thereby changing its physicochemical properties (Bove et al., 2012).

Specific gene transcription regulators have been reported to influence biofilm formation capacity. The catabolite control protein A (CcpA) is a global regulator of gene expression in Gram positive bacteria regulating carbon and nitrogen metabolism (Fujita, 2009; Zomer et al., 2007). Muscariello et al. (2013) suggested a role of CcpA in biofilm formation of *L. plantarum* LM3 since deletion of its gene resulted in lower biofilm formation measured as CV compared to the WT under static conditions in polystyrene wells. The authors suggested a role of CcpA controlled genes *flmA*, *flmB*, and *flmC* in this process. The identified Flm proteins may be involved in autolysis and cell wall integrity and belong to the LytR family (Muscariello et al., 2013). In addition, by analysing exponentially growing cells, Vastano et al. (2015) showed by transcriptional analysis that CcpA might be responsible for the regulation of capsular polysaccharides clusters in *L. plantarum* WCFS1. Moreover, CcpA also affects biofilm formation of *Bacillus subtilis* (Stanley et al., 2003), *Streptococcus mutans* (Wen and Burne, 2002) and *S. aureus* (Seidl et al., 2008).

Proteomic approaches have been used to identify candidate genes or proteins which are related to biofilm formation. Using a proteomics approach, De Angelis et al. (2015) studied the differences between planktonic and biofilm grown *L. plantarum* DB200 cells. In total 115 proteins were differentially expressed showing either higher or lower levels in biofilm growth with predicted roles distributed in almost all functional categories. Most of the redox and stress response proteins (14 out of 18) were overexpressed, indicating the relevance of stress response activation during biofilm formation. Interestingly, the major sortase A (SrtA) was the only representative of the group of cell wall and catabolic processes overexpressed during biofilm formation. In *L. plantarum* WCFS1, this protein was predicted to mediate covalent binding of 27 target proteins encoded in the genome including cell surface protein precursors, mucus binding proteins, hydrolase, mannose-specific adhesin and surface proteins with collagen binding domains (Boekhorst et al., 2005; Kleerebezem et al., 2010). The role of this enzyme in *L. plantarum* WCFS1 surface adhesion and biofilm formation is further discussed in chapter three of this thesis.

Outline of the thesis

This thesis focuses on the environmental factors and mechanisms influencing biofilm formation of *L. plantarum*. Additionally, population dynamics have been analysed in a dual and multi-strain setting. Mixed strain conditions allow to obtain a deeper understanding of the impact of several environmental conditions in population dynamics and to determine the individual contribution to biofilm formation.

Chapter 2 describes the impact of environmental factors such as nutrient sources, maturation time and temperature, on biofilm formation of *L. plantarum* WCFS1 and six *L. plantarum* food isolates FBR1-6. Furthermore, the composition of the biofilm matrix was analysed with enzymatic treatments where eDNA and proteinaceous material were found to be the main components. Moreover, it was shown that for *L. plantarum* biofilms the crystal violet (CV) assay, which is routinely used to quantify total biofilm formation, correlates poorly with the number of culturable cells in the biofilm. These results stress the relevance to use combined approaches in *L. plantarum* biofilm research.

Chapter 3 provides new insights into biofilm development by *L. plantarum* WCFS1 through comparative analysis of wild type and selected mutants with defects in cell surface composition including $\Delta srtA$ which codes for Sortase A, an enzyme responsible for the covalent attachment of surface proteins to the cell wall peptidoglycan, and $\Delta cps1-4$ affected in the production of one or more capsular polysaccharides. Furthermore, the importance of autolysis and the role of *acm2* the major autolysin in the development of the biofilm matrix is further analysed as well as other mutants with affected lysis properties.

In **Chapter 4**, the performance of six food spoilage related strains FBR1-6 and WCFS1 was studied in single and multi-strain competitive static biofilm models using strain-specific quantitative PCR. Propidium monoazide (PMA) enabled quantification of the viable cell fraction that following dispersal, determines the outgrowth potential and food spoilage risk. The obtained results show that the performance of individual strains in multi-strain cultures generally correlates with their performance in pure culture, and relative strain abundance in multi-strain biofilms positively correlated with the relative strain abundance in the respective suspended (planktonic) cultures. Notably, *L. plantarum* FBR5 was able to outcompete all other strains and showed the highest relative abundance in multi-strain biofilms. All the multi-strain biofilms contained a considerable number of viable cells, representing a potential source of contamination.

Chapter 5 describes the influence of maturation time, temperature and medium composition on the population dynamics of a mixture of 12 *Lactobacillus plantarum* strains with different origins, in competitive planktonic and surface-attached biofilm growth models. A next generation sequencing approach based on detection of strain specific alleles was used to determine the relative abundance of each strain in the different conditions. Data were obtained in the presence and absence of PMA, thus allowing for identification and quantification of relative contributions of each individual *L. plantarum* strain to the fraction of viable cells in planktonic and biofilm phase and the fraction of dead cells (with compromised membranes) and levels of eDNA in the biofilm matrix, respectively. The genome content of the two groups of dominating strains was explored to identify genetic factors that potentially contribute to biofilm forming capacity under static and dynamic flow conditions, respectively.

Chapter 6 discusses the results presented in this thesis that contribute to the understanding of mechanisms underlying biofilm formation and matrix composition of *L. plantarum* which allowed proposing a model for static biofilm formation. New insights obtained on performance in multi-strain static and dynamic flow competitive biofilm models are discussed, next to biofilm control strategies, and a final section on future perspectives of *L. plantarum* biofilm research.

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CHAPTER 2

Characterisation of biofilms formed by *Lactobacillus plantarum* WCFS1 and food spoilage isolates

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Abstract

Lactobacillus plantarum has been associated with food spoilage in a wide range of products and the biofilm growth mode has been implicated as a possible source of contamination.

In this study we analysed the biofilm forming capacity of *L. plantarum* WCFS1 and six food spoilage isolates. Biofilm formation as quantified by crystal violet staining and colony forming units was largely affected by the medium composition, growth temperature and maturation time and by strain specific features. All strains showed highest biofilm formation in Brain Heart Infusion medium supplemented with manganese and glucose. For *L. plantarum* biofilms the crystal violet (CV) assay, that is routinely used to quantify total biofilm formation, correlates poorly with the number of culturable cells in the biofilm. This can in part be explained by cell death and lysis resulting in CV stainable material, conceivably extracellular DNA (eDNA), contributing to the extracellular matrix. The strain to strain variation may in part be explained by differences in levels of eDNA, likely as result of differences in lysis behaviour. In line with this, biofilms of all strains tested, except for one spoilage isolate, were sensitive to DNase treatment. In addition, biofilms were highly sensitive to treatment with Proteinase K suggesting a role for proteins and/or proteinaceous material in surface colonisation. This study shows the impact of a range of environmental factors and enzyme treatments on biofilm formation capacity for selected *L. plantarum* isolates associated with food spoilage, and may provide clues for disinfection strategies in food industry.

Introduction

Lactic acid bacteria (LAB) have been used by the food industry for centuries as starter cultures in food fermentation processes for the production of dairy, meat and vegetable products (Buckenhüskes, 1993; Caplice and Fitzgerald, 1999; Leroy and De Vuyst, 2004) and are widely researched for its probiotic properties (Boesten and de Vos, 2008; Lee et al., 2013; Turrone et al., 2013). Besides their beneficial role, LAB also pose a challenge to food producing industries as they can be associated as spoilage microorganisms in a wide variety of products (Bartowsky and Henschke, 2008; Samelis et al., 2000) including ketchup (Bjorkroth and Korkeala, 1996), marinated herring (Lyhs et al., 2001), sliced meat products (Bjorkroth and Korkeala, 1997; Chenoll et al., 2006) and salad dressings (Kurtzman et al., 1971). LAB associated with food spoilage generally belong to the genus *Lactobacillus* and display a large variability in behaviour. The latter was also shown in a recent phenotype characterisation study with *Lactobacillus* spoilage strains investigating its response to organic acids used in food preservation (Sanders et al., 2014).

A potential cause of contamination of food products by lactobacilli is via their presence in biofilms either on the raw material (Kubota et al., 2008) or formed during processing in a food production environment as for example shown for nonstarter LAB on stainless steel process equipment in a cheese factory (Somers et al., 2001). Biofilms are composed of microorganisms attached to a substratum and embedded in a matrix of extracellular polymers (Watnick and Kolter, 2000). Within this extracellular matrix, cells are protected from harsh environmental conditions including processing and disinfectant treatments used by food processors. Biofilm development can be divided in distinct stages and include an initiation stage by adherence of single cells to a surface, followed by micro colony formation and development to mature biofilms with concomitant production and/or release of extracellular polymeric substances including polysaccharides (Abee et al., 2011; O'Toole et al., 2000).

Biofilm formation by lactobacilli has been studied mostly in the context of survival and interaction with the host as relevant properties for probiotic bacteria (Lebeer, Verhoeven, et al., 2007) but the number of studies with focus on food spoilage is limited. A study on biofilm formation by LAB isolated from onions indicated that most isolates were capable to form a biofilm, albeit that the amount of biofilm formed varied from strain to strain (Kubota et al., 2008). The biofilm matrix for LAB has so far been poorly characterized, however, a protective function in resistance was shown against common disinfecting agents such as ethanol, sodium hypochlorite and organic acids used for food preservation such as acetic, citric, lactic and malic acids (Kubota et al., 2008; Kubota et al., 2009).

Most of the biofilm research has been carried out with motile bacteria such as *Salmonella*, *Listeria*, *Bacillus* and *Pseudomonas* (Davey and O'Toole, 2000; Simões et al., 2010; van

Houdt and Michiels, 2010) and the role of flagella in initial attachment to surfaces has been extensively reviewed (Pratt and Kolter, 1998; Stoodley et al., 2002; Vatanyoopaisarn et al., 2000). Non-motile bacteria cannot move actively to a surface but depend on sedimentation processes for adhesion to a surface (Lemon et al., 2008). In general LAB are described as non-motile microorganisms with the exception of some species such as *Lactobacillus ruminis* (Neville et al., 2012). In this context, the initiation of biofilm formation and maturation of non-motile bacteria is expected to be different from that reported for motile bacteria and underpins the relevance of understanding the mechanisms of biofilm formation by LAB.

The aim of this study was to analyse the biofilm forming capacity of *L. plantarum* WCFS1 and food spoilage isolates using a range of techniques including crystal violet (CV) staining, plate counting to quantify the number of culturable cells in the biofilms and fluorescence staining. DNase and Proteinase K treatments were used to assess the possible contribution of extracellular DNA (eDNA) and proteins and/or proteinaceous material to the *L. plantarum* biofilm matrix. This approach revealed that biofilm formation as determined by CV is not proportional to the number of culturable cells in *L. plantarum* biofilms and indicates the importance of plate counting in addition to CV measurements.

Materials and methods

Bacterial strains and culture conditions

Seven *Lactobacillus plantarum* strains including food spoilage isolates obtained from food manufacturers and *L. plantarum* WCFS1 as a reference strain were used in this study (Table 2.1). *L. plantarum* strains were streaked from a -80°C stock in De Man-Rogosa-Sharpe (MRS) agar (Merck) and incubated for 48 h at 30°C. Single colonies were inoculated in 10 ml of MRS at 30°C for 18 h to prepare a starting culture. In an initial screening for biofilm forming capacity, five different media were used: MRS medium, *Lactobacilli* AOAC medium (Difco), modified trypticase soy broth (Oxoid) supplemented with 2 % neutralised peptone (mTSB, Oxoid) (Lebeer, De Keersmaecker, et al., 2007), brain heart infusion (Becton Dickinson) supplemented with 0.005% manganese sulphate and 2 % glucose (Merck) (BHIMnG) to optimize growth of *L. plantarum* (van der Veen and Abee, 2011), and a chemically defined media (PMM7) described previously (Wegkamp et al., 2010).

Table 2.1. *L. plantarum* strains used in the present study

Strain designation	Alternative designation	Isolation source	Reference
FBR1	L21	Salad dressing	Sanders et al., 2014
FBR2	L30	Salad dressing	
FBR3	JH2	Salad dressing	
FBR4	TC157	Cheese with garlic	
FBR5	JV5	Salad dressing	
FBR6	La 10-11	Onion ketchup	
WCFS1		Human saliva	Kleerebezem et al., 2003

Biofilm assay

Screening experiments using different types of media

Biofilm formation was measured under static conditions as described elsewhere (Merritt et al., 2005). Briefly, 96-well polystyrene microtiter plates (Greiner Bio-One) were filled with 200 µl of either MRS, AOAC, mTSB, BHIMnG or PMM7 and inoculated with 1 % (v/v) of an overnight grown culture (18 h at 30°C) containing 8.5 log₁₀ colony forming units (cfu) per ml (18 h). The microtiter plates were incubated for either 24, 48 or 72 h at 30°C and biofilm formation was quantified using crystal violet (CV) assay and by plate counting to determine the number of culturable cells in the biofilm. For the CV assay (Merritt et al., 2005), the medium was carefully removed with the aid of a micropipette and the biofilms were washed three times by adding 225 µl of phosphate buffered saline (PBS, NaCl 8 g/L; KCl 0.2 g/L; Na₂HPO₄ 1.44 g/L; KH₂PO₄ 0.24 g/L; pH 7.4 (Merck)) to remove unattached cells. The resulting biofilm was stained for 30 minutes with 200 µl of 0.1 % (w/v) of CV (Merck), and excess of CV was removed by washing three times with 225 µl PBS as described above. The dye attached to the biofilm was solubilized in 70% ethanol for 30 minutes and the optical density (OD) was measured at 595 nm (SpectraMax, Molecular Devices). When OD values exceeded 1, samples were diluted in 70 % ethanol and resulting OD measurements were corrected for the dilution factor and by subtracting the average of the blank. The resulting measurement was defined as the total biofilm. Each plate was prepared in triplicate and three independent biological replicates were analysed per condition.

Time and temperature influence

The biofilm formation was assessed on 12 well polystyrene (PS) plates (Greiner Bio-One). Each well was filled with 1.5 ml of BHIMnG inoculated with *L. plantarum* as described in the above paragraph and incubated up to 72 h at 30°C sealed with parafilm to avoid evaporation. The biofilm formation was determined both by the CV assay and plate counting to determine the number of culturable cells in the biofilm. For this purpose, the

medium was removed and the adhering cells in each well were washed three times with 2 ml of PBS and the remaining biofilm was resuspended by scraping and rigorous pipetting in 2 ml of PBS (Kubota et al., 2009; van der Veen and Abee, 2011). In addition, the absence of clumps and cell aggregates was confirmed by phase contrast microscopy. Serial dilutions were prepared in PBS and plated on MRS agar (Oxoid) and incubated at 30°C for 48 h. Biofilm formation was followed as a function of time (24, 48 or 72 h at 30°C) and temperature (20, 25, 30 and 37°C for 72 h). All experiments involved three independent biological replicates and each replicate included three technical replicates.

Enzymatic treatment with DNase I and Proteinase K

Mature biofilms (48 h, 30°C, BHIMnG) were washed once with 1 ml PBS prior to the addition of either 100 µg/ml of DNase I (final concentration in PBS; Sigma-Aldrich), 10 µg/ml of Proteinase K (Qlagen) or PBS (as a control) and incubated at 30°C for one hour. After the treatment, the biofilms were washed three times to remove enzymes prior to biofilm quantification by both the CV assay and cell enumeration.

The influence of Proteinase K on the initial attachment was tested by addition of Proteinase K (final concentration of 10 µg/ml) along with the inoculated BHIMnG and after one or 2.5 hours incubation at 30°C, the medium was removed and the wells were washed three times with 2 ml of PBS to remove the unattached cells. Attached cells were enumerated on MRS agar.

The LIVE/DEAD® Bacterial Viability Kit (BacLight™) was used to visualise the biofilms and the effect of the enzymatic treatments following the supplier's protocol. For the microscopy experiments, the fluorescent filters UMN BV (SYTO9) and U-MWIG (PI) were used in a magnification of 1000x with a BX41 microscope (Olympus).

Data analysis

The effects of temperature, incubation time and strain on biofilm formation capacities of the tested *L. plantarum* strains were compared by univariate analysis of variance and by a multiple regression model in SPSS. These variables were treated as qualitative or quantitative variables, considering a $P < 0.001$ as significantly different (Kadam et al., 2013).

Results

Diversity in biofilm formation

Biofilm formation can be affected by numerous factors including the composition of the growth medium. Therefore, we used five different media to select a medium that supports *L. plantarum* biofilm formation in an initial screening using crystal violet for quantification of total biofilm. This screening included six different *L. plantarum* spoilage isolates (FBR1 - FBR6) and *L. plantarum* WCFS1 with known biofilm forming capacity (van der Veen and Abee, 2011) as a reference. Media tested included the standard media for *Lactobacillus* cultivation (MRS and AOAC), a modified TSB medium (mTSB, see materials and methods), a meat-based medium supplemented with glucose and manganese to support growth of lactobacilli (BHIMnG, see materials and methods section) and a chemically defined medium supporting *L. plantarum* growth that was described previously (Wegkamp et al., 2010). In these experiments, the biofilm was measured using the CV assay that is typically applied to quantify total biofilm. The data revealed that biofilm formation was variable among the seven strains tested (Figure 2.1) but for all strains, highest CV reads were reached in BHIMnG. Based on this screening, BHIMnG was selected as medium for further biofilm experiments.

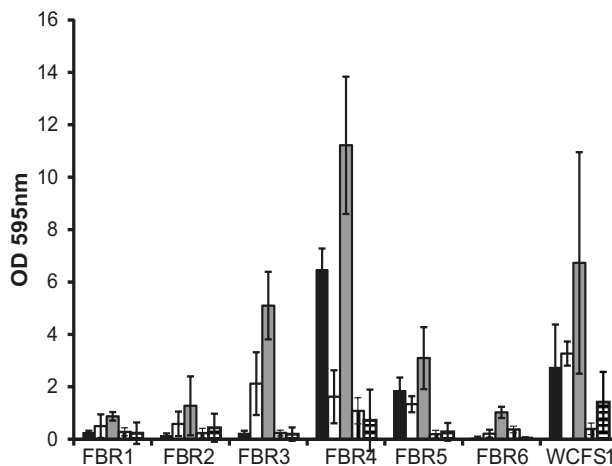


Figure 2.1. Biofilm formation by *L. plantarum* WCFS1 and different *L. plantarum* spoilage strains. Biofilms formed at 30°C for 72h in either PMM7 (black bars), MRS (white bars), BHIMnG (grey), TSB (vertical pattern) or AOAC (horizontal pattern) were quantified by the CV assay at 595nm. The data represents the average of three biological replicates and the standard deviation is indicated by vertical bars. The threshold value for the CV assay was established as two times the standard deviation of the blank.

Effect of time and temperature on biofilm formation

In a food processing environment, different temperatures may be encountered and time allowed for biofilm formation varies depending on the interval between cleaning regimes and design of the equipment. Both parameters are expected to influence growth and biofilm forming capacity. Therefore, biofilm formation of the seven *L. plantarum* strains was tested at four different temperatures (20, 25, 30 and 37°C) on polystyrene coupons (Figure 2.2) as described in the Materials and Methods section. For these experiments, the biofilm was grown in 12 wells plates which have a larger surface area compared to the 96 wells plates used in the screening described above, resulting in larger values for the CV assay.

The effect of temperature on biofilm formation varied between the *L. plantarum* strains tested. For strains FBR3, FBR4, FBR5 and WCFS1 the number of cells in the biofilm decreased with increasing temperatures. However, CV staining was higher at elevated temperature. This shows that although the total biomass increased with temperature, the number of living cells was reduced by 2 to 3 log units. A similar trend was observed for FBR2, albeit that the total biofilm and number of culturable biofilm cells were low compared to FBR3, FBR4, FBR5 and WCFS1. For FBR2, an increase in temperature resulted in a decrease in the culturable cell numbers as observed for the above mentioned strains. However, CV staining values reflecting total biofilm formation remained just above the background. For FBR6, cell numbers decreased at 37 °C with a concomitant decrease in CV signal.

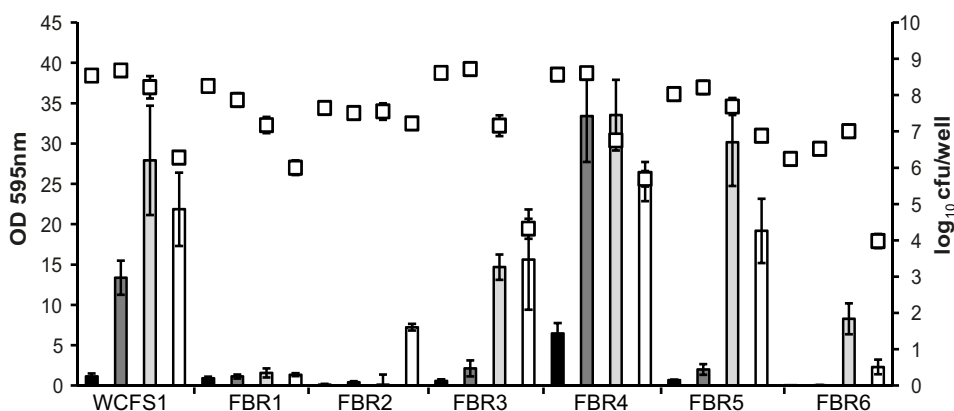


Figure 2.2. Biofilms formed by *L. plantarum* on polystyrene surfaces at either 20°C (solid black), 25°C (dark grey), 30°C (light grey), and 37°C (white column). The data represents the average of three biological replicates and the standard deviation of biofilm formation CV assay (columns) and number of cells in the biofilm (squares) of selected *L. plantarum* strains grown in BHIMnG for 72h.

For most strains, highest culturable cell numbers in the biofilm (of 8-9 log per well) were reached already within 24 h (Figure 2.3) and remained constant for 48 h, but after 72 h

culturable cell numbers in the biofilm decreased by 1 to 3 log units per well. Similar to the observed temperature effect on biofilm formation, the drop in cell counts in the biofilm coincided with an increase in CV staining for strain WCFS1, FBR3, FBR4, and FBR5. For FBR6, cell numbers in the biofilm were significantly lower compared to the other strains and extension of the incubation time led to an increase in cell numbers albeit that final numbers remained approximately 2 log lower compared to the other strains.

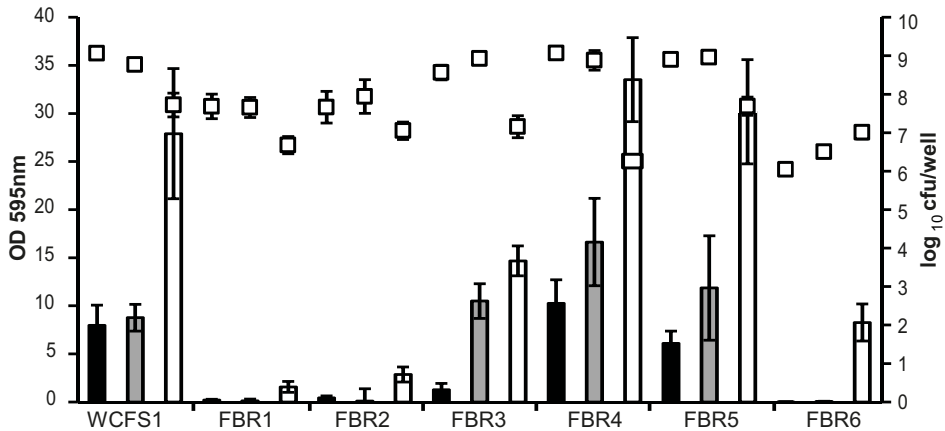


Figure 2.3. Biofilm formed on polystyrene by different *L. plantarum* strains for either 24 (black), 48 (grey), and 72 h (white) incubation in BHIMnG at 30°C. The data represents the average of three biological replicates and the standard deviation of biofilm formation CV assay (columns) and number of cells in the biofilm (squares) of selected *L. plantarum* strains.

Both an increase in the incubation temperature and time displayed comparable effects on FBR3, FBR4, FBR5 and WCFS1 where a drop in cell counts corresponds to an increase in total biofilm formation determined with CV staining. These data show that for *L. plantarum*, there is a poor correlation between CV staining and cells in the biofilm. This discrepancy between CV staining and cell counts is even more apparent when all data points for each individual strain are displayed in a scatter plot (Figure 2.4). The statistical analysis showed that the impact of strain, time and temperature on biofilm formation was similar.

Based on these results it can be hypothesized that an increase in temperature or incubation time results in cell death and conceivably lysis followed by release of DNA. Biofilm matrix-associated eDNA and released proteins and/or proteinaceous material may explain the relatively high CV reads under these conditions.

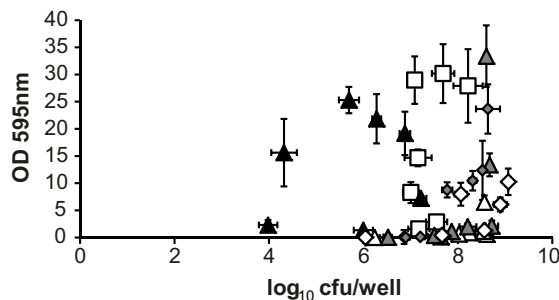


Figure 2.4. Scatter plot of biofilms formed by *L. plantarum* spoilage isolates and WCFS1 measured as OD 595nm (CV assay) versus the \log_{10} cfu/well. The plotted data includes the points obtained for each strain grown at 30°C for 72 h (open squares); the temperature effect 20°C, 25°C, 37°C (white, grey and black triangles respectively); and time effect for 24 and 48 h (white and grey diamonds respectively). All data points are a result of triplicates and three biological replicates and grown in BHIMnG using PS as contact surface.

Matrix resistance to Proteinase K and DNase treatment

To substantiate whether DNA and proteins are structural components of the biofilm matrix, mature biofilms were treated with either DNase I or Proteinase K (Figure 2.5).

An indication for the presence of extracellular DNA (eDNA) was inferred from the results described above and it was further supported by fluorescence microscopy with propidium iodide (PI) that specifically stains DNA (Figure 2.5 B). When the mature biofilm produced by strain WCFS1 was treated with either Proteinase K or DNase I, both CV staining and culturable cell numbers in the biofilm decreased (Figure 2.5 A). It can be excluded that the addition of Proteinase K affected the initial attachment of cells since its addition along with the inoculum did not influence in the initial attachment up to 2.5 hours (data not shown). Similar effects after Proteinase K and DNase I addition were observed for strains FBR3, FBR5 and FBR6 (Figure 2.5 A). Strains FBR1 and FBR2 showed no detectable level of CV staining; however, the culturable cell numbers in the biofilm were reduced. Remarkably, the biofilm produced by strain FBR4, was only reduced after addition of Proteinase K, while DNase I treatment did not have a significant effect. It cannot be excluded that other components in the biofilm matrix produced by these strains interfere with the accessibility of the eDNA. Proteinase K treatment resulted in a large decrease in attached cells for all the *L. plantarum* strains resulting in CV staining below detection level.

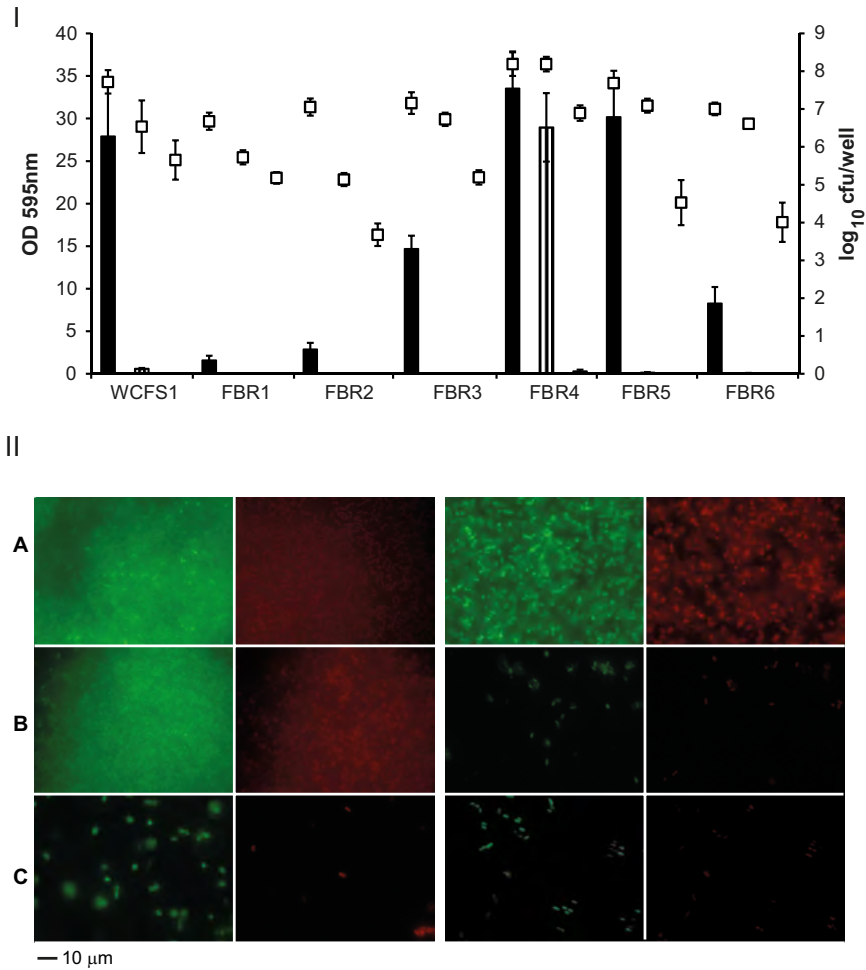


Figure 2.5. Effect of DNase and Proteinase K treatment on mature biofilms formed on polystyrene. (I) Biofilms were quantified by both CV (bars) and enumeration of cell counts (squares). The graph represents the average of three biological replicates of mature biofilms treated for 1h at 30 °C with either PBS (control, black solid bars), 10μg/ml Proteinase K (grey bars) or 100μg/ml DNase I (pattern). (II) Representative fluorescent images of biofilms stained with LIVE/DEAD BacLight bacterial viability kit: (A) Mature Biofilm after 48h incubation at 30°C, (B) Mature biofilm after treatment with 100μg/ml DNase I or (C) 10μg/ml Proteinase K for 1h at 30°C. Left FBR4, Right WCFS1.

Discussion

Although spoilage bacteria generally pose no risk for illnesses, their presence in food has an economic impact as it may lead to premature spoilage of food. According to the Food and Agriculture Organization of the United Nations (FAO, 2011) approximately one third of food for human consumption is wasted or lost causing an annual economic loss of US\$ 680 billion in industrialized countries and US\$ 310 billion in developing countries. These figures include food loss due to spoilage, physical and chemical damaged food. Biofilm formation in raw materials and food processing environments are important sources of contamination, leading to food spoilage or in some cases outbreaks (Carpentier and Cerf, 1993; Chmielewski and Frank, 2003; van Houdt and Michiels, 2010). There are only a limited number of studies describing biofilm formation of food spoilage lactobacilli (Kubota et al., 2008; Kubota et al., 2009).

In this study we show that biofilm formation by *L. plantarum* is strongly affected by the type of growth medium, temperature and by strain specific features. The meat based medium BHI supplemented with additional glucose and Mn(II), provided optimum conditions for the tested strains to form dense biofilms as measured by CV staining and was superior to MRS medium which is routinely used for *L. plantarum* cultivation in laboratory experiments. Two media of plant origin (mTSB and AOAC) were evaluated. AOAC medium is tomato based and mimics a tomato-based food matrix. Most of the strains showed capacity to form dense biofilms in the meat based medium; however, also media of plant origin supported biofilm formation. The fact that biofilm formation is supported by a diverse range of media with different nutrient composition suggests that *L. plantarum* biofilm formation may occur in food processing environments posing risks for recontamination of food products.

The CV assay is typically used for quantification of total biofilm since it binds non-specifically to cells as well as matrix components and therefore is a high throughput method to quantify total biomass. This technique is widely used for various bacterial species (Merritt et al., 2005). Notably, the CV data correlate poorly with the number of culturable cells in the *L. plantarum* biofilm. This is for example illustrated by the data for strain FBR1 and FBR2, although relatively high cell numbers were reached (up to 10^7 - 10^8 cells per well), CV staining was close to the detection limit. By contrast, CV staining increased upon maturation of the biofilm with concomitant reduction in live cell counts for some strains (FBR3, FBR4, FBR5, FBR6 and WCFS1) with time and/or temperature increase. A high density biofilm may thus contain relatively low numbers of culturable cells, but since these are embedded in a thick layer of matrix components they may be protected against antimicrobial treatments, for example by diffusion limitation of cleaning and disinfection agents (Bridier et al., 2011; Lequette et al., 2010).

This implies that biofilm assays based on CV staining do not provide an estimation of the number of culturable cells attached to a surface, since CV may also bind to dead cells, eDNA, proteins and/or exopolysaccharides. The finding that dead cells contribute to the *L. plantarum* biofilm matrix was further supported by fluorescence microscopy which revealed that the reduced cell counts coincided with an increase in the number of dead cells. The presence of eDNA in the biofilm matrix of some *L. plantarum* strains was further supported by DNase treatment of the mature biofilms showing a large reduction in both CV staining and number of culturable cells for WCFS1, and FBR3 and FBR5. The mature biofilm produced by FBR4 was resistant to DNase treatment, suggesting that the matrix contains other structural components and/or that eDNA was not accessible to the DNase. Fluorescent microscopy of the FBR4 biofilm revealed that the biofilm was not disturbed after the DNase treatment, but the matrix could be stained with propidium iodide suggesting that eDNA was present in the biofilm. For strains FBR1, FBR2 and FBR6 the CV staining was below detection limit, but also here DNase and Proteinase K treatments reduced the number of biofilm associated cells, with the latter treatment showing the largest effect.

The observed effect of cell lysis may be more pronounced in high glucose containing media as addition of glucose to BHI resulted in an increased acidification as shown previously by van der Veen and Abee (2011) or *L. plantarum* WCFS1. The presence of glucose allows the pH to drop to pH 3.5 and this may increase cell death in time and favour cell lysis. In this scenario, the strains which are more prone to autolysis are likely to be qualified as better biofilm formers as cell lysis results in more eDNA, proteins and other intracellular components that can bind CV. The lack of CV staining of FBR1 and FBR2 possibly results from the limited lysis of aged cells or synthesis of other extracellular polymeric substances with limited CV binding capacity.

The participation of eDNA as part of the biofilm matrix was previously shown for other species including *Listeria monocytogenes* (Harmsen et al., 2010), *Streptococcus pneumoniae* (Hall-Stoodley et al., 2008), *Staphylococcus aureus* (Mann et al., 2009) and for *L. plantarum* LM3 (Muscariello et al., 2013), however, culturable cells in the biofilm were not quantified and neither an assessment of the correlation, if any, between CV staining and cfu's.

For all seven strains evaluated the total biofilm and cell numbers were reduced after Proteinase K treatment, suggesting that *L. plantarum* biofilms typically contain proteins and/or proteinaceous material cementing the biofilm cells to the surface. *Lactobacillus* spp. have predominantly been studied for interactions with biotic surfaces such as epithelial cells and mucus as relevant traits for their probiotic properties. In host-microbe interaction, surface proteins play an important role in the attachment because treatments with lithium chloride or proteases such as trypsin and pepsin reduced the attachment capacity of *Lactobacillus* species (Adlerberth et al., 1996; Lorca et al., 2002; Tallon et

al., 2007; Tuomola et al., 2000). Proteins have been described previously as relevant matrix components for *Bacillus subtilis* (Branda et al., 2006) and *Pseudomonas putida* (Jahn et al., 1999) biofilms. Studies in other species point to the importance of biofilm associated proteins (bap) for the substratum-bacteria and bacteria-bacteria interactions in *Staphylococcus*, *Enterococcus*, *Salmonella* and *Pseudomonas* (Cucarella et al., 2001; Espinosa-Urgel et al., 2000; Latasa et al., 2005; Tendolkar et al., 2004; Toledo-Arana et al., 2001). The type of proteins involved in *L. plantarum* spp. biofilm formation remains to be characterised.

Most biofilm studies have been performed with motile bacteria and motility is considered an important aspect in the initial stage of attachment (Lemon et al., 2008; O'Toole and Kolter, 1998; Pratt and Kolter, 1998). Most *Lactobacillus* spp. are non-motile microorganisms including *L. plantarum* strains used in the study (Kleerebezem et al., 2003), and consequently the biofilm is initiated from cells that sediment to a surface. Once sedimented, *L. plantarum* is capable to form biofilms on materials relevant in the food industry such as SS and PS but the capacity to form high density biofilms as determined by CV staining is strain dependent. The strain to strain variation could not be explained by differences in initial attachment and final cell counts but may in part be explained by differences in levels of eDNA, likely as result of differences in lysis behaviour. Depending on the strain, incubation time and temperature, *L. plantarum* may form low and high density biofilms. Even in low density biofilms, still a high number of culturable cells may be present. High density biofilms consist of a large contribution of CV stainable matrix components conceivably composed of proteins, eDNA and proteinaceous material. Information on environmental conditions that influence biofilm formation by lactobacilli is of relevance to food producing industries and could add to the design of new intervention strategies to prevent biofilm formation.

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CHAPTER 3

Role of cell surface composition and lysis in static biofilm formation by *Lactobacillus plantarum* WCFS1

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Abstract

Next to applications in fermentations, *Lactobacillus plantarum* is recognized as a food spoilage organism, and its dispersal from biofilms in food processing environments might be implicated in contamination or recontamination of food products. This study provides new insights into biofilm development by *L. plantarum* WCFS1 through comparative analysis of wild type and mutants affected in cell surface composition, including mutants deficient in the production of Sortase A involved in the covalent attachment of 27 predicted surface proteins to the cell wall peptidoglycan ($\Delta srtA$) and mutants deficient in the production of capsular polysaccharides (CPS1-4, $\Delta cps1-4$). Surface adhesion and biofilm formation studies revealed none of the imposed cell surface modifications to affect the initial attachment of cells to polystyrene while biofilm formation based on Crystal Violet (CV) staining was severely reduced in the $\Delta srtA$ mutant and significantly increased in mutants lacking the *cps1* cluster, compared to the wild-type strain. Fluorescence microscopy analysis of biofilm samples pointed to a higher presence of extracellular DNA (eDNA) in *cps1* mutants and this corresponded with increased autolysis activity. Subsequent studies using $\Delta acm2$ and $\Delta lytA$ derivatives affected in lytic behaviour revealed reduced CV staining of biofilms, confirming the relevance of lysis for the build-up of the biofilm matrix with eDNA.

Introduction

Lactobacilli are Gram positive, generally non-motile bacteria which can be found in a diverse range of habitats. They are widely used in the food industry as probiotics (Boesten and de Vos, 2008; Turrone et al., 2014) and starter cultures for the production of fermented food products (Caplice and Fitzgerald, 1999; Leroy and De Vuyst, 2004). However, besides their desired properties, they are also associated with food spoilage (Bartowsky and Henschke, 2008; Bjorkroth and Korkeala, 1996; Lyhs et al., 2001; Samelis et al., 2000). One contamination route with *Lactobacillus plantarum* is via the presence of biofilms in the food production environment. Biofilms are defined as microorganisms attached to a surface embedded in a matrix of extracellular polymeric substances (O'Toole et al., 2000). *L. plantarum* has been shown to form submerged biofilms, both as single species but also in multispecies biofilms (Fernández Ramírez et al., 2015; Kubota et al., 2008; Kubota et al., 2009; Metselaar et al., 2015; van der Veen and Abee, 2011). Several stages in biofilm development can be recognized including surface adhesion, microcolony formation, biofilm growth, matrix formation, and biofilm dispersion as the final stage (Abee et al., 2011; Watnick and Kolter, 2000).

Surface components of the cell envelope of bacteria have been shown important for interaction with the environment (Kleerebezem et al., 2010; O'Toole et al., 2000; Pratt and Kolter, 1998). The main constituents of the Gram positive cell envelope are peptidoglycan, teichoic acids, proteins and polysaccharides (Kleerebezem et al., 2010; Silhavy et al., 2010). Especially, polysaccharides were found to play a role in biofilm formation contributing to the formation of the biofilm matrix (Branda et al., 2005; Stewart and Franklin, 2008). Four different gene clusters encoding capsular polysaccharide biosynthesis are located in the *L. plantarum* WCFS1 genome and the role of these cell surface polysaccharides in probiotic functionality was studied in deletion mutants lacking either individual or multiple *cps* gene clusters (Remus et al., 2012). In addition, some of the cell surface proteins present in *L. plantarum*, such as mannose-specific adhesins, have been reported to play a role in attachment to biotic surfaces including host epithelial cells (Pretzer et al., 2005). Notably, the major sortase SrtA mediates covalent binding of 27 predicted cell-surface proteins encoded in the *L. plantarum* WCFS1 genome, including mucus-binding proteins, a hydrolase, as well as mannose-specific and collagen-binding adhesins (Boekhorst et al., 2005; Kleerebezem et al., 2010). Among these, the function of three sortase dependent proteins (SDPs) has been experimentally validated (Du et al., 2015; Pretzer et al., 2005; Sturme et al.). The deletion of sortase A in *Enterococcus faecalis* resulted in a lower initial attachment and to defective biofilm formation under static and dynamic conditions, showing its relevance in initial attachment and biofilm formation (Guiton et al., 2009). Although the exact matrix composition of *L. plantarum* biofilms remains to be determined, enzyme treatments have shown proteins and eDNA to be part of the biofilm

matrix (Fernández Ramírez et al., 2015). DNA can be released into the biofilm matrix either by active secretion or cell lysis (Jakubovics et al., 2013). The latter has been reported for different species including enterococci (Guiton et al., 2009; Thomas et al., 2008) and staphylococci (Qin et al., 2007; Rice et al., 2007) conceivably caused by autolysins that play a role in cell wall degradation (Bayles, 2007; Frese et al., 2013).

The current study focuses on the role of capsular polysaccharides and cell-wall associated proteins in biofilm formation of *L. plantarum* WCFS1. Comparative analysis of *L. plantarum* WCFS1 (wildtype) and selected mutants affected in cell surface composition (Δ srtA, Δ cps1-4) and cell lysis activity (Δ acm2, Δ lytA) provided evidence that cell wall autolysis and release of DNA are major determinants of *L. plantarum* WCFS1 static biofilm formation.

Materials and methods

Strains and media

The bacterial strains used in the present study are listed in Table 1. The strains were streaked from a -80°C glycerol stock on De Man-Rogosa-Sharpe (MRS) agar (Merck) plates and incubated for 48 h at 30°C. A single colony was inoculated in 10 ml of MRS at 30°C for 18 h to prepare a starting culture. The media were supplemented with 10 µg/ml chloramphenicol when appropriate. Biofilms were grown in brain heart infusion (BHI; Becton Dickinson) supplemented with 0.005% manganese sulphate and 2% glucose (Merck) (BHIMnG) as it has been shown previously that this medium favours biofilm formation of *L. plantarum* (Fernández Ramírez et al., 2015; van der Veen and Abee, 2011). A concentration of 0.01 mg/ml of chicken egg lysozyme (Sigma) was added to BHIMnG when indicated.

Biofilm formation

Biofilms were formed under static conditions as described previously (Merritt et al., 2005). The biofilms were grown in polystyrene (PS) 12 well plates (Greiner Bio-One). Each well was filled with 1.5 ml of BHIMnG inoculated with 1% (v/v) of an overnight grown culture (18 h at 30°C) containing approximately $9.2 \log_{10}$ colony forming units (cfu) per ml. The biofilms were grown at 30°C for either 1 h (for determination of initial attachment) or 48 h and the plates were sealed with parafilm to avoid evaporation. The initial attachment of strain WCFS1 and its Δ acm2 derivative was also evaluated in the presence of lysozyme (0.01 mg/ml) in BHIMnG. From the overnight culture, 1 ml was taken and centrifuged for 10 min $5,000 \times g$ and washed once with phosphate buffered saline (PBS, NaCl 8 g/l; KCl 0.2 g/l; Na_2HPO_4 1.44 g/l; KH_2PO_4 0.24 g/l; pH 7.4) (Merck). Resulting cells were resuspended in 1 ml of PBS with 0.01 mg/ml lysozyme and incubated for one hour at 30°C. From this

suspension, 0.01% (v/v) was inoculated as described above in BHIMnG containing 0.01 mg/ml lysozyme. The control sample was treated in the same way but without 0.01 mg/ml lysozyme in PBS or BHIMnG.

Table 3.1. Strains used in the present study

Strain	Description	Notation	Reference
WCFS1	<i>L. plantarum</i> NCIMB8826		Kleerebezem et al. (2003); Siezen et al. (2012)
NZ3548Cm	Cm ^r ; WCFS1 derivative; chromosomal replacement of the $\Delta cps1A$ -I gene cluster	$\Delta cps1$	Remus et al. (2012)
NZ3533ACm	Cm ^r ; WCFS1 derivative; chromosomal replacement of the $\Delta cps2A$ -J gene cluster	$\Delta cps2$	
NZ3549Cm	Cm ^r ; WCFS1 derivative; chromosomal replacement of the $\Delta cps3A$ -J gene cluster	$\Delta cps3$	
NZ3534Cm	Cm ^r ; WCFS1 derivative; chromosomal replacement of the $\Delta cps4A$ -J gene cluster	$\Delta cps4$	Andre et al. (2011)
NZ3550Cm	Cm ^r ; WCFS1 derivative; chromosomal replacement of the $\Delta cps1A$ -3J cluster	$\Delta cps1$ -3	
NZ3680Cm	Cm ^r ; WCFS1 derivative; chromosomal replacement of the $\Delta cps1A$ -3J, $\Delta cps4A$ -J cluster	$\Delta cps1$ -4	
NZ3513Cm	$\Delta srtA$	$\Delta srtA$	Remus et al. (2013)
NZ3557	Cm ^r ; WCFS1 derivative; <i>acm2::cat</i>	$\Delta acm2$	Fredriksen et al. (2012)
TR0011	NZ7100 derivative; <i>lp_3093 (lys2)::lox72</i>	$\Delta lys2$	Rolain et al. (2012)
TR006	NZ7100 derivative; <i>lp_3421 (lytA)::lox66-P32-cat-lox71</i>	$\Delta lytA$	

The biofilm formation was measured both by the Crystal Violet (CV) assay and plate counting to determine the total biofilm formation and number of culturable cells in the biofilm, respectively. For cell enumeration, the biofilms were washed three times with 2 ml of PBS to remove unattached cells. Next, the remaining biofilm was resuspended in 2 ml of PBS by scraping the surface with the tip of the pipette and vigorous pipetting. Additionally, the absence of clumps or cell aggregates was analysed by phase contrast microscopy (Fernández Ramírez et al., 2015). Serial dilutions were prepared in PBS and plated on MRS agar and incubated at 30°C for 48 h.

For the CV assay, the medium was carefully removed with a pipette and the biofilm was washed as described above. The attached biofilm was stained for 30 min with 1.5 ml of 0.1% (w/v) of CV (Merck). The excess of CV was removed by washing three times with 2 ml of PBS as described above. The dye attached to the biofilm was solubilized in 70% ethanol for 30 min at room temperature and the optical density (OD) was measured at 595 nm (SpectraMax, Molecular Devices). When OD₅₉₅ values exceeded a value of 1, the samples were diluted in 70% ethanol and the resulting OD measurements were corrected for the dilution factor and by subtracting the average of the blank. The resulting measurement

was defined as the total biofilm formed. Each plate was prepared in triplicate and three independent biological replicates were analysed.

The LIVE/DEAD® Bacterial Viability Kit (BacLight™) was used to visualise the biofilms following the supplier's protocol. For microscopy experiments, the fluorescent filters UMN BV (SYTO9) and U-MWIG (PI) were used at a magnification of 1,000 times with a BX41 microscope (Olympus).

Triton X-100 induced autolysis in buffer solution

This assay was carried out as previously described (Rolain et al., 2012) with some modifications. Briefly, *L. plantarum* strains were grown to mid exponential phase in BHIMnG ($OD_{600} = 0.8$) without (all strains) and with lysozyme (0.01 mg/ml) in the case of WCFS1 (WT) and its $\Delta acm2$ derivative. Bacteria were harvested by centrifugation (5,000 x g, 10 min, 4°C) and washed once with PBS. Washed cells were resuspended at a final OD_{600} of 1 in PBS containing 0.05% Triton X-100 (Sigma-Aldrich). Aliquots of 200 µl were placed in 96-well polystyrene microtiter plates (Greiner Bio-One) and incubated at 30°C, and the OD_{600} was determined every 20 min (SpectraMax, Molecular Devices).

Results

Cell surface composition does not affect initial attachment

Initial attachment of cells to the surface is important in the early stages of biofilm formation. Cell surface characteristics involved in initial attachment are expected to affect biofilm development. Mutants affected in cell-surface protein composition ($\Delta srtA$) and capsular polysaccharide production (Δcps) were examined for their capacity to interact with polystyrene (PS) surfaces. First, cell morphology of *L. plantarum* WCFS1 (WT), $\Delta srtA$ and Δcps mutants was analysed using phase contrast microscopy following incubation of the respective cultures for 18 h in BHIMnG at 30°C.

Mutants with a deletion of the *cps1* cluster (either alone or in combination with other *cps*) were found to form chains of cells, with $\Delta cps1-4$ forming cell aggregates (Figure 3.1A). Free cells were obtained from all mutants by vigorous pipetting and samples were processed within one hour for cell enumeration, a time frame in which no aggregation was observed (data not shown). Subsequent analysis of the initial attachment of WT, $\Delta srtA$ and the Δcps mutants revealed no significant differences in adhesion capacity after one hour of incubation at 30°C (ANOVA, Dunett's test $P < 0.005$; Figure 3.1B). This shows that the changes in cell-surface composition of the tested mutants do not affect initial adhesion capacity to PS.

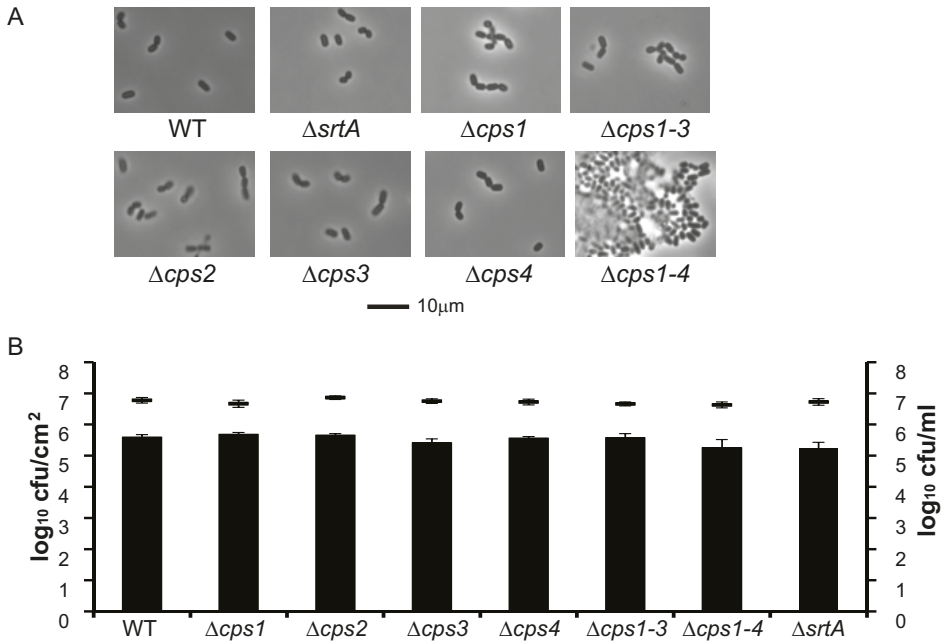


Figure 3.1. Morphology and initial attachment of *Lactobacillus plantarum* WCFS1 and its mutants affected in cell surface properties (A) Morphology of *L. plantarum* WCFS1 (WT) and mutants affected in cell surface properties grown in BHIMnG at 30°C for 18 h. (B) The number of cells attached to polystyrene after one hour incubation at 30°C in BHIMnG was determined and represented by the columns with the lines on top of each columns marking the initial inoculum (\log_{10} cfu/ml). The error bars display the standard deviation of the experiment. The experiment was performed in triplicate and with three biological replicates. No significant difference from the WT was found (ANOVA, $P < 0.001$).

Biofilm formation is affected by *cps1* and *srtA* deletions

The biofilm forming capacity of WT, $\Delta srtA$, $\Delta cps1$, $\Delta cps2$, $\Delta cps3$, $\Delta cps4$, $\Delta cps1-3$, and $\Delta cps1-4$ was tested to determine the influence of the surface modifications on biofilm formation following initial adhesion. The biofilm forming capacity was analysed after 48 h of incubation at 30°C by both CV staining and enumeration of culturable cells in the biofilm (Figure 3.2A). The number of culturable cells in the biofilms formed by the mutants was comparable to the number obtained with the WT strain, except for the $\Delta srtA$ mutant. The $\Delta srtA$ mutant biofilm contained an approximately 100-fold reduced level of culturable cells. Notably, CV staining for this mutant was also below the detection limit, suggesting that this mutant strain is severely hampered in biofilm formation. For the *cps* mutants, CV staining showed a remarkable increase in total biofilm formation for the strains lacking the *cps1* gene cluster (i.e., $\Delta cps1$, $\Delta cps1-3$ and $\Delta cps1-4$) relative to the WT strain (Figure 3.2A). The biofilms were stained with propidium iodide (PI) which binds to intracellular DNA of cells with damaged membranes but also to eDNA released into the matrix, conceivably following lysis of cells. The images obtained by fluorescence microscopy of propidium iodide (PI)-stained biofilms, show denser agglomerates in biofilms formed by strains lacking the $\Delta cps1$ gene cluster, suggesting higher eDNA levels in the biofilm matrix (Figure 3.2B), conceivably linked to increased autolysis activity in these strains.

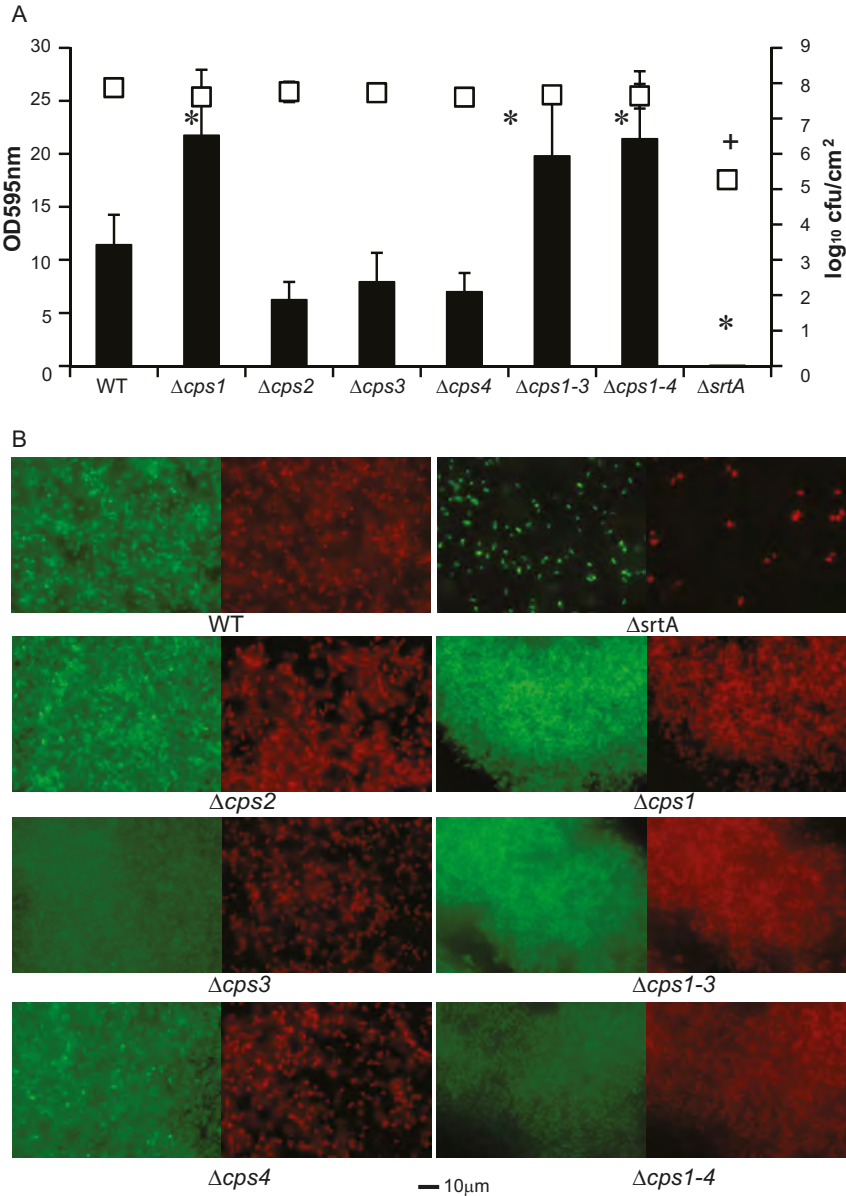


Figure 3.2. Biofilm formation of WCFS1 and its mutants affected in cell surface properties. Biofilms were grown in BHIMnG for 48 h at 30°C on polystyrene. (A) The data represent the average of three biological replicates in triplicate and the standard deviation of biofilm formation measured by CV assay (columns) and number of cells in the biofilm (squares). Statistical difference (ANOVA, Dunett's test $P < 0.005$) is marked * for CV and + for number of cells in the biofilm compared to the WT. (B) Representative fluorescent images of biofilms formed by the different strains stained with LIVE/DEAD BacLight bacterial viability kit: Syto9 (left) and propidium iodide (right).

Sensitivity of $\Delta cps1$ mutants to chemically-induced autolysis differs from the WT strain

To analyse the autolysis capacity, exponentially growing cells of WT and mutant strains were exposed to triton X-100 and the degree of cell lysis was determined (Figure 3.3). Autolysis under these conditions was comparable to the WT for all tested strains with the exception of the $\Delta cps1$ and $\Delta cps1-3$ mutant strains, which showed a higher degree of lysis. The increased lysis activity of these two mutants corroborates the increased CV and PI staining of the biofilms of these strains relative to the WT (see above; Figure 3.2B), which supports an role for eDNA in matrix formation in the biofilms of these mutants. The lysis behaviour of the $\Delta cps1-4$ mutant could not be determined by this method as a consequence of cell aggregation at later stages in the assay.

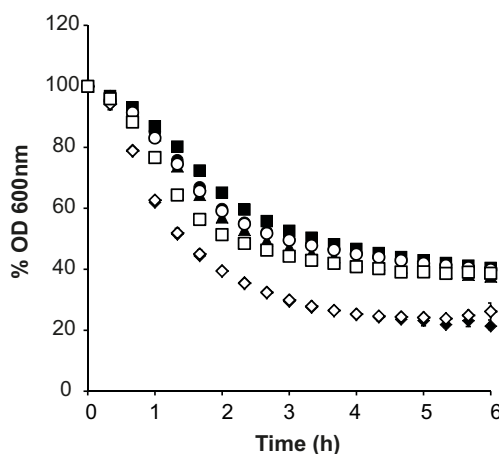


Figure 3.3. Triton X-100 (0.05 %)-induced autolysis of WCFS1 and its mutants affected in cell surface properties. The data represent the average and the standard deviation of three biological replicates of residual OD 600 (%) after treating exponentially growing cells of WT (filled circle), $\Delta cps1$ (filled diamonds), $\Delta cps2$ (filled squares), $\Delta cps3$ (filled triangles), $\Delta cps4$ (open circles), $\Delta cps1-3$ (open diamonds) and $\Delta srtA$ (open squares) with 0.05% Triton X-100. %OD 600nm for $\Delta cps1$ and $\Delta cps1-3$ is significantly different from the WT after 40 min; $\Delta srtA$ is significantly different from the WT only between 1 and 2.6 h; $\Delta cps2$ is significantly different between 1.3 and 2.3 h (ANOVA, Dunett's test $P < 0.005$).

Cell lysis is important for biofilm development

To further explore the importance of cell lysis in biofilm formation, we characterised *L. plantarum* mutants deficient for specific peptidoglycan hydrolases ($\Delta acm2$, $\Delta lys2$, $\Delta lytA$) that are potentially affected in cell lysis during biofilm formation. Microscopy analysis of BHIMnG grown cultures showed that the $\Delta acm2$ mutant form chains of cells and cells of the $\Delta lytA$ mutant display slight aggregation and loss of rod shaped morphology, confirming previously reported phenotypes for these mutants after growth in MRS medium (Rolain

et al., 2012) (Figure 3.4A). The initial attachment of the WT and mutant strains to PS was evaluated after one hour of incubation in BHIMnG and no significant difference in adhesion capacity could be observed (ANOVA, Dunett's test $P < 0.005$; Figure 3.4B). The $\Delta lytA$ mutant strain showed a slightly reduced inoculum size, which is likely due to increased cell death in the stationary phase, which is in agreement with the previously reported 25 % reduced viability of this mutant (Rolain et al., 2012). The slightly lower colony counts observed in the initial inoculum of the $\Delta acm2$ mutant is likely explained by the formation of cell-chains that lead to an underestimation of the viable cells using colony forming unit enumeration. It was not possible to separate these chains into single cells by vigorous pipetting. However, the average chain length of the $\Delta acm2$ mutant has been reported to be approximately 4 cells (Rolain et al., 2012) and correction of the obtained colony counts for this factor results in similar number of attached and initial inoculum cell numbers for this mutant as compared to WT (Figure 3.4B and D). Notably, it has previously been shown that inactivation of the major autolysin AcmA of *Lactococcus lactis* also resulted in chain formation and it was suggested that a reduced number of peptidoglycan breaks affected adhesion capacity to surfaces (Mercier et al., 2002). Therefore, adhesion was also studied with *L. plantarum* $\Delta acm2$ cells grown in the presence of 0.01 mg/ml of lysozyme using WT as a control. This concentration of lysozyme does not damage the cells as reflected in the similar numbers of culturable cells for both strains (Figure 3.4D). Notably, lysozyme treatment of the $\Delta acm2$ mutant resulted in the disintegration of the chains with a cell morphology comparable to that observed for the WT (Figure 3.4C). The WT strain showed similar PS adhesion efficiency when grown in absence or presence of lysozyme, whereas lysozyme treatment of the $\Delta acm2$ mutant led to higher cell numbers in both the initial inoculum and the PS-attached fraction (Figure 3.4D), confirming that the chain formation affected initial attachment of the $\Delta acm2$ mutant.

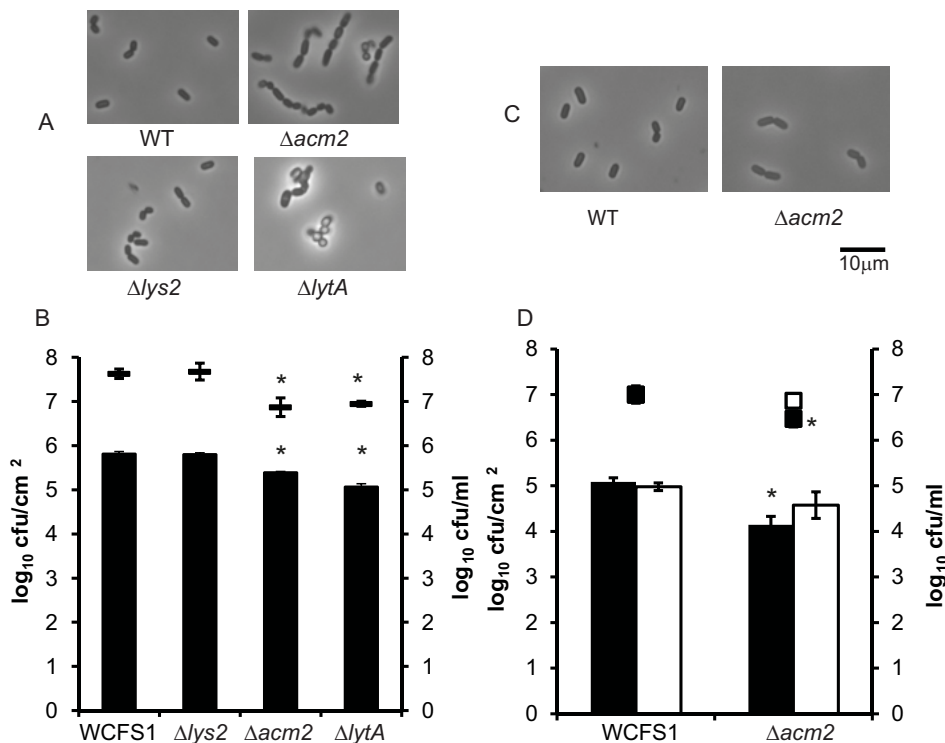


Figure 3.4. Morphology and initial attachment of *L. plantarum* WCFS1 and mutants affected in peptidoglycan hydrolases. (A) The morphology of the strains grown in BHIMnG at 30°C for 18 h is shown. (B) Shows the number of cells attached to polystyrene (\log_{10} cfu/cm²) after one hour incubation at 30°C in BHIMnG (black bars) and on top of each bar the initial inoculum (\log_{10} cfu/ml) is indicated. (C) Shows the morphology of WT and $\Delta acm2$ strains grown in BHIMnG at 18 h at 30°C with 0.01 mg/ml lysozyme. (D) Shows adhesion capacity, with the initial inoculum pre-incubated for one hour with PBS (black squares) or PBS with 0.01 mg/ lysozyme (open squares) in \log_{10} cfu/ml, and initial attachment after one hour incubation at 30°C in BHIMnG without (black bars) or with 0.01 mg/ml lysozyme (open bars). The error bars display the standard deviation of the experiment. The experiment was performed in triplicate and with three biological replicates. Statistical differences were determined by comparing the results to the WT (ANOVA, Dunett's test $P < 0.005$) and are marked (*).

Next, induced autolysis behaviour was analysed in BHI supplemented with Mn(II) and glucose (BHIMnG) that was used in adhesion and biofilm formation experiments for the mutants (Figure 3.5A), as well as for WT and $\Delta acm2$ mutant cells grown in the presence of lysozyme (Figure 3.5B). In BHIMnG, $\Delta lytA$ and $\Delta acm2$ mutants showed a lower degree of lysis as compared to the WT, whereas the $\Delta lys2$ mutation did not significantly affect lysis behaviour. While these results are in concordance with previously reported results obtained in MRS medium (Rolain et al., 2012), it is notable that the $\Delta lytA$ mutant displayed a lower degree of lysis in BHIMnG compared to MRS. In addition, the lysis behaviour of the

$\Delta acm2$ mutant was not affected by lysozyme treatment (Figure 3.5B). This indicates that although the chaining is disrupted, the degree of lysis is not influenced.

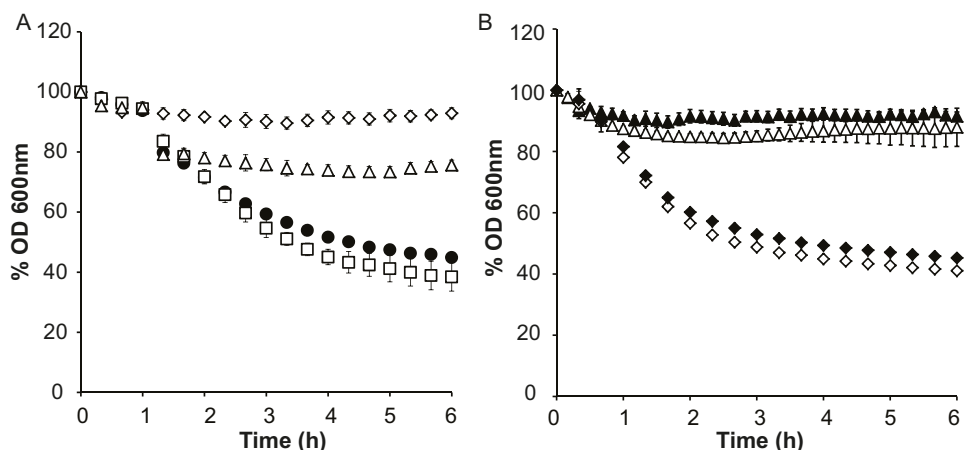


Figure 3.5. Triton X-100 (0.05%)-induced autolysis of WCFS1 and mutants affected in peptidoglycan hydrolases. (A) Shows OD 600 (%) for WT (filled circle), $\Delta acm2$ (diamonds), $\Delta lys2$ (squares), and $\Delta lytA$ (triangles). %OD 600nm for $\Delta acm2$ and $\Delta lytA$ are significantly different from the WT after 1.3 h and 2 h respectively (ANOVA, Dunett's test $P < 0.005$). (B) Shows additional data for strains WCFS1 (diamonds) and $\Delta acm2$ (triangles) grown in the absence (black) or presence (open) of 0.01 mg/ml lysozyme. %OD 600nm for lysozyme treated strains was not significantly different from untreated strains (Student's t- test $P < 0.005$). The data represent the average and the standard deviation of three biological replicates.

The biofilm forming capacity of WT and $\Delta lytA$, $\Delta lys2$, $\Delta acm2$ mutants was further characterised by enumeration of the culturable biofilm cells and CV staining using BHIMnG (Figure 3.6A) and for the WT and $\Delta acm2$ mutant also using BHIMnG containing 0.01 mg/ml lysozyme (Figure 3.6C). Although WT and the $\Delta lytA$ mutant had a comparable number of culturable cells in the biofilm, the biofilm produced by the $\Delta lytA$ mutant showed a lower level of CV binding as compared to the WT. Without lysozyme, the $\Delta acm2$ mutant did not form measurable biofilms based on CV staining, but appeared to develop a submerged pellicle that was not attached to the PS surface (data not shown). However, the biofilm formation capacity of the $\Delta acm2$ mutant significantly increased in the presence of lysozyme (Figure 3.6C), containing similar numbers of culturable cells and an approximate 50% reduced CV staining compared to biofilms formed by WT. These observations were supported by fluorescence microscopy using LIVE/DEAD staining (Figure 3.6B). Images of the Syto9-stained samples showed that very few cells of the $\Delta acm2$ mutant were attached when incubated in BHIMnG without lysozyme, while the images obtained for the $\Delta acm2$ mutant in BHIMnG with lysozyme were similar to those obtained for the WT (without and with lysozyme) and the $\Delta lytA$ and $\Delta lys2$ mutants. PI-staining of biofilms of the $\Delta acm2$ and

Δ *lytA* mutant strains show only dead cells and reduced presence of eDNA compared to the WT and Δ *lys2* mutant biofilms (Figure 3.6B). This observation is in agreement with the reduced CV staining indicative of lower total biofilm formation for the Δ *acm2* and Δ *lytA* mutants (Figure 3.6A).

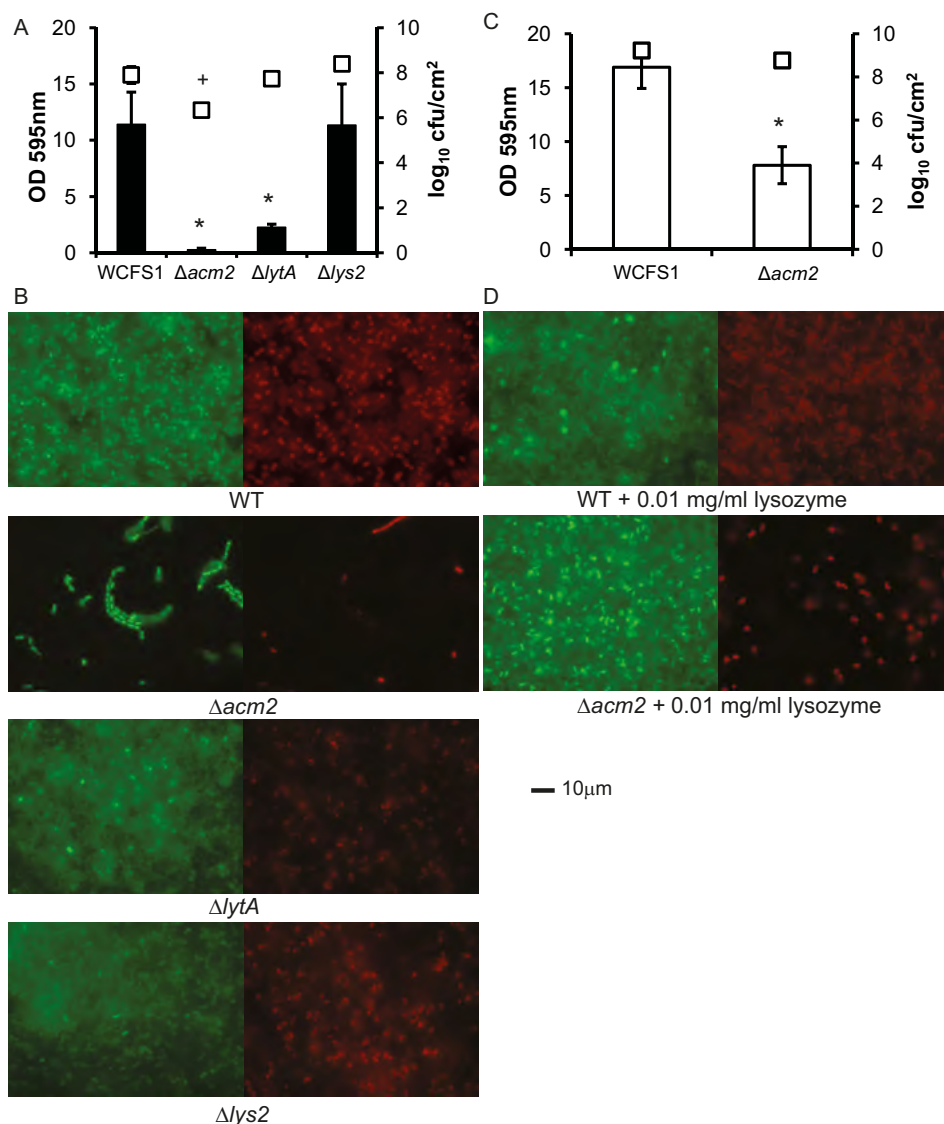


Figure 3.6. Biofilm formation of WCFS1 and mutant derivatives. Biofilms were grown in BHIMnG for 48 h at 30°C on polystyrene. The data represent the average of three biological replicates in triplicate and the standard deviation of biofilm formation CV assay (columns) and number of cells in the biofilm (squares) without (A) or with lysozyme (C) for WT and Δ *acm2*. (B and D) Representative fluorescent images of biofilms stained with LIVE/DEAD BacLight bacterial viability kit: Syto9 (left) and propidium iodide (right). Statistical difference (ANOVA, Dunnett's test $P < 0.005$) is marked * for CV and + for number of cells in the biofilm when compared to the WT.

Discussion

This study has provided new insights into biofilm development by *L. plantarum* WCFS1 through comparative analysis of wild type and selected mutants affected in cell surface composition and cell lysis, establishing an important role of eDNA in the biofilm matrix.

The initial attachment of *L. plantarum* to PS surfaces was not affected in mutant strains lacking genes in either one or multiple combinations of the four clusters encoding CPS production or the sortase gene (*srtA* gene). Notably, deletion of *srtA* resulted in a severe impairment of subsequent biofilm development by *L. plantarum*, which was apparent from a severe drop in both culturable cells (300-fold decrease relative to WT after 48 h) and corresponding CV values that remained below the detection limit. The importance of sortase A in biofilm formation was previously described for *Enterococcus faecalis* and both initial attachment and biofilm development were affected in this species (Guiton et al., 2009). SrtA covalently anchors proteins with a LPTXG motif, collectively called sortase dependent proteins (SDPs), to the cell wall peptidoglycan (Fischetti et al., 1990). These results indicate that one or more of the *L. plantarum* WCFS1 SDPs are involved in biofilm development. Previously, it was shown that *srtA* was significantly upregulated during biofilm formation in *L. plantarum* DB200, supporting a possible role for one or more SDP's in biofilm formation in this strain as well (De Angelis et al., 2015). Further work will be required to identify which of the *L. plantarum* SDPs play(s) a role in cell-cell interactions and/or biofilm formation.

The impact of capsular polysaccharides on *L. plantarum* WCFS1 surface adhesion and biofilm formation was studied using strains lacking the *cps1* cluster, either individually or in combination. Notably, the $\Delta cps1$ mutant displayed a chaining phenotype, whereas the $\Delta cps1-4$ mutant formed cell aggregates (cell clumping) that could be dissociated by vigorous pipetting. Chaining and clumping phenotypes are indicators of cell envelope modification, which could cause the observed differences in biofilm formation. Biofilm formation analysis revealed that strains lacking the *cps1* gene cluster showed increased CV staining, whereas the number of culturable cells was comparable to the WT. Previous studies showed that deletion of *cps1* resulted in a significant decrease of the amount of galactose and rhamnose (> 99 and 95 %, respectively) and reduced chain length of the capsule polysaccharides (Remus et al., 2012). Deletion of *cps3* or *cps4* in *L. plantarum* WCFS1 did not have an impact on the monosaccharide composition of the capsular polysaccharides whereas for mutants lacking *cps2*, the relative amount of galactose and rhamnose in capsular polysaccharides was also found to be significantly decreased by approximately 50% (Remus et al., 2012). Since the initial attachment of none of the *cps* mutants was affected, it was possible to reach similar numbers of culturable cells in the biofilm as compared to the WT. The numbers of culturable cells in the $\Delta cps1$ mutant

biofilm were only slightly lower (approximately 20%) than the WT but given the fact that CV measurement is expressed on a linear scale, this slight reduction resulted in double the amount of total biofilm as quantified by CV, suggesting that the increase in CV staining is best explained by the enhanced lysis capacity of single and multiple *cps1* deletion mutants resulting in higher amounts of eDNA in the biofilm matrix. The mechanism underlying enhanced cell lysis of these mutants remains to be elucidated. Notably, several studies have reported that the lack of capsular polysaccharides in *Streptococcus pneumoniae* increased the total biofilm amount (Moscoso et al., 2006; Muñoz-Elías et al., 2008), but did not provide mechanistic insight into how the loss of capsule affects biofilm formation.

Current data obtained with *cps* mutants support our previous study that showed *L. plantarum* biofilms to contain eDNA as matrix component (Fernández Ramírez et al., 2015). A role for eDNA in *L. plantarum* biofilm formation was further confirmed by biofilm formation analysis of mutants deleted in the major cell wall autolysin *Acm2*, or the redundant peptidoglycan hydrolase *Lys 2*, and the D, L-endopeptidase *LytA*. None of these enzymes are predicted to be sortase dependent (Boekhorst et al., 2005; Kleerebezem et al., 2010) and therefore do not play a plausible role in the impaired biofilm formation of the *srtA* mutant. Reduced lytic behaviour of the autolysin mutants correlated with decreased biofilm formation of the mutants as measured by CV staining. The deletion of *lys2* did not have an impact on biofilm formation which is in line with the observation that its lytic behaviour is similar to the WT. The Δ *lytA* mutant displayed a lower degree of lysis compared to the WT and although the number of culturable cells of the Δ *lytA* mutant attached to the surface in the mature biofilm was comparable to the WT, the total biofilm amount as measured by CV staining was 5-fold lower compared to the WT. It is conceivable that reduced levels of eDNA in the Δ *lytA* biofilm as observed using fluorescence microscopy, result in lower biofilm formation as measured by CV staining.

Notably, the Δ *acm2* mutant strain displayed extensive cell chaining, typically observed for mutants lacking major cell wall hydrolase activity since these enzymes have been found to be responsible for cell separation and the introduction of peptidoglycan breaks (Mercier et al., 2002; Rolain et al., 2012). Therefore the biofilm performance of the Δ *acm2* mutant was analysed also in the presence of low levels of lysozyme. This condition was shown to prevent cell-chain formation by complementing the cell separation defect by exogenously introducing peptidoglycan breaks. Notably, in presence of lysozyme, the biofilm formation of Δ *acm2* mutant differed significantly from the WT. In addition, the Δ *acm2* mutant showed virtual absence of autolysis induced by Triton X-100 even when treated with lysozyme. This lack of autolysis behaviour coincided with reduced biofilm formation capacity compared to WT as measured by CV staining. These observations are in line with the previously suggested role for autolysins in cell lysis and DNA release to the

biofilm matrix reported for *E. faecalis* and *Staphylococcus epidermidis* (Bayles, 2007; Frese et al., 2013; Qin et al., 2007; Thomas et al., 2008).

Taken together, results obtained with single and multiple $\Delta cps1$ mutants (enhanced lysis), and $\Delta lytA$ and $\Delta acm2$ mutants (reduced lysis) indicate that there is a correlation between the lytic behaviour of *L. plantarum* and total biofilm formation measured by CV staining with only slightly lower numbers of culturable biofilm cells. The absence of peptidoglycan hydrolase LytA and the major autolysin Acn2 proved to affect biofilm formation as the lack of cell lysis resulted in a lower contribution of eDNA to the matrix. In addition, a role for sortase A dependent proteins in biofilm formation was identified conceivably supporting cell-cell interactions as hypothesized in previous work that showed biofilm formation to be sensitive to Proteinase K treatment (Fernández Ramírez et al., 2015). Further work will be required to determine the role in *L. plantarum* biofilm formation of sortase A dependent protein(s) that may contribute to cell-cell interactions and biofilm formation.

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CHAPTER 4

Quantitative assessment of viable cells of *Lactobacillus plantarum* strains in single and multi-strain biofilms

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Abstract

Biofilms of *Lactobacillus plantarum* are a potential source for contamination and recontamination of food products. Although biofilms have been mostly studied using single species or even single strains, it is conceivable that in a range of environmental settings including food processing areas, biofilms are composed of multiple species with each species represented by multiple strains. In this study six spoilage related *L. plantarum* strains FBR1-FBR6 and the model strain *L. plantarum* WCFS1 were characterised in single and multiple strain competition models. A quantitative PCR approach was used with added propidium monoazide (PMA) enabling quantification of cells in the biofilm without membrane damage, representing the viable cell fraction that determines the food spoilage risk. Our results show that the performance of individual strains in multi-strain cultures generally correlates with their performance in pure culture, and relative strain abundance in multi-strain biofilms positively correlated with the relative strain abundance in suspended (planktonic) cultures. The total biofilm quantified by CV staining of the multi-strain biofilms formed was mainly correlated to CV values of the dominant strain obtained in single strain studies. However, the combination of FBR5 and WCFS1 showed significantly higher CV values compared to the individual performances indicating that total biofilm formation was higher in this specific condition. Notably, *L. plantarum* FBR5 was able to outgrow all other strains and showed the highest relative abundance in multi-strain biofilms. All the multi-strain biofilms contained a considerable number of viable cells, representing a potential source of contamination.

Introduction

Lactic acid bacteria (LAB), including lactobacilli, are widely used as starter culture for diverse fermented foods and beverages (Buckenhüskes, 1993; Caplice and Fitzgerald, 1999; Hammes et al., 1990; Leroy and De Vuyst, 2004). However, certain *Lactobacillus* species also cause food spoilage of a wide range of food products (Bjorkroth and Korkeala, 1996; Gram et al., 2002) such as beer (Sakamoto and Konings, 2003; Simpson and Fernandez, 1992), sliced meat (Bjorkroth and Korkeala, 1997; Chenoll et al., 2006) and salad dressings (Kurtzman et al., 1971).

Lactobacillus plantarum is frequently encountered as spoilage contaminant of food products, with biofilms present in food processing environments conceivably acting as a source of contamination and/ or recontamination (Kubota et al., 2008). Biofilms consist of microorganisms attached to a surface embedded in a matrix generally composed of polymeric substances including polysaccharides (O'Toole et al., 2000; Watnick and Kolter, 2000). Unlike motile microorganisms which depend on structures such as flagella to actively attach to the surface, non-motile microorganisms rely on passive processes such as sedimentation and/or electrostatic interactions to reach for a surface (Lemon et al., 2008). *L. plantarum* has been shown to form biofilms on abiotic surfaces (Fernández Ramírez et al., 2015; Kubota et al., 2008; Sturme et al., 2005) and raw materials such as onions and olives (Domínguez-Manzano et al., 2012; Kubota et al., 2008). Six *L. plantarum* food isolates and the model strain *L. plantarum* WCFS1 were previously analysed for biofilm forming capacity. A substantial level of strain diversity with respect to biofilm formation was observed. Moreover, dense biofilms formed by specific strains were found to be sensitive to Proteinase K and/or DNase treatments (Fernández Ramírez et al., 2015), suggesting roles for proteins and extracellular DNA (eDNA) as structural matrix components. Recently, a more detailed analysis of cellular parameters contributing to biofilm development by the fully sequenced *L. plantarum* WCFS1 strain was conducted via comparative analysis of wild type and mutants affected in cell surface composition, including mutants deficient in (i) the production of Sortase A involved in the covalent attachment of surface proteins to the cell wall peptidoglycan ($\Delta srtA$), (ii) the production of capsular polysaccharides (CPS1-4; $\Delta cps1-4$), and (iii) the production of cell wall lytic enzymes ($\Delta acm2$, $\Delta lytA$). It was shown that sortase-dependent proteins (SDPs) and cell wall autolysis with concomitant release of DNA (eDNA) are major determinants of *L. plantarum* WCFS1 static biofilm formation (Chapter 3).

Although a wealth of information is available on biofilm formation by single strains, it is conceivable that in a range of environments including food processing areas, biofilms are composed of multiple species and/or strains (Elias and Banin, 2012; Yang et al., 2011). Microbial interactions have for example been studied for biofilms formed by *L. plantarum*

/ *Listeria monocytogenes* (van der Veen and Abee, 2011), *Pseudomonas* / *L. monocytogenes* (Fatemi and Frank, 1999; Saá Ibusquiza et al., 2012), and *Streptococcus mutans* / *Veillonella parvula* (Kara et al., 2006). In multi-species biofilms, both stimulatory and inhibiting effects can be expected as a result of competition for space and nutrients. Notably, studies on biofilm formation using mixtures of strains belonging to the same species have not been reported and therefore the aim of this study is to analyse the performance of six *L. plantarum* food spoilage isolates and *L. plantarum* WCFS1 (Fernández Ramírez et al., 2015) in single and multiple strain biofilms using strain-specific quantitative PCR (qPCR) in the presence of propidium monoazide (PMA). Previously, the use of PMA was applied to monitor population dynamics of LAB during cheese ripening (Erkus et al., 2016) and for quantification of selected species in oral biofilms (Álvarez et al., 2013; Yasunaga et al., 2013), showing that PMA can be used in samples from complex matrices. Application of qPCR in the presence of PMA enables quantification of *L. plantarum* cells in the biofilm without membrane damage, representing the viable cell fraction that following dispersal, determines the outgrowth potential and food spoilage risk. The current study will provide insight in population dynamics in multi-strain biofilms at the level of viable cells of individual strains and will add to a more comprehensive model of static biofilm formation in conditions mimicking natural settings including food processing environments.

Materials and methods

Lactobacillus plantarum WCFS1 and six *L. plantarum* food isolates (Fernández Ramírez et al., 2016; Fernández Ramírez et al., 2015) were used in this study (Table 4.1). The strains were streaked from a -80°C stock in De Man-Rogosa-Sharpe (MRS) agar (Merck) and incubated for 48 h at 30°C. Single colonies were inoculated in 10 ml of MRS at 30°C for 18 h to prepare the starting culture. Planktonic and biofilm growth was performed in Brain heart infusion (Becton Dickinson) supplemented with 0.005% manganese sulphate and 2 % glucose (Merck) (BHIMnG) which was shown previously to support *L. plantarum* biofilm formation (Fernández Ramírez et al., 2015; van der Veen and Abee, 2011).

Table 4.1. *L. plantarum* strains used in the present study

Strain designation	Alternative designation	Isolation source	Reference
FBR 1	L21	Salad dressing	Fernández Ramírez et al., 2015
FBR 2	L30	Salad dressing	
FBR 3	JH2	Salad dressing	
FBR 4	TC157	Cheese with garlic	
FBR 5	JV5	Salad dressing	
FBR 6	La 10-11	Onion ketchup	
WCFS1		Human saliva	Kleerebezem et al., 2003

Table 4.2. Primers used in this study.

Strain identified	Target	Forward primer (5→3')	Reverse primer (5→3')	Amplicon length (bp)
FBR1	LH21_sc3_peg.212	AACCCAACTACCGCGATCAAT	TCATTGAGCCAATCAGCGACT	176
FBR2	L30_2890	GAAACAAGCACAGTCTGCCG	ACGTCTTGCAATGCTGAATAGC	97
FBR3/ FBR6	JH2_3060/ FBR6_2541	CCCTGGTTCGTATGCCCCG	CTTCTGGCCAACTCTGCATTT	145
FBR4	FBR4_2479	AAGAAATCCGCTGGGACGTT	ACAGTCTTGCCCTTAGCGAC	125
FBR5	FBR5_0394	TGGTCAATCTCCGAAGCAA	GTCAGCAGGATCAGTGGACG	118
WCFS1	lp_0375	ACCGACTGATAATTCTGGGCA	GCTCAATCAACCCTAACTCGC	116

Planktonic growth and biofilm formation

Equal mixtures of the individually grown overnight cultures were used for inoculation of BHIMnG to a final concentration of 1 % for biofilm and planktonic growth studies. The equality in culture composition for each strain was confirmed by cell enumeration of overnight cultures. The overnight cultures were plated on MRS agar, and after incubation for 48 h at 30°C the colonies were enumerated.

Formation of biofilms under static conditions was assessed in polystyrene wells plates as described by Merritt et al. (2005). Each well of a polystyrene 12 well plate (Greiner Bio-One) was filled with 1.5 ml of culture. The plates were sealed with parafilm to avoid evaporation. The biofilms were incubated at 30°C in static conditions and samples were collected after 24, 48 and 72 h. For this purpose, the medium was carefully removed with a pipette followed by three times washing with 2 ml of phosphate buffered saline (PBS, NaCl 8 g/L; KCl 0.2 g/l; Na₂HPO₄ 1.44 g/l; KH₂PO₄ 0.24 g/l; pH 7.4 (Merck)) to remove unattached cells. The resulting biofilms were quantified using the crystal violet (CV) assay. After washing, the biofilm was stained for 30 minutes with 1.5 ml of 0.1 % (w/v) of CV (Merck). Excess of CV was removed by washing three times with 1.5 ml PBS as described above. The dye attached to the biofilm was solubilized in 1.5 ml 70 % ethanol for 30 minutes and the optical density (OD) was measured at 595 nm (SpectraMax, Molecular Devices). When OD values exceeded a value of one, samples were diluted in 70 % ethanol. The resulting OD measurements were corrected by the dilution factor and by subtracting the average of the blank. The resulting measurement was defined as the total biofilm formed. Each plate was prepared in triplicate and three independent biological replicates were analysed per condition.

For cell enumeration by qPCR, biofilms formed in parallel experiments were washed as described above, and the remaining biofilm was resuspended by scraping and vigorous pipetting in 1 ml of PBS (Fernández Ramírez et al., 2015; Kubota et al., 2009; van der Veen and Abee, 2011). The samples were used to extract DNA for qPCR analysis as described below.

DNA extraction

Cells originated from biofilms and planktonic and suspensions were centrifuged 10 min at 5,000 x g and resuspended in 1 ml of 50 μ M of propidium monoazide (PMA; Biotium) dissolved in PBS according to the manufacturer's instructions and based on the optimized PMA treatment conditions as described by Erkus et al. (2016). The use of PMA allows selective amplification of DNA from intact cells in the (biofilm) cultures thereby avoiding interference of extracellular DNA present in the biofilm matrix or from dead or injured cells with a compromised membrane. DNeasy Blood and tissue kit (Qiagen) were used according to the manufacturer's instructions with some modifications in used quantities of the kit's buffer solutions AL, AW1 and AW2. The cells were resuspended in 300 μ l of lysis buffer; 62.5 μ l Proteinase K (Qiagen, Germany) was added before the addition of 500 μ l of AL buffer. Five hundred μ l of absolute ethanol was added. The washing steps were applied two times with AW1 and AW2 buffers to remove residual biofilm matrix. An additional step was added for the incubation of the samples with 10 mg/ μ l of RNase A (Qiagen). The DNA concentration was measured using an Eppendorf Biophotometer (Eppendorf, The Netherlands).

Quantitative PCR to determine the presence of individual *L. plantarum* strains

Quantitative PCR (qPCR) was used to determine the number of viable cells in planktonic or biofilm phase (Bio – Rad CFX96). qPCR was performed in triplicate for each sample using Power SYBR green (Applied Biosystems) and two biological replicates. The reactions were initiated at 95°C (10 min) followed by 40 amplification cycles with a denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min. The amplification of a single product was confirmed by running a melting curve analysis for all the samples (65 – 95°C with 0.5°C intervals). Specific primers for each strain were designed with the NCBI primer design tool using available genome sequence information (Table 4.2) (Fernández Ramírez et al., 2016). No specific primers could be designed for strains FBR3 and FBR6 since their genome sequences are highly similar and differ mainly in the presence of an additional plasmid in strain FBR6. Since plasmid stability and maintenance is not secured in prolonged growth and biofilm formation experiments, we decided to use the selected primers for FBR3 and FBR6, and therefore this combination of strains was not considered in the dual strain experiment. For the multi-strain experiment, two mixtures were prepared, one containing FBR3, the other FBR6. To correlate target copy numbers to colony forming units (cfu), DNA from PMA treated samples was extracted from cultures with known numbers of culturable cells (Figure S4.1). The C_q values obtained were corrected depending on the efficiency (in all cases > 91 %) as previously described (Pfaffl, 2004).

Results and discussion

Performance of single strains in biofilm

First, biofilm formation of the individual strains was tested and the number of viable cells was determined by quantitative PCR (qPCR). Despite comparable inoculation levels at time 0 for all strains (approximately $7.5 \log_{10}$ cfu/ml) (data not shown) at the start of the experiment, significant differences in individual strain performance were observed in the number of viable cells in the biofilms at the different incubation times (Figure 4.1A), with FBR1, FBR2 and FBR6 showing low numbers of viable cells (approximately $7 \log_{10}$ cfu/ml; except time point 72 h for FBR6 with approximately $8 \log_{10}$ cfu/ml), strain FBR3 with intermediate levels (approximately $8 \log_{10}$ cfu/ml), and strains FBR4, FBR5 and WCFS1 with the highest numbers of viable cells in the biofilm (approximately $9 \log_{10}$ cfu/ml). Cell numbers in the biofilm were subsequently plotted as a function of corresponding cell numbers in the planktonic phase for all strains (Figure 4.1B) which allowed for discrimination of described groups. A clear trend can be observed showing increasing numbers of viable cells in the biofilm with increasing numbers of viable planktonic cells. In the planktonic phase, strain FBR1 reached the lowest number of viable cells and strains FBR4, FBR5, and WCFS1 the highest. Strain FBR2 displayed the lowest number of viable cells in the biofilm, and again FBR5 the highest. Notably, viable cell numbers in biofilms based on qPCR data in the presence of PMA, are in agreement with previously determined levels of culturable cells in biofilms formed by these strains where we showed that highest and lowest culturable cell numbers in the biofilm were found for FBR4, FBR5 and WCFS1 versus strains FBR1, FBR2 and FBR6, respectively (Fernández Ramírez et al., 2015).

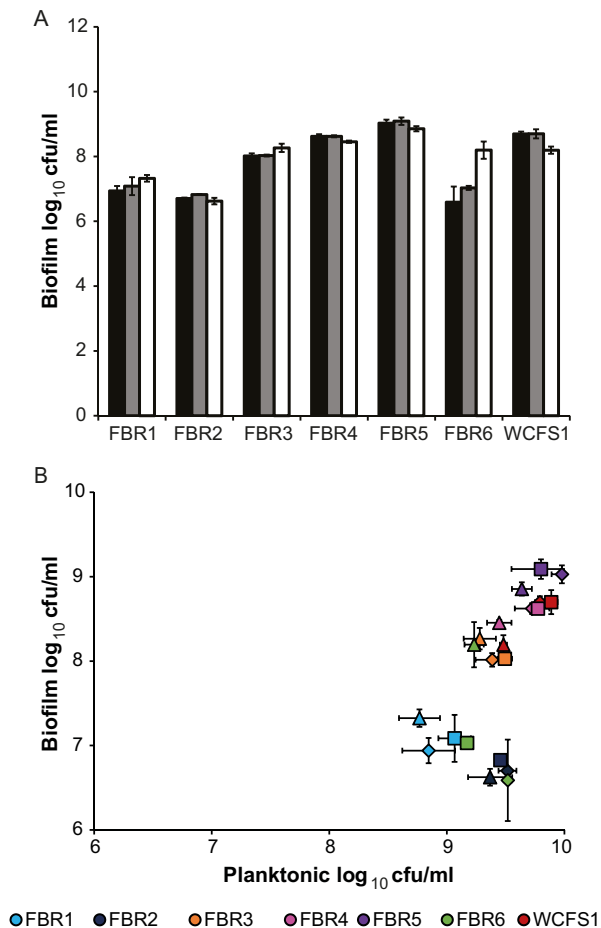


Figure 4.1. Biofilm formation of individual strains. (A) The single strain performance in the biofilm was quantified by qPCR after incubation in BHIMnG after 24 h (black), 48 h (grey) and 72 h (white) at 30°C. (B) The number of viable cells in the biofilm and in the planktonic phase were calculated based on qPCR Cq values using the calibration curve (S1) as described in the materials and methods section. Different time points after growth at 30°C in BHI MnG are included: 24 h (diamonds); 48 h (squares); 72 h (triangles). The data represent the average of two biological replicates and technical triplicates. The horizontal and vertical lines indicate the standard deviation.

Relative contribution of individual strains in dual strain biofilm formation

Assessment of relative contributions of individual strains to dual strain biofilms was based on the total number of cells in dual biofilms obtained by summation of cell numbers following quantification of individual strains in the mixtures by qPCR in the presence of PMA. Figure 4.2 presents a scatter plot of dual strain performance in biofilms compared to performance in planktonic conditions. Performance in dual-strain biofilms was highly strain dependent and influenced by the presence of the secondary strain. Moreover, in most cases, no correlation between the relative contributions of viable planktonic cells and viable cells in the biofilm was noted, except for strain FBR5 which showed highest viable cell numbers both in planktonic and biofilm phase.

Strains FBR1, FBR2 and FBR6, which displayed low numbers of viable cells in planktonic and in biofilm phase in single strain experiments, showed variable performance in planktonic phase constituting fractions ranging from 2 - 50%, 10 - 98% and 10 - 95% with a range of secondary strains (Figures 4.2 A, B and F). It is noteworthy that strains FBR2 and FBR6, despite significant contributions to planktonic fractions, in most cases hardly contribute to the population of cells found in the biofilm (< 10%). Strain FBR1 shows good performance in biofilms in dual strain cultures with FBR2, FBR3 and FBR6 (> 45%), respectively. The reduced performance of strain FBR1 in biofilms in dual strain biofilms with FBR4, FBR5 and WCFS1, matches its lower contribution to the fraction of viable planktonic cells. Strain FBR3 (Figure 4.2C) showed in most conditions a modest contribution to the viable cell population in the dual strain biofilms, with very low contribution to the dual strain biofilm formed with WCFS1 despite over 90% contribution to viable planktonic phase cells. Strain FBR4 (Figure 4.2D) generally showed good performance in the biofilm (> 35%) except in combination with FBR5, where the low fraction of viable cells in the biofilm matches the low fraction of viable planktonic cells. Strain FBR5 (Figure 4.2E) displayed a remarkable performance as viable cells of this strain dominated in both planktonic and biofilm fractions in the tested conditions (most cases > 80%). This indicates that FBR5, next to single strain experiments, also in competition with the selected secondary *L. plantarum* strains performs best in the conditions used, i.e. incubation at 30°C in BHIMnG medium.

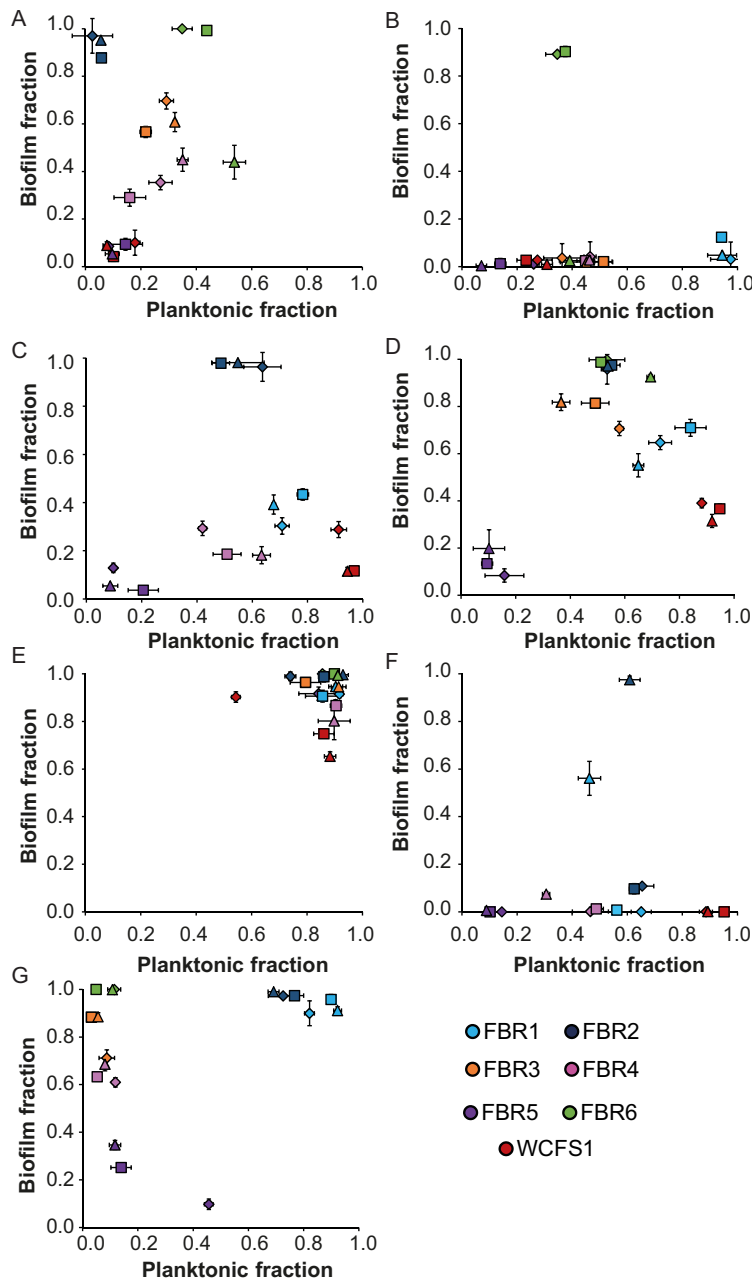


Figure 4.2. Relative abundance in the biofilm and planktonic growth of each *L. plantarum* strain. The relative abundance of each strain in all the combinations was determined by qPCR after growth in BHIMnG at 30°C: (A) FBR1; (B) FBR2; (C) FBR3; (D) FBR4; (E) FBR5; (F) FBR6, (G) WCFS1, at 24 h (diamonds), 48 h (squares), and 72 h (triangles). Vertical and horizontal bars represent the standard deviation of two biological replicates in triplicate.

The participation of viable FBR6 cells in the planktonic phase is quite variable, but contribution to the population of viable cells in the biofilm is very low (< 10%) except for two 72 h data points obtained from mixed cultures with strains FBR1 and FBR2. The described increase in relative abundance of FBR6 may be related to delayed maturation of the biofilm as observed also in single strain conditions.

Finally, strain WCFS1 (Figure 4.2G) displayed a relatively poor performance in dual-strain planktonic conditions (except when combined with either strain FBR1 or FBR2) whereas its performance in a biofilm was remarkably better, and in all cases exceeding the corresponding fraction in viable planktonic cells.

Total biofilm formation in single and dual strains biofilms using CV staining

The total biofilm formed at 48 h in dual strain experiments was measured using the crystal violet (CV) assay and compared to single strain performance (Figure 4.3). Single strain experiments showed no measurable CV staining for strains FBR1, FBR2, and FBR6, low level staining for strain FBR3, and somewhat higher staining for strains FBR5 and WCFS1, with the highest CV staining for the biofilm of strain FBR4. Notably, in dual strain conditions with either strain FBR1, FBR2 or FBR6 as the secondary strain, significant CV values were only obtained in combinations with FBR3, FBR4, FBR5 and WCFS1, although the values did not exceed those obtained in their respective single strain performances. It is conceivable that CV stainable biofilm material in dual strain conditions is contributed by the dominant strains only. Notably, total biofilms for strains FBR3, FBR4 or FBR5 were significantly lower (ANOVA, Dunett's test $P < 0.005$) in dual-strain biofilms with strain FBR1, compared to their single strain performance. The combination of strains FBR3 or FBR5 with strain FBR2 also resulted in lower total biofilm formation and this was also observed for FBR4 mixed with FBR3 or FBR6. Notably, synergy in total biofilm formation was only observed for the combination of strains FBR5 and WCFS1 (OD 595nm: added individual performance 16 ± 1.9 , FBR5 and WCFS1 in combination, 30 ± 3.8). We have previously characterised the biofilm matrix of *L. plantarum* biofilms for these strains providing evidence for a role of eDNA as well as protein-protein interactions and/or proteinaceous material in the biofilm matrix (Chapters 2 and 3). Fluorescence microscopy of sampled biofilm material using LIVE/DEAD staining did not provide evidence of higher levels of eDNA in these dual-strain biofilms (data not shown). It is conceivable that other matrix components contributed to the higher CV staining, but putative components remain to be identified.

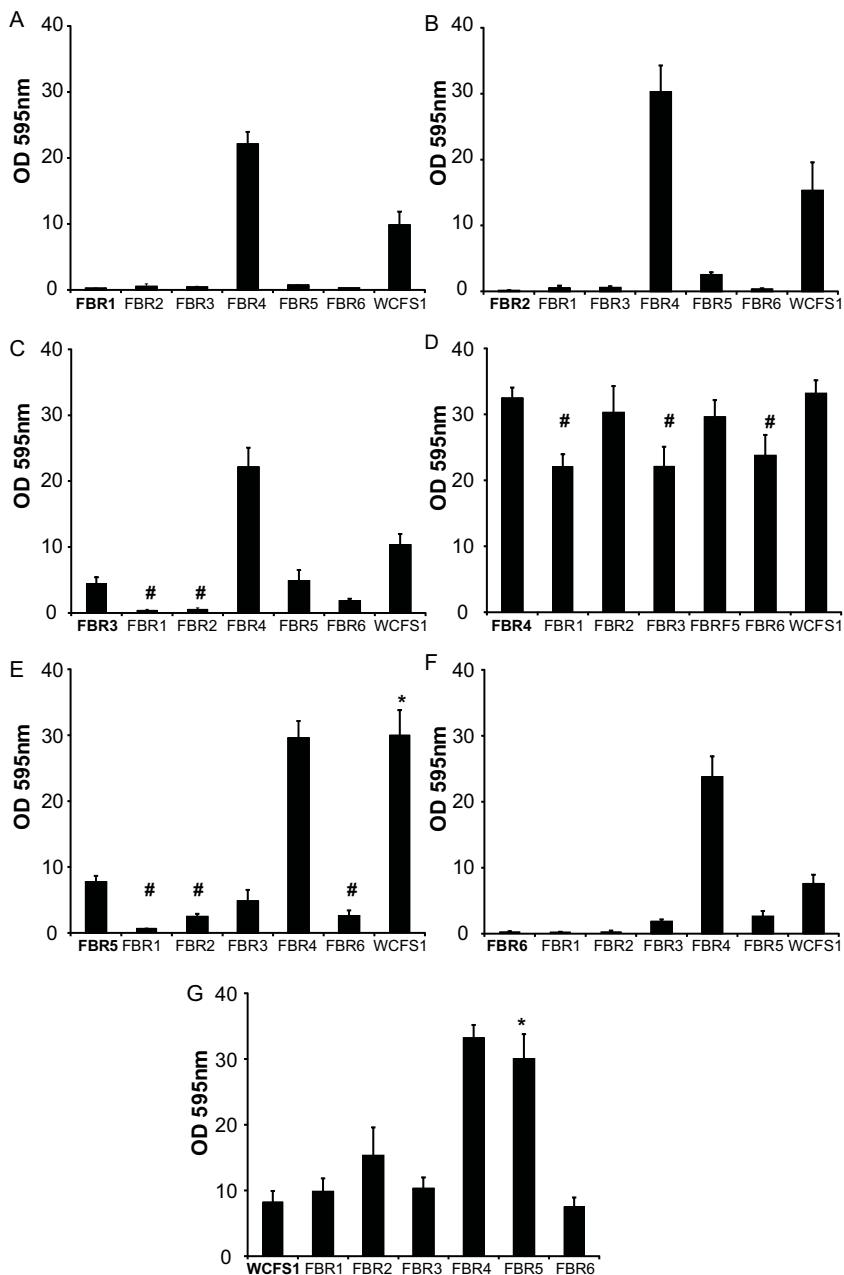


Figure 4.3. Total biofilm formation in single and dual strain combinations. The total biofilm formation was quantified by the crystal violet assay after 48 h incubation at 30°C in BHIMnG for (A) FBR1, (B) FBR2, (C) FBR3, (D) FBR4, (E) FBR5, (F) FBR6, and (G) WCFS1, in single (bold letters) and in dual strain biofilm cultures. The data represent the average of three biological replicates and the standard deviation is indicated by vertical bars. Significant differences (ANOVA, Dunett's test $P < 0.005$) are marked for higher (*) or lower (#) CV compared to the single strain performances presented in the respective A-G Figures.

Relative contributions of individual strains in multi-strain biofilm performance

To further study interactions between the strains, biofilm formation was analysed in two multi strain models, with either strain FBR3 or strain FBR6, since these two strains could not be distinguished in the qPCR approach used. Viable planktonic and biofilm associated cell fractions were determined (Figure 4.4), and unlike the dual strain biofilms, the multiple strain interaction setting shows a correlation between the relative abundance of viable cells in planktonic and biofilm fractions (Figure 4.4A). Overall, viable cells of strain FBR5 dominated again both in planktonic and biofilm fractions in the multi strain model, followed by strains WCFS1 and FBR4 (Figure 4.4A, B). Notably, strain FBR3 performs better than strain FBR6 (approximately 3% and 0.5% respectively to the fraction of viable cells; significantly different Student's t-test, $P < 0.01$), in the multistrain biofilm. Both strains are virtually identical when presence and order of orthologous groups (OGs) were compared. Strain FBR6 has 4 large plasmid fragments in the range of 5 kb up to 21 kb which are not present in strain FBR3. These FBR6 plasmid fragments include genes with predicted function in extracellular polysaccharides (EP) synthesis and heavy metal transporters. Since gene expression studies were not performed, and EP production has not been assessed, a role of EP in reduced performance of strain FBR6 remains to be investigated. Although it is generally assumed that EP contributes to biofilm formation (Branda et al., 2005; Flemming and Wingender, 2010), negative effects on adhesion capacity and negative effects on cell-cell interactions have been reported (Lebeer et al., 2009). Finally, the CV assay shows that both mixtures of multiple strains resulted in a comparable total biofilm formed, although CV values were approximately 2-fold lower compared to the values observed in single and in general dual biofilms containing strain FBR4, conceivably due to the lower fraction of this strain in both mixtures of the multiple strain biofilm formation experiments. In dual strain biofilms, strain FBR4 contributed to more than 40% to the number of viable cells in the biofilm whereas for multi-strain biofilm formation it only contributed 10%.

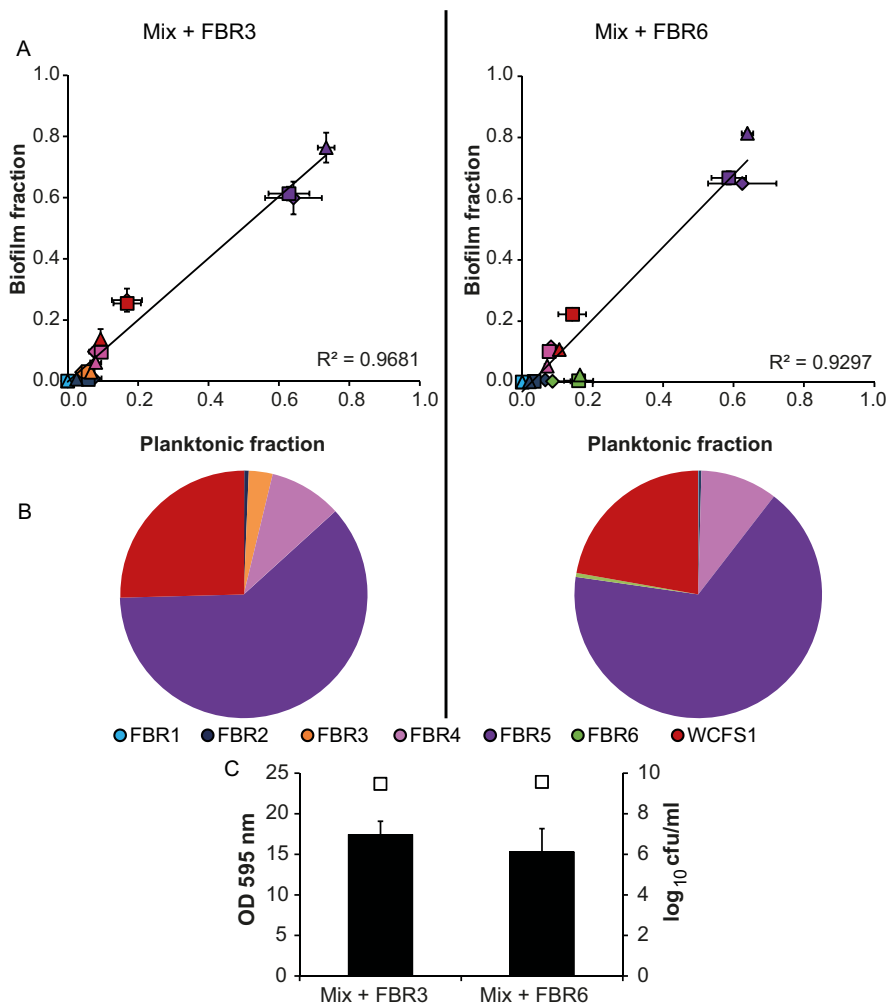


Figure 4.4. Multi strain biofilm formation of *L. plantarum* spoilage strains and WCFS1. (A) The correlation between relative abundance of the biofilm and planktonic fractions based on the determination by qPCR is displayed and the relative abundance of each strain is shown after incubation in BHIMnG at different time points: 24 h (diamonds); 48 h (squares); 72 h (triangles). (B) The relative abundance of strains in the two mixtures is shown, and (C) shows the total biofilm formation quantified by the crystal violet assay OD 595 nm and qPCR after 48 h incubation at 30°C in BHIMnG. The data represent the average of two biological replicates in triplicates and the standard deviation is indicated by vertical bars. No significant differences in OD595 nm and \log_{10} cfu/ml were found (Student's t-test $P < 0.005$)

Comparison of viable planktonic and cells in biofilms for the individual strains in single, dual and multiple strain experiments

An overview of all data on viable cells is presented in Figure 4.5, enabling linking of this dataset to the relative performance of the individual strains (Figures 4.2 and 4.4) and to total biofilm formed measured by CV staining (Figure 4.3). This section thus provides insight in putative (re)contamination risks of the tested strains related to the levels of viable cells in biofilms of the tested *L. plantarum* strains. In general, the average cell numbers in dual planktonic growth were not significantly different from their respective numbers in single strain growth, with strains FBR4, FBR5 and WCFS1 showing highest average cell numbers, followed by FBR2, FBR3 and FBR6, and FBR1 with significantly different lowest average cell numbers (ANOVA, Dunett's test $P < 0.005$) (Figure 4.5A). Notably, in multi-strain cultures, most strains had significantly lower viable cell numbers (approximately 10-fold) compared to single and dual strain conditions, except strains FBR5 and FBR6, which showed similar mean values in all three conditions. It is conceivable that increased competition for nutrients and/or interactions between strains resulted in the observed reduced viable cell numbers for most strains, except FBR5 and FBR6, in multi-strain cultures.

Notably, the performance of individual strains in corresponding single, dual and multistrain biofilms shows a different pattern compared to the corresponding viable planktonic cells (Figure 4.5A and B). Single mean viable cell numbers in the biofilm was comparable to dual biofilm formation for all strains except for strains FBR3 and FBR5 which had higher average cell numbers as single strains (Figure 4.5B). Nevertheless, strain FBR5 was the dominant strain in all dual combinations. In contrast to planktonic growth, the cell numbers in multi-strain biofilm formation were comparable to those of single strain biofilm. However, *L. plantarum* strains FBR2 and FBR5 had higher mean cell numbers in multi-strain biofilm formation (approximately 5 and 4-fold higher, respectively), with FBR5 showing the highest number of viable cells in the biofilm ($9.5 \log_{10}$ cfu/ml). Despite the increase in cell numbers of strain FBR2, this strain did not represent more than 1% of the total viable cells in the multi-strain biofilm, whereas strain FBR5 dominated the multi-strain biofilms. Notably, previous studies focusing on comparative analysis of heat resistance of a collection of 20 *L. plantarum* food spoilage isolates, showed strain FBR5 to be the most heat resistant strain with the highest *D*-value at 55°C and 58°C (Aryani et al., 2016). Next to an excellent performer in single, dual and multi-strain biofilms, this strain is highly heat stress resistant, conceivably posing an elevated spoilage risk due to persistence of high viable cell numbers in food processing environments.

Total biofilm formation capacity is another relevant parameter, and the corresponding data were obtained using CV staining of biofilms produced in single and dual strain cultures (Figure 4.3) and in multi-strain conditions (Figure 4.5). Although a range of interactions including production of matrix components including eDNA, and inter-strain interactions could affect the individual performance of the selected strains in dual and multiple strain

conditions, the current study shows that those strains showing low levels of viable cells in the biofilm as single strains, also appeared the least abundant in viable cell fractions of dual and multiple strain mixtures (Figure 4.5).

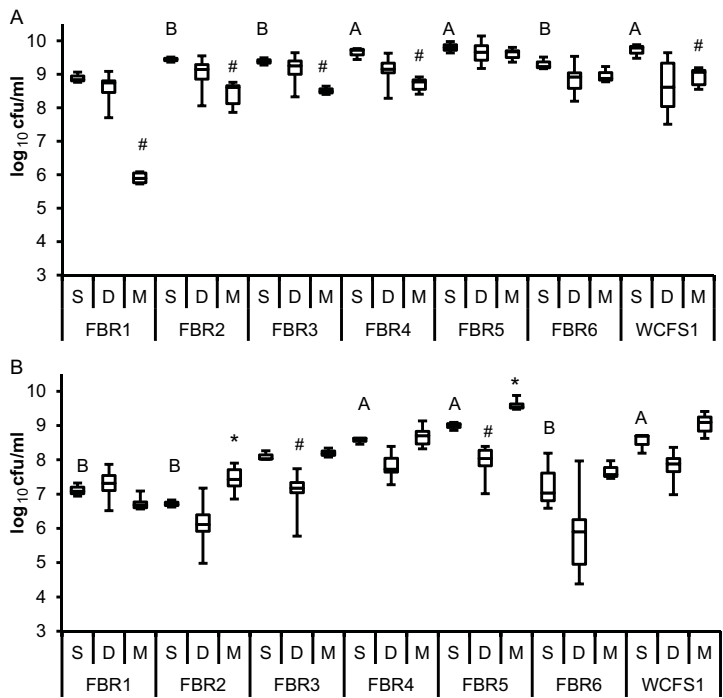


Figure 4.5. Viable cell numbers quantified by qPCR for each individual strain in all tested conditions. All data points are represented for single strain (S), dual strain (D) and multiple strain (M) mixtures for planktonic (A) and biofilm growth (B). The data represent the average of two biological replicates in triplicates and the standard deviation is indicated by vertical bars. Letters are used to indicate the comparison among single strains (ANOVA, Tukey test $P < 0.005$). Significant differences (ANOVA, Dunett's test $P < 0.005$) are marked for lower (#) or higher (*) average dual and/or multi-strain performance in comparison to their respective single strain.

Compared to previous single strain studies, performance in multi-strain conditions may be affected due to strain specific differences in cell surface protein composition and/or levels and autolysis capacity affecting intra- and inter-strain cell-cell interactions and matrix production. Successful performance in single, dual and multi-strain biofilms showed some correlation with performance in planktonic phase where mean viable cell numbers of strains FBR3, FBR4, FBR5 and WCFS1 are slightly higher (but not significantly different) than for the other strains. Especially, at later time points (48 and 72 h) the number of viable cell may have decreased due to sedimentation (and possible association in the biofilm) and/or lysis of cells. The variations in biofilm formation measured as total biofilm by CV staining could not be directly correlated to viable cell numbers, conceivably due to variations in matrix composition including differences in levels of eDNA.

A range of factors can affect competition between strains in planktonic and/or in biofilms including competition for certain nutrients or production of other compounds such as polysaccharides or antimicrobial compounds such as bacteriocins. The production of bacteriocins can aid a specific strain to dominate in a community. On the other hand, other microorganisms can co-exist when they are not sensitive to the bacteriocins produced (Smid and Lacroix, 2013). Söderling et al. (2011) reported that the biofilm formation of *Streptococcus mutans* was inhibited when it was in co-culture with *L. plantarum* 299v or *L. rhamnosus* GG due to antimicrobials produced by the lactobacilli. Kim et al. (2009) found that released exopolysaccharides from *L. acidophilus* A4 were able to inhibit biofilm formation by *Escherichia coli* and Gram positive microorganisms such as *Listeria monocytogenes* and *Bacillus cereus*. Since performance in planktonic and biofilm phase might be affected by a range of general and niche-specific parameters, the underlying mechanisms contributing to competitive fitness and robustness remain to be elucidated using a systems biology approach.

The findings of this research provide insights into single, dual and multi-strain planktonic and biofilm growth of selected *L. plantarum* strains. It was previously hypothesised that in environmental conditions such as food processing facilities, biofilms *in situ* may be composed of multiple strains of the same species which share the same niche. Based on novel insights obtained in the current study, we conclude that our approach can be used in future studies to test this hypothesis using isolates from specific fouling sites and determine their performance, including disinfectant resistance, in multi-strain experiments using appropriate conditions including selected surface material(s), temperature(s) and (model) food media.

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Supplemental data

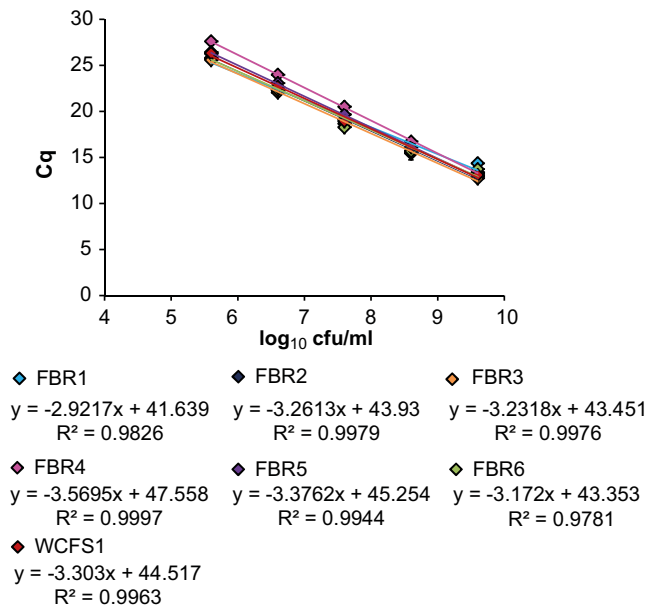


Figure S4.1. Calibration curves for individual strains. The DNA extraction of a known number of culturable cells was performed to obtain the calibration curves and determine the number of cells for each strain for single, dual and multi-strain planktonic and biofilm samples.

CHAPTER 5

Strain-specific tracking of *Lactobacillus plantarum* in competitive multi-strain static and dynamic flow biofilm models

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Abstract

In nature, several strains from the same species can share the same niche. The environment can influence the population dynamics of such multi-strain communities. We studied the influence of maturation time, temperature and medium composition with low/high Mn(II) without/with haem and vitamin K2, on the population dynamics of a mixture of 12 *Lactobacillus plantarum* strains with different origins, in competitive planktonic and surface-attached biofilm growth models. A next generation sequencing approach based on detection of strain specific alleles was used to determine the relative abundance of each strain in the different conditions. Data were obtained in the presence and absence of propidium monoazide, thus allowing for identification and quantification of relative contributions of each individual *L. plantarum* strain to the fraction of viable cells in planktonic and biofilm phase and the fraction of dead cells (compromised membrane) and levels of eDNA in the biofilm matrix, respectively. This approach revealed that the relative abundance of each strain in the static biofilm positively correlates with its performance in static planktonic conditions.

Environmental stresses such as absence of Mn(II) and increased temperature affected the relative abundance of strains both in planktonic and biofilm growth, and also the release of eDNA. The genome content of two groups of dominating strains was explored to identify genetic factors that potentially contribute to specific features in relation to biofilm forming capacity under static and dynamic flow conditions.

Introduction

The success of a bacterial strain within a community in complex environments can be attributed to deterministic factors (for example competition and antimicrobial activity) in which the combined traits of each strain determine the prevalence in a specific niche (Polchan et al., 2013; Vanwonterghem et al., 2014). In addition, stochastic processes such as death and proliferation can play a role in shaping the composition of the community (Bell, 2001; Sloan et al., 2006). In real life, the population dynamics are governed by the product of stochastic and deterministic factors (Caruso et al., 2011; Stegen et al., 2012).

Lactic acid bacteria (LAB) are widely distributed in the environment occupying several niches. LAB have been isolated from soil (Kawasaki et al., 1996; Klijn et al., 1995; Magnusson et al., 2002), plants (Barth et al., 2009; Yang et al.) as well as from fermented food (Buckenhüskes, 1993; Leroy and De Vuyst, 2004; Stiles and Holzapfel, 1997) and feed (Ennahar et al., 2003; Lindgren and Pleje, 1983) products. LAB can also be found in the oral cavity (Paster et al., 2006) and in the gastrointestinal tract (Gill et al., 2006; Hayashi et al., 2002; Wang et al., 2003) where *Lactobacillus* spp. represent less than 1% of the total bacterial population (Kimura et al., 1997; Sghir et al., 2000). Notably, it has been shown that the undefined starter culture Ur, used for Gouda cheese manufacturing, is composed of 7 lineages of *Lactococcus lactis* and one lineage of *Leuconostoc mesenteroides*. The population dynamics of this complex starter culture has been assessed and it was found that bacteriophage predation played a role in maintaining the strain diversity following the 'kill-the-winner principle' (Erkus et al., 2013; Smid et al., 2014). Besides the biotechnological role of LAB as starter cultures, they can also have detrimental effects in food products such as beer (Sakamoto and Konings, 2003), sliced meat (Chenoll et al., 2006), salad dressings (Kurtzman et al., 1971) and other foods. Furthermore, *Lactobacillus* spp. capable of biofilm formation have been isolated from a range of raw materials including onions and olives (Domínguez-Manzano et al., 2012; Kubota et al., 2008).

The biofilm forming capacity of LAB has been studied in single strain setting for several species, including *Lactobacillus plantarum* (Fernández Ramírez et al., 2015; Kubota et al., 2009; Muscariello et al., 2013), *Lactobacillus rhamnosus* (Lebeer et al., 2007) and *Lactobacillus curvatus* (Somers et al., 2001). Characterization of biofilm material sampled from natural environments has generally shown the presence of multiple species of Gram-negative and/or Gram-positive bacteria (Besemer et al., 2012; Li et al., 2004; Lin et al., 2013).

In mixed species biofilms, competition for space and nutrients can take place and positive, synergistic or negative interactions can occur among the different species (Elias and Banin, 2012; Foster and Bell, 2012; Rendueles and Ghigo, 2012). Several studies describe the population dynamics in biofilms composed of two or more species; however, studies on multi-strain behaviour are very limited. For example, dual strain biofilm formation using

Pseudomonas fluorescens D3-348 and D3-350 has been investigated and resulted in co-existence of both strains in biofilms formed under dynamic flow conditions (Simões et al., 2008). Intrinsic to biofilm formation is the formation of microenvironments. For example, cells in the lower layers of the biofilm can experience oxygen and nutrient limitation. These conditions are not necessary detrimental as metabolic cooperation can take place between the species or strains, whereby metabolic (by-) products may be exchanged in the respective niches or microenvironments (Elias and Banin, 2012; Moons et al., 2009).

Here we report how different growth conditions representing different environmental niches affect the composition of the microbial community of 12 *L. plantarum* strains in competitive static and dynamic flow biofilm models. Individual strains exhibited a specific degree of biofilm formation and autolysis. These individual characteristics can be influenced by co-cultivation with other strains due to cooperative and/or inhibitory interactions between community members. The parameters tested include time, temperature, presence or absence of Mn(II) and static and dynamic flow conditions in planktonic and biofilm growth. To identify and quantify individual strains from the same species in the community, the natural genetic variation of a discriminatory allele in their genomes was used in a next-generation sequencing approach (van Bokhorst-van de Veen et al., 2012). The study of population dynamics in this range of conditions together with the presence/absence of propidium monoazide (PMA) revealed strains dominating the viable cell fractions in the static and dynamic flow biofilm communities, whereas others, especially in static biofilms, were found to contribute to a larger extent to the biofilm matrix by DNA release. With this approach, the relative abundance of individual strains within the community has been monitored in a wide range of environmental conditions. Finally, genomic content of the strains has been correlated with their relative community dominance to identify candidate genetic marker genes that contribute to their niche-specific competitiveness.

Materials and methods

Lactobacillus plantarum strains and growth conditions

Each strain (Table 5.1) was grown in De Man, Rogosa and Sharp broth (MRS, Merck) at 30°C for overnight cultures. One ml from overnight grown cultures was centrifuged at 5 000 x g for 10 min and washed two times with phosphate buffered saline (PBS; NaCl 8 g/L; KCl 0.2 g/L; Na₂HPO₄ 1.44 g/L; KH₂PO₄ 0.24 g/L; pH 7.4 (all from Merck)) to remove residual nutrients. Prior to mixing cultures, individually grown cultures were resuspended at an optical density (OD) at 600 nm of 10. This OD corresponded to approximately 9.2 log₁₀ cfu/ml of each individual strain before being mixed. Different media were used for

growth and biofilm formation: Brain Heart Infusion (Becton Dickinson; intrinsic Mn(II) concentration: 427 nM) supplemented with 2% (w/v) glucose (Merck) without (BHIG) or with 0.005% (w/v) manganese sulphate (Merck) (BHIMnG, Mn(II) 265µM). In addition, to study the respiration growth mode 10 µg/ml hemin (from bovine, Sigma) and 50 µg/ml Vitamin K2 (Menaquinone4, Sigma) (Brooijmans, Smit, et al., 2009; Brooijmans, de Vos, et al., 2009) were added to BHIMnG (BHIMnGHK).

Table 5.1. Strains used in the present study

Strain	Alternative designation	Origin	Source* or Reference
WCFS1		Human saliva, UK	Kleerebezem et al. (2003)
ATCC14917	LMG 6907	Pickled cabbage, Denmark	ATCC
NCTH19-2		Pickled sour sausage, Vietnam	NIZO
CIP104440	61A	Human stool, France	CIP
NC8		Grass silage, Sweden	Aukrust and Blom (1992)
CIP104448	61BB	Human stool, France	CIP
SF2A35B		Sour cassava, South America	Figueroa et al. (1995); Siezen et al. (2010)
FBR1	L21	Salad dressing	Sanders et al. (2015)
FBR2	L30	Salad dressing	
FBR4	TC157	Cheese with garlic	
FBR5	JV5	Salad dressing	
FBR6	La 10-11	Onion ketchup	

* NCIMB, National Collections of Industrial, Marine and Food Bacteria, United Kingdom; ATCC, American Type Culture Collection, USA; NIZO, NIZO food research collection, the Netherlands; CIP, Collection of Institute Pasteur, France; and BCCM, Belgian Coordinated Collections of Micro-organisms, Belgium.

Biofilm and planktonic growth

The mixed culture prepared as described above was 100 fold diluted in each medium. The overview of the experimental set-up can be found in Figure 5.1A. For planktonic growth in static conditions, 10 ml of inoculated BHIG and BHIMnG were incubated at 20, 25, 30 and 37°C. Additionally, 20 ml of BHI, BHIMnG and BHIMnHK were incubated at 20°C with shaking at 200 rpm in 100 ml Erlenmeyer flasks for aerobic and respiration conditions. Biofilms in static conditions were grown in 12 well polystyrene plates (Greiner Bio-One) as described previously (Fernández Ramírez et al., 2015) and incubated at 20, 25, 30 and 37°C for the media BHI and BHIMnG. The medium BHIMnHK was used to study the competition model under respiration growing conditions to investigate whether growth performance is affected in these conditions. Additionally, results can be linked to performance in the competitive biofilm model in dynamic flow conditions. Samples were collected in duplicate, one sample for the determination of the relative community-contribution of

each strain and the other sample for the determination of the contribution of cells that were considered 'viable' (with an intact cell membrane) after 24, 48 and 72 h. Biological duplicates for each sample were included. To collect the samples from the biofilm, the medium was removed with the aid of a pipette and the biofilm was washed two times with PBS to remove unattached cells. Finally, the biofilm was resuspended with vigorous pipetting in 1.5 ml of PBS. Continuous flow biofilms were grown in three channel flow cells (channel dimensions 40 x 4 x 1 mm) (Systems Biology, Technical University of Denmark) at 20°C. The procedure followed to set up the flow cell was performed as described previously Cruzs et al. (2012). Each channel was injected with 0.5 ml of the different media, i.e. BHI, BHIMnG and BHIMnHK, inoculated with 1 % of the prepared strain mixture. The media was pumped at a dilution rate (D) of 10 h⁻¹. The biofilms were harvested after 24, 48 or 72 h and dispersed in 2 ml of PBS.

All collected samples were washed once with PBS. The samples intended for viable cell quantification were treated with propidium monoazide (PMA; Biotium) following the manufacturer's instructions to cross link PMA to DNA (extracellular or in cells with leaky membranes that are considered non-viable). All cell pellets were frozen until DNA extraction.

DNA isolation and PCR

The DNeasy Blood and tissue kit (Qiagen) was used according to the manufacturer's instructions with some modifications described in the supplemental materials and methods. The DNA concentration and quality were measured by NanoDrop (ND-1000 spectrophotometer, NanoDrop Technologies, Wilmington, USA). The DNA samples were used for PCR and quantitative PCR (Primers listed in ST5.1). The Ip_1173 reverse primers included a unique 6 nucleotide barcode for the discrimination of all the samples derived from different time points, media, temperature and growing conditions (ST5.2). All PCR reactions were performed using REDTaq DNA Polymerase (Sigma-Aldrich) as follows: 94°C for 4 min followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1.3 min and a final extension at 72°C for 4 min in a Veriti® thermal cycler (Applied Biosystems). The PCR products (528bp) were purified by QIAquick PCR Purification Kit (Qiagen, The Netherlands). The concentration of each sample was measured with Quant-it dsDNA Assay Kit (Molecular probes, Life Technologies) following the manufacturer's instructions and measuring excitation at 510 nm and emission at 527 nm with the plate reader SpectraMax M2 (Molecular Devices). All samples were pooled in equimolar amounts. The pooled sample was ran in a 1.5 agarose gel, the band of 528 bp was purified from gel with the QIAquick gel extraction kit (Qiagen) and sent for Next Generation Sequencing to Base Clear B. V. (Leiden, The Netherlands) where paired-end sequence reads were generated using the Illumina MiSeq system.

Quantitative PCR

Quantitative PCR was used to determine the total numbers of viable cells and to estimate the total quantity of DNA originated from viable, dead cells with compromised membranes and extracellular DNA (eDNA). The quantification was performed using Power SYBR green (Applied Biosystems) for each sample in triplicate and two biological replicates and was measured in a Bio-Rad CFX96 device. The reactions were initiated at 95°C (10 min) followed by 40 amplification cycles with a denaturation at 95°C for 15 s, annealing and extension at 61.4°C for 1 min for the primer pair targeting *groEL* (ST5.1); followed by a melting curve analysis from all the samples (65 – 95°C with 0.5°C intervals). Additionally, the PCR efficiency was tested and calibration curves were obtained by extracting DNA from cultures with known cell concentrations.

Target identification and validation for single-locus sequencing typing

Target identification and validation for single-locus sequencing typing (SLST) was performed following the principle from Scholz et al. (2014). The genome assemblies of the 12 *L. plantarum* strains were annotated with the RAST automated annotation engine, using default settings (Aziz et al., 2008). Predicted open reading frames (ORF) of protein-coding genes were used for gene orthology calculation by OrthoMCL (Li et al., 2003), using a Markov Cluster Algorithm (MCL) inflation index of 1.5 for building of ortholog clusters. Single-copy one-to-one ortholog clusters were selected in search for a genetic region able to discriminate the 12 *L. plantarum* strains. For this, we used an in-house Perl-based custom Bio-IT pipeline that allowed us to identify a 480 bp long variable region (Figure 5.1B) within the open reading frame lp_1173 present in *L. plantarum* WCFS1 and annotated as a gene encoding UDP-N-acetylglucosamine 2-epimerase, with conserved flanking regions allowing for manual primer design. The final amplicon size is 522 bp long, and can be retrieved using the primers listed in ST5.1. To prove primer specificity and to exclude potential mis-priming or non-specific off-target binding, this primer pair was *in silico* validated using Primer Prospector (Walters et al., 2011) on the full set of complete *L. plantarum* genomes, using default author-advised settings, prior to PCR- and Sanger sequencing wet-lab validation on *L. plantarum* isolates.

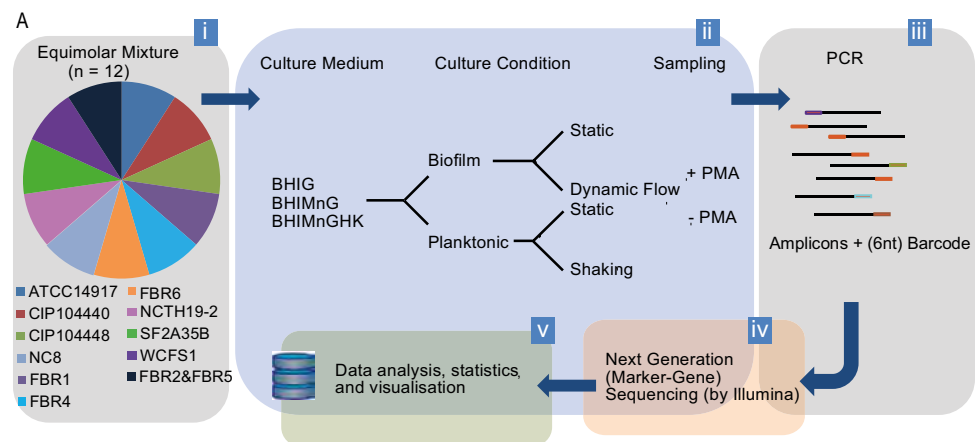
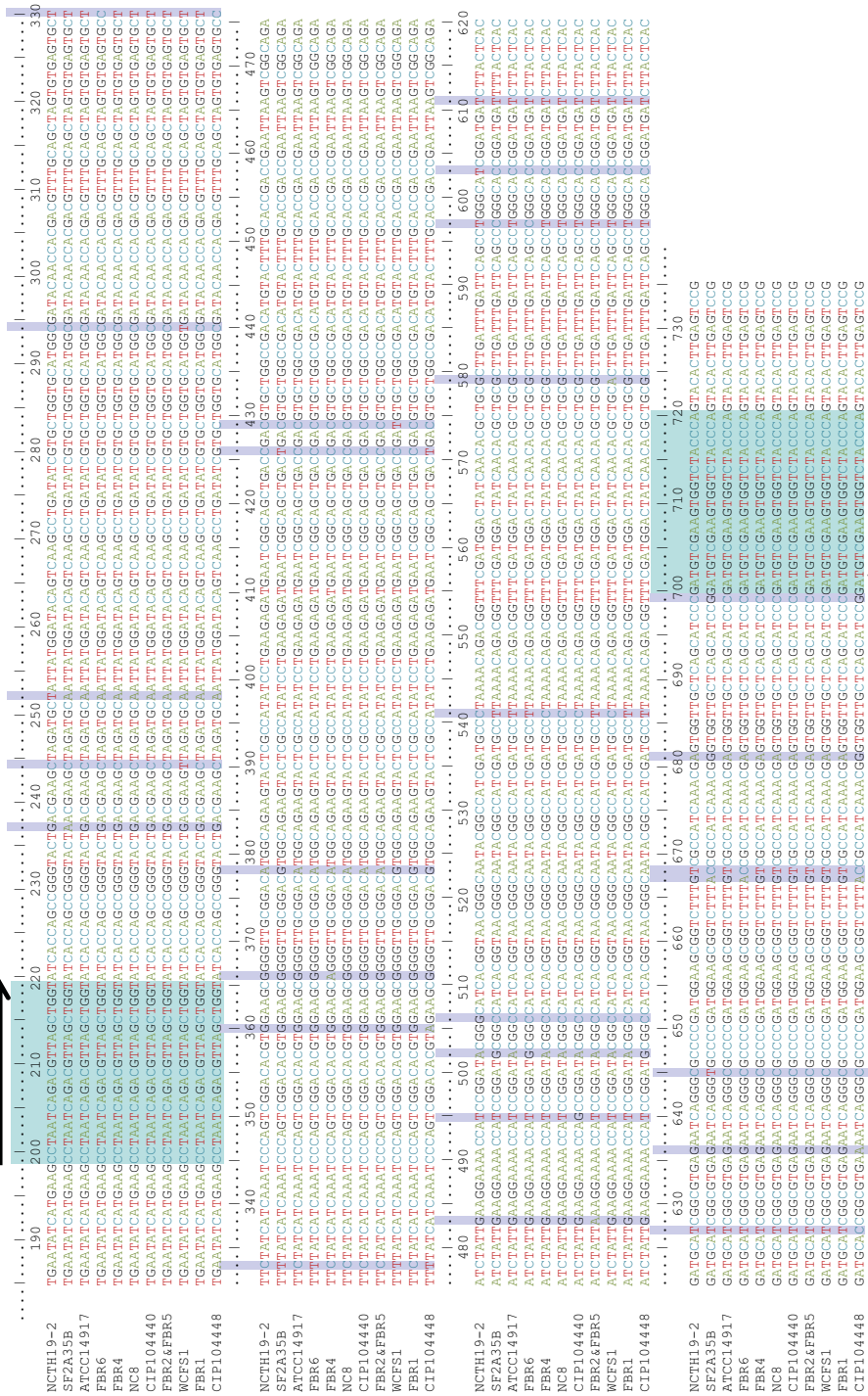


Figure 5.1. Experimental design and variable regions. (A) An overview of the experimental design is shown and the details of the conditions per sample can be seen in ST5.1. (B) (next page) Sequence comparison of variable region *lp_1173* of all strains used.

Forward primer



Marker gene sequencing data quality control and (pre-)processing

Next-generation paired-end sequence reads of the lp_1173 marker gene (amplicon) were generated by BaseClear B. V. (Leiden, The Netherlands) using the Illumina MiSeq PE300 system. Multiplexed FASTQ files as provided by BaseClear were first used to generate Illumina paired-end sequence pseudoreads by PEAR (Zhang et al., 2014), using the default settings. A total of 215k reads (1.9%) were unable to assemble, leaving 11.1M assembled sequencing reads for further downstream processing. In order to filter all non-target (lp_1173 marker gene) sequences, reads were BLAST to a database of the putative 12 unique *L. plantarum* reference lp_1173 marker gene sequences by BLASTn, using its default parameters; and all reads without a BLAST hit were discarded (15k reads in total only). Thereafter, reads were demultiplexed (including removal of barcodes and primer sequences) using the split libraries python script from the QIIME sequencing data analysis pipeline (Caporaso et al., 2010), which was successful for 10.1M reads. Finally, reads were stringently selected for the exact length of 480 bp (as there are no indels in the target region of our strains-of-interest), discarding only 66k reads in total, and thereby yielding more than 10M reads in 300 samples for biological analysis ($33.7k \pm 17.5k$ SD reads per sample on average; with exception of 3 dropout samples with <150 reads per sample: samples from biofilm formed under dynamic flow conditions in absence of PMA in BHIG at 24 h (one sample) and 72 h (two samples)).

Sequencing-based *L. plantarum* strain typing and analysis

From the target variable region of the 12 *L. plantarum* strains-of-interest, sequence types were constructed based on all (27) SNP positions in the reference alignment (see Figure 5.1A). The sequence types are unique for each strain-of-interest (with exception of FBR2 and -5, which will be referred as FBR2-5), and are defined as short pseudosequences based on the 27 concatenated SNP position nucleotides of each strain. For each sequencing sample, pseudosequences were constructed from the reads surviving quality control as described above, and were mapped to a reference sequence type, accepting perfect matches only (on average, $59.4\% \pm 14.1\%$ SD of the total reads per sample could be mapped to a reference strain). Relative abundances for each strain-of-interest to the total mapped pseudosequences per sample were calculated, and reported in a compositional matrix for further downstream (statistical) analysis and biological interpretation. In addition, primers were designed to selectively and specifically quantify DNA derived from the strains FBR2 and FBR5 (ST5.1, S5.5A), since these strains share the same allele sequence that was used for strain discrimination. The strains were quantified as described above using an annealing and extension temperature of 64.5°C providing information on the relative contribution of FBR2 and FBR5 in planktonic and biofilm fractions. Using qPCR it was established that FBR5 was the dominant strain in the FBR2-5 fraction (relative

contribution > 80%) in the static and dynamic flow biofilms, whereas contribution of FBR2 and FBR5 was similar (each approximately 50%) in the planktonic phase (S5.5A). Based on this information, we assume that FBR2-5 contributions in static and dynamic flow biofilm fractions determined by NGS represent performance of FBR5, whereas in planktonic conditions relative contributions of the two strains are assumed to be similar.

Data analysis

The Statistical software Canoco 4.5 (Braak and Smilauer, 2012) was used to perform the principle component analysis.

The effects of temperature, media, incubation time, and growth mode on performance of the tested *L. plantarum* strains were compared by univariate analysis of variance and by a multiple regression model in Excel. These variables were treated as qualitative or quantitative variables, considering a $P < 0.001$ as significantly different.

Results and discussion

The selected *L. plantarum* strains used were individually characterized and exhibited differences in biofilm forming capacity, sensitivity to Proteinase K and DNase I (S5.1) and autolysis (S5.2). In total, 12 *L. plantarum* strains from different origins, including faeces, fermented foods and spoiled foods were mixed and their ability to proliferate in the multi-strain microbial community was monitored in a range of environmental conditions. In mixed species communities, predation, cooperation or competition can occur and the current study provides insight in the interactions taking place in communities in biofilm and planktonic niches composed of a variety of strains belonging to the same species. Population dynamics was monitored as a function of temperature, absence/presence of added Mn(II) (427nM versus 265μM), and growth mode i.e. planktonic (static, aerobic or respiration conditions) and as integral part of a biofilm under static or dynamic flow conditions. Furthermore, each condition was analysed in the presence and absence of PMA to assess the contribution of individual strains to the fraction of viable cells, and the fraction of viable and dead cells (with compromised membranes) or eDNA (lysed cells), respectively.

Discrimination of individual *L. plantarum* strains

To assess the detection limit of strain quantification by next generation sequencing based on the *lp_1173* locus, five mixtures of eleven strains with a variable amount of strain WCFS1 (10-fold dilution range) were quantified. The results showed that within a range of 120-fold dilution the relative abundance of an individual strain in the mixture can be determined accurately. As demonstrated for strain WCFS1, higher dilutions result in overestimation of the relative abundance of the strain present at a lower abundance (S5.3 and ST5.3). All samples were within the range to be accurately quantified by next generation sequencing. Moreover, the use of PMA could efficiently avoid the quantification of DNA originated from dead cells (compromised membrane) and/or eDNA (S5.4). A scheme of the experimental set up can be found in Figure 5.1A. The sequence comparison of the variable region is shown in Figure 5.1B.

Environmental impact in the multi-strain competition model

Quantitative PCR (qPCR) was used to determine the total *L. plantarum* population by using the primers of the conserved gene *groEL*. The total numbers of viable cells in the biofilm were found to range from 7.0 to 10.0 log₁₀ cfu/ml, whereas for planktonic conditions it was between 7.7 and 9.2 log₁₀ cfu/ml. In general, the absence of Mn(II) resulted in lower total cell numbers of viable cells whereas the highest numbers were found in biofilms growing under dynamic flow conditions with added Mn(II) (BHIMnG and BHIMnGHK). The data are in line with the previously described role of high intracellular concentrations of Mn(II) (up to 0.24 mM) in *L. plantarum* providing resistance to oxidative stress because of the lack of superoxide dismutase (Archibald and Duong, 1984; Archibald and Fridovich, 1981; Nierop Groot et al., 2005).

Furthermore, increased numbers of total viable cells can be explained by the respiration growth mode. *L. plantarum* lacks of a functional electron transport chain (ETC); however, it has been shown that upon addition of haem and vitamin K2 the electron transport chain can be functionally reconstituted (Brooijmans, Smit, et al., 2009; Brooijmans, de Vos, et al., 2009).

The Principal component analysis (PCA) in Figure 5.2 provides the visualization of the 10 *L. plantarum* strains and the grouped strains FBR2-5 abundance determined under all the conditions analysed. The arrows point in the direction of the steepest increase of the corresponding strain. The direction of each arrow shows the conditions in which a determined strain is favoured. The variable of time is concentrated on the intersection of the principal components indicating that they do not explain the variability of the data. Growth mode (biofilm or planktonic grown under static or dynamic conditions) explains most variation (67%). The strains NC8, CIP104440 and WCFS1 and the grouped strains FBR2-5 are favoured during static biofilm growth. We tentatively assume that the FBR2-5 position represents performance of FBR5 (see methods section), which is in line with previous biofilm formation studies where FBR5 outperformed FBR2 (chapters 2 and 4). The impact of the biofilm growth under dynamic flow conditions on FBR4, NCTH19-2 and especially for CIP104448 is relatively high ($P < 0.001$). These three strains dominated the biofilm growing under dynamic flow conditions where strain CIP104448 was the most abundant. Moreover, the addition of Mn(II) and vitamin K2 and haem influenced the outcome of the relative abundance of strains FBR4, NCTH19-2 and CIP104448. The biofilm and planktonic growth were not significantly different ($P < 0.001$) under static conditions for almost all strains except for FBR6 and SF2A35B. The impact of planktonic static growth on FBR6 and SF2A35B is relatively high ($P < 0.001$). In fact, strain FBR6 performs better in the planktonic phase and was not dominating in any of the static biofilms. The planktonic growth with shaking conditions was found to be significantly different to growth in dynamic flow biofilms ($P < 0.001$).

This study showed that there is no significant difference in composition of the microbial population between static planktonic and static biofilm growth ($P > 0.001$). The relative abundance of each strain in biofilms developing under dynamic flow conditions was significantly different to that of static biofilms ($P < 0.001$). Data obtained without and with PMA showed that the relative abundance for strains FBR1, SF2A35B and ATCC14917 is higher in the absence of PMA ($P < 0.001$), suggesting that these three strains contribute to the biofilm by providing eDNA. DNA can be released into the biofilm matrix by cell lysis as a result of increased activity of autolysins (Jakubovics et al., 2013; Okshevsky and Meyer, 2015). Cell lysis is the most common mechanism of DNA release for different species including enterococci (Guiton et al., 2009) and staphylococci (Qin et al., 2007; Rice et al., 2007). The absence of Mn(II) favoured the abundance of strains ATCC14917 and FBR1 in static biofilms ($P < 0.001$). It is possible that these two strains are more robust in low Mn(II) concentrations. The increase of temperature favours the abundance of strains WCFS1 and FBR6 ($P < 0.001$), whereas for strains NCTH19-2 and FBR4 a temperature increase results in a decrease of relative abundance. For the rest of the strains the temperature does not affect performance significantly.

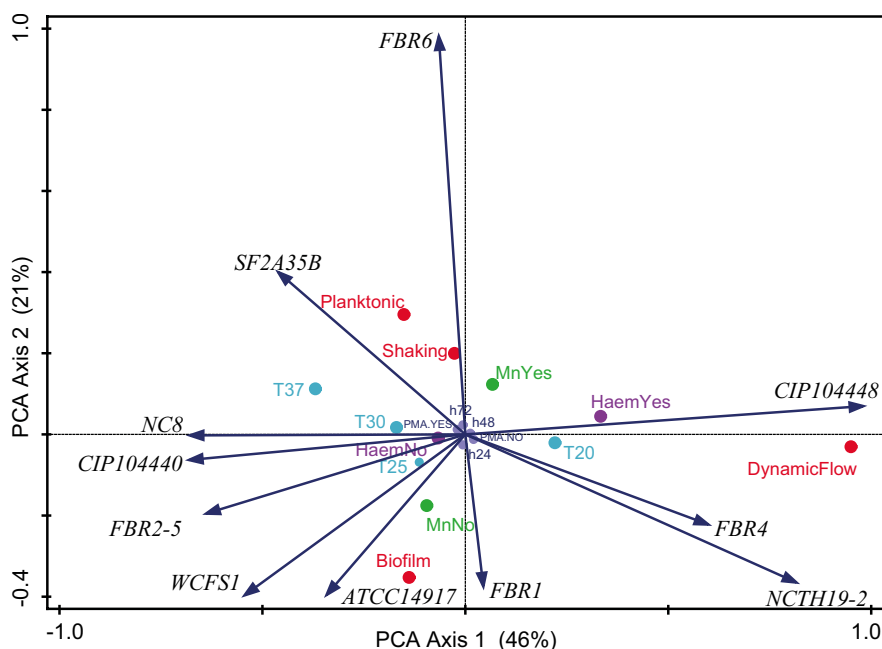


Figure 5.2. Principal component analysis of different growth modes of a mixture of 10 *L. plantarum* strains and grouped FBR2-5. Samples are distributed based on relative abundance of the *L. plantarum* strains. The arrows point in the direction of the steepest increase of the corresponding strain. Coloured symbols are centroids of sample classes. Growth mode (red): planktonic static (Planktonic), static biofilm (Biofilm), planktonic with shaking conditions (Shaking), biofilm under dynamic flow conditions (Dynamic flow). Temperature (blue): 20°C (T20), 25°C (T25), 30°C (T30) and 37°C (T37). Presence of Mn(II) (green): absent (MnNo) and present (MnYes). Haem and vitamin K2 (purple): absent (HaemNo) and present (HaemYes). Figure was generated with Canoco 5.0 (Braak and Smilauer, 2012). For details concerning grouped FBR2-5, see methods.

Extracellular DNA release is affected by temperature and Mn(II)

The addition of propidium monoazide (PMA) to the samples allows for the quantification of viable cells, without interference of DNA from lysed or dead cells. The latter category includes cells with a compromised membrane and extracellular DNA (eDNA) released from lysed cells. eDNA has been found in soil (Levy-Booth et al., 2007; Pietramellara et al., 2009), deep sea sediments (Dell'Anno and Corinaldesi, 2004; Dell'Anno and Danovaro, 2005) and it has also been described as a component of the biofilm matrix of several microorganisms including *Listeria monocytogenes* (Harmsen et al., 2010), *Streptococcus pneumoniae* (Hall-Stoodley et al., 2008), *Staphylococcus aureus* (Mann et al., 2009) and *L. plantarum* (Fernández Ramírez et al., 2015; Muscariello et al., 2013).

The contribution of eDNA was analysed in the competition models. The contribution per strain was calculated by the determination of total DNA concentration per sample obtained by qPCR (targeting *groEL*) multiplied by the relative abundance values per

strain obtained by NGS without and with added PMA. After this calculation, each value without PMA (-PMA) was divided by the corresponding value in the presence of PMA (+PMA). The \log_2 transformed ratios ($\log_2(-\text{PMA}/+\text{PMA})$ -values) provide the quantitative contribution of each strain to the presence of eDNA in the biofilm (Table 5.2) and under planktonic conditions both static and aerated cultures (ST5.4). In general, under static conditions as the temperature increased, more eDNA was released and the release was more pronounced in the absence of Mn(II). In contrast to biofilms formed under static conditions, eDNA release does not seem to contribute substantially in biofilms formed under dynamic flow conditions. The strains FBR1, SF2A35B and ATCC14917 had the highest contribution to eDNA release. Previously, we have linked the degree of induced autolysis to biofilm forming capacity in which cells having a high degree of autolysis were those with higher total biofilm forming capacity (Fernández Ramírez et al., unpublished results; Fernández Ramírez et al., 2015). Remarkably, strains FBR1 and ATCC14914 display lysis already after 24 h in static biofilm and planktonic growth whereas in general the rest of the strains display lysis upon extended incubation (48 and 72 h). The release of eDNA is not only relevant to form the biofilm matrix and provide protection to the cells within the biofilm but it can also aid the incorporation of planktonic cells to the biofilm. The role of early lysis in biofilm development has been shown for *Staphylococcus epidermidis* and *S. aureus*, both species benefit from eDNA for cell attachment (Qin et al., 2007; Rice et al., 2007). The results in the present study emphasize the relevance of cell lysis for the release of eDNA into the biofilm matrix in static biofilm formation.

Table 5.2. Extracellular DNA contribution in static biofilms. The log₂(-PMA/+PMA) is shown per strain and per condition and is the result of the average of the two biological replicates and signifies the relative contribution of individual strains to the fraction of dead cells and/or extracellular DNA. The scale ranges from blue (lowest values) to red (highest values).

Culture Type	Temperature	Medium	Time Point (h)	FBR4	CIP104448	NCTH19-2	CIP104440	NC8	ATCC14917	FBR1	FBR6	SF2A35B	WCF51	FBR2-5
Static biofilm	20°C	BHIG	24	0.91	1.96	1.32	0.93	0.43	0.34	0.79	0.85	1.87	1.51	0.71
			48	-0.2	1.39	-0.12	0.42	-0.07	0.6	3.34	0.09	0.24	0.59	-0.5
			72	-0.53	0.74	-0.55	-0.44	-1.3	-0.41	1.59	-1.08	0.29	-0.03	-1.07
		BHI MnG	24	0.28	1.59	0.75	0.01	-0.53	-0.9	1.81	0.65	1.17	1.41	-0.02
			48	-0.09	0.4	-0.35	-0.1	-0.17	0.54	0.74	-0.31	0.68	-0.02	-0.42
			72	0.18	2.13	0.49	0.43	0.17	0.93	2.33	0.46	2.08	1.06	0.51
		BHI MnGHK	24	1.02	0.69	0.51	0.61	0.87	1.56	2.34	0.69	1.06	0.68	0.63
			48	0.07	0.15	-0.05	-0.13	-0.18	0.87	2.46	-0.17	0.92	-0.17	-0.29
			72	0.54	1.7	0.76	0.74	0.53	1.76	3.36	0.86	1.68	0.89	0.9
	25°C	BHIG	24	1.03	1.73	1.09	1.45	1.11	1.61	2.45	1.21	0.71	1.09	0.75
			48	1.04	1.97	0.6	0.78	0.94	2.68	3.09	1.27	1.88	1.53	0.9
			72	-0.53	1.5	-0.36	0.61	-0.6	2.05	2.51	-1.04	1.1	-0.51	-0.32
		BHI MnG	24	0.01	0.3	-0.18	-0.05	-0.14	1.56	2.03	-0.09	0.17	-0.28	-0.18
			48	0.73	2.23	1.34	0.86	0.57	1.14	2.37	0.94	1.71	1.07	0.49
			72	1.62	3.03	1.92	1.71	1.24	1.89	2.96	1.7	2.42	1.7	1.4
		BHIG	24	0.44	1.73	0.83	0.49	0.5	1.7	1.24	0.5	1.1	0.58	0.21
			48	1.9	3.43	2.64	2.39	2.15	5.33	4.63	2.14	3.07	3.23	2.56
			72	0.97	2.53	1.69	1.83	1.07	4.2	4.58	0.62	4.28	1.86	1.3
	30°C	BHI MnG	24	-0.09	0.74	-0.34	0.32	0.3	2.48	2.27	-0.38	-0.74	-0.44	-0.14
			48	0.87	3.21	1.44	0.93	0.78	1.85	3.34	0.89	0.98	1.14	0.53
			72	0.80	2.27	1.7	1.11	0.83	1.62	2.19	1.11	1.35	1.36	1.1
	37°C	BHIG	24	0.92	-0.64	1.5	0.76	0.87	3.88	5.89	0.26	2.35	0.99	0.79
			48	5.62	3.37	6.36	4.71	4.26	5.96	7.22	4.41	6.75	2.71	2.37
			72	5.42	3.77	6.35	5.49	6.02	6.56	7.57	6.35	7.05	0.58	1.24
		BHI MnG	24	0.95	2.03	-0.03	0.53	0.69	3.41	4.99	0.31	-0.57	0.09	0.54
			48	2.11	3.77	4.17	2.16	1.49	2.61	5.54	3.14	2.85	2.17	2.22
			72	3.82	4.98	6.69	3.63	3.67	4.93	5.8	4.72	6.75	4.66	4.38
Biofilm in dynamic flowing conditions	20°C	BHIG	24	0.12	-0.17	0.67	1.32	0.55	0.81	1.22	0.18	1.87	0.14	0.18
			48	0.71	0.56	0.74	0.6	0.41	0.77	1.05	0.34	0.09	0.59	0.46
		BHI MnG	24	-2.23	-2.84	-1.52	-3.55	-1.08	-2.01	-1.33	-2.19	-1.96	-1.29	-2.22
			48	0.43	0.02	0.13	-0.38	0.11	-0.18	0.43	-0.01	0.43	0.23	-0.1
			72	-0.29	-0.3	-0.37	-0.28	-0.35	0.14	0.14	-0.4	-0.26	-0.41	-0.22
		BHI MnGHK	24	0.94	1.12	1.17	0.36	0.76	0.17	0.09	1.3	0.68	0.62	0.69
			48	0.92	0.69	0.91	0.54	0.56	0.43	1.41	0.92	0.22	0.7	0.45
			72	0.13	-0.04	-0.19	-0.09	-0.15	-0.17	-1.42	-0.23	-0.19	-0.22	-0.09

Multi-strain biofilm formation is affected by the growth mode

PMA treated samples were analysed to determine the contribution of viable cells of the strains to the two types of biofilms. Strains FBR4, NCTH19-2 and CIP104448 dominated the biofilm in dynamic flow conditions (Figure 5.3I) whereas strains NC8, CIP10440, WCFS1 and FBR5 were dominant in static biofilms (Figure 5.3II). In biofilms formed under dynamic flow conditions, strain CIP104448 showed a greater presence than in any of the static biofilms and even dominated when Mn(II) was supplemented to the medium (Figures 5.3B and 5.3C). This strain did not show abundance higher than 5 % in any of the static biofilm or planktonic conditions regardless of the media or temperature (S5.6).

From our results, it is clear that environmental conditions have an impact on the composition of the multi-strain *L. plantarum* community. The formation of *L. plantarum* biofilms under static conditions depends on sedimentation to the surface and the presence of extracellular polymeric solutes including eDNA. Notably, in these multi-strain models eDNA may be donated by strains lysing at an early stage, supporting the incorporation of planktonic cells including that of other strains, into the biofilm. Moreover, some strains might take advantage by co-aggregating with other strains that are already attached to the surface and adhere to the cells or to the biofilm matrix. Co-aggregation has been described for oral biofilms, for example, *Treponema denticola* co-aggregates with the initial surface colonizer *Porphyromonas gingivalis* (Yamada et al., 2005). As another example, *Streptococcus gordonii* produces extracellular polysaccharides which promote co-aggregation with *Actinomyces naeslundii* which expresses an adhesin to bind to the polysaccharides (Cisar et al., 1997). Moreover, the aggregative phenotype of *L. plantarum* CMPG5300 promoted adhesion to vaginal epithelial cells and biofilm formation on polystyrene (37°C, MRS) (Malik et al., 2013). Interestingly, the aggregating phenotype was associated to sortase A (SrtA) dependent proteins since the *srtA* mutant derivative had impaired attachment to epithelial cells and impaired biofilm formation in polystyrene (Malik et al., 2013). The relevance of aggregation for initial attachment was also shown for *L. lactis* (Oxaran et al., 2012). For this strain the overexpression of the *pil* operon was required to cause aggregation and favoured adhesion and biofilm formation to polystyrene (30°C, M17) (Oxaran et al., 2012).

In static biofilm formation and planktonic growth, strain NC8 dominates in almost all conditions (S5.6). The strains dominating the static growth (NC8, CIP104440, WCFS1 and FBR5) showed highest viable cell numbers in single strain biofilms, indicating that single strain performance can predict behaviour in a competitive static biofilm model.

Biofilm formation under dynamic flow conditions can be different from biofilm development under static conditions. Notably, the time is limited for initial attachment as the flow in the present study was stopped for one hour in the beginning and non-adhered

cells are washed away after activation of the flow. Additionally, in this experimental design, more oxygen is supplied in the continuously supplied fresh medium conceivably increasing oxidative stress, although it cannot be excluded that cells in the lower layers of the biofilm experience oxygen and nutrient limitation. Our studies reveal that in contrast to static biofilm formation, early cell lysis may not play a major role in dynamic flow conditions. The $\log_2(-PMA/+PMA)$ -values (Table 5.2) are very low in comparison to the ratios obtained in static conditions which may point to a role for other matrix components in surface adhesion and biofilm formation.

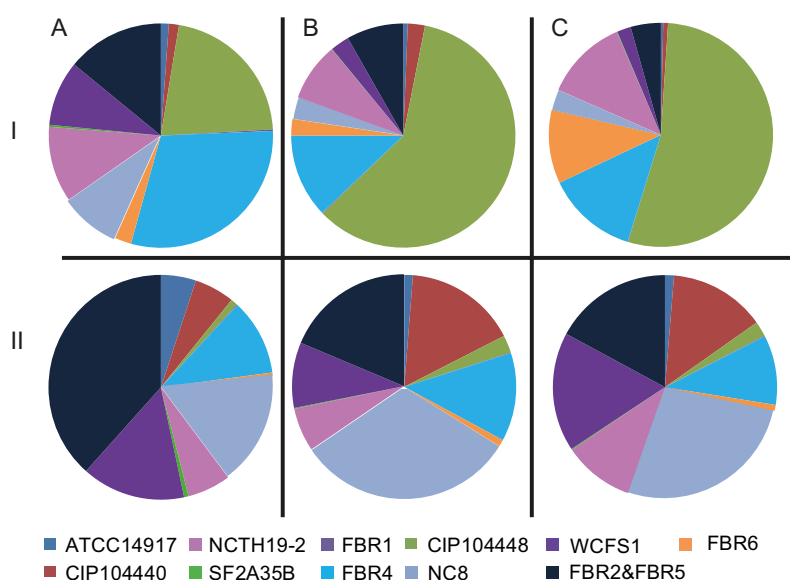


Figure 5.3. Strain specific relative abundance of viable cells in biofilms detected by next generation sequencing. The samples represent the average of two biological replicates of biofilms grown under (I) dynamic flow and (II) static conditions in (A) BHIG, (B) BHIMnG and (C) BHIMnGHK at 20°C for 48h.

Link between genome content and robustness in a community

The gene content of the strains dominating the strain community in biofilms formed in static or dynamic flow conditions was analysed to find candidate genes which could explain their success in the competition model. For strains NC8, CIP104440, WCFS1 and FBR5, dominating in static biofilms, no genes were exclusively shared or lacking in the remaining eight strains. Dominant strains in the competitive static biofilms showed highest viable cell numbers in single strain biofilms, indicating that single strain performance can predict behaviour in a competitive static biofilm model. In static conditions high cell numbers in the culture are key to prominent contributions in static biofilms in multi-strain competition models. This may offer an explanation for the fact that comparative genome

analysis of the dominant strains in static biofilms compared to the remaining strains did not reveal unique shared features.

Notably, FBR4, NCTH19-2 and CIP104448 which dominated biofilms developed in dynamic flow conditions, showed highest CV staining (total biofilm) in single strain performance pointing to a role for biofilm matrix components in the competitive dynamic biofilm model. In addition, the biofilm formed by strains FBR4, NCTH19-2 and CIP104448 under static conditions as individual strains could not be dispersed by DNase I treatment (S5.1), and biofilms formed by strain CIP104448 as a single strain could not be dispersed by Proteinase K treatment (S5.1).

To explain the dominance of strains FBR4, NCTH19-2 and CIP104448 in biofilms formed under dynamic flow conditions we compared their genome content to that of the other nine strains. Notably, in these three strains, only a single gene encoding a site-specific recombinase, DNA invertase Pin-related protein (strain NCTH19-2 has two paralogues) clustered separately (based on DNA and protein sequences) from the invertases present in the other strains (data not shown). Site-specific recombinases, DNA invertases, belong to the serine recombinase superfamily. Serine recombinases promote DNA rearrangements in which two sites are broken and joined again after being exchanged (Hallet and Sherratt, 1997; Stark, 2014). DNA invertases have been described to affect the expression of capsular polysaccharides and outer membrane proteins in *Bacteroides fragilis* (Coyne et al., 2003; Kuwahara et al., 2004; Weinacht et al., 2004). Furthermore, Weinacht et al. (2004) showed that the deletion of one of the DNA invertases (*aapl*) resulted in both higher auto aggregation and biofilm formation, indicating this DNA invertase has a role in modulation of cell surface properties in *B. fragilis*. Furthermore, in *L. casei*, the recombinase LSEI_1403 is required for gut establishment since it can modulate the cell surface and aid the attachment to epithelial cells (Licandro-Seraut et al., 2014). We analysed the gene context of the DNA invertases in strains FBR4, NCTH19-2 and CIP104448 (ST5.5), but noted that the gene context was not conserved in the three strains. Future studies are required to elucidate the role of the identified invertase in the performance of *L. plantarum* strains in dynamic flow biofilm formation.

Concluding remarks

This study shows that strain-specific tracking of *Lactobacillus plantarum* using NGS provides novel insights in the performance of individual *L. plantarum* isolates in competitive multi-strain static and dynamic flow biofilms. This work unveils large strain variability with respect to performance in different types of biofilm promoting niches and identified different sets of strains dominating in either static or dynamic flow biofilms. Furthermore, a subset of strains was identified that conceivably contributed to release of eDNA in the biofilm. Analysis of genome content of the strains dominating in dynamic flow biofilms, identified the presence of a unique homologue of a DNA invertase. Further research is needed to determine the possible role of this DNA invertase in biofilm formation in dynamic flow conditions.

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Supplemental data

Materials and methods

Single biofilm characterisation

Biofilm formation and quantification. Biofilm formation and quantification by the crystal violet assay and cell enumeration were performed as described previously (Fernández Ramírez et al., 2015). Briefly, overnight cultures of each strain were grown in De Man, Rogosa and Sharp (MRS; Merck) and then 100 fold diluted in BHIMnG. Subsequently 1.5 ml were placed in triplicate in 12 well polystyrene plates (Greiner Bio-One). The biofilm was quantified after 48 h incubation at 30°C.

Biofilm dispersion. The established biofilms formed were treated with 1 ml of either 100 µg/ml DNase I (Sigma-Aldrich) or 10 µg/ml of Proteinase K (Qiagen) as described previously (Fernández Ramírez et al., 2015).

Triton X-100 induced autolysis. Autolysis properties of the 12 individual strains were determined as described previously (Rolain et al., 2012) with few modifications. The growth medium was BHIMnG, the OD₆₀₀ drop, representing the lysing properties, was measured at 20 min intervals (SpectraMax, Molecular Devices) at 30 °C.

Preparation of titration curve. Five mixtures were prepared with a ten-fold dilution range of strain WCFS1 in a mixture with the other 11 strains. This mixture was done in technical duplicate and the preparation was done from the overnight cultures as described in the main article. Validation of the PMA treatment was done through five mixtures containing a ten-fold dilution of dead cells of WCFS1. Once the overnight culture was adjusted to OD₆₀₀ 10 it was treated with 70 % propanol (Merck) for 10 min as described previously (Taskin et al., 2011). A sample was taken to be plated directly in MRSA plates to confirm no more culturable cells were present. In addition, a sample was taken for a preparation with LIVE/DEAD® BacLight™ Bacterial Viability kit (Invitrogen) was made according to the manufacturer's instructions. Several fields were observed with the fluorescent filters UMN BV (SYTO9) and U-MWIG (PI) were observed in a BX41 microscope (Olympus) to make sure all cells had a compromised membrane. The 11 strain cultures were mixed and ten-fold dilutions of WCFS1 were added; then 1 ml was processed without PMA whereas 1 ml was treated with PMA as described above.

Modifications for DNA extraction. The cells were resuspended in 300 µl of lysis buffer instead of 180 µl to obtain a higher DNA yield; the amount of Proteinase K was increased from 25 to 62.5 µl to degrade proteinaceous material from the biofilm matrix; 500 µl of AL buffer were added instead of 200 µl to enhance lysis; the added absolute ethanol was 500 µl instead of 200 µl. The washing steps were applied two times with AW1 and AW2 buffers

to remove residual biofilm matrix. An additional step was added for the incubation of the samples with 10 mg/μl of RNase A (QIAGEN).

Tables

ST5.1. Primers used for PCR and quantitative PCR used for the current study

Strain	Target	Forward primer (5→3')	Reverse primer (5→3')
All	lp_1173	CCTAATCAGACGTTAGCTGGT	(barcode)TGGGTAGACCACTTCGACATC*
All	<i>groEL</i>	GGTGGT GGTACTGCT TTG	CGATTT GGCGAACTG GTTCT
FBR2	L30_2967	AAACTGTTGAAATGGGCGGC	CGATGCCTGAGATCCCGTTT
FBR5	FBR5_2965	TGAAAGCATCGCAGCCACTA	GCTCCCGTAGCACTAGAAGC

*The barcodes used for each sample are listed in below. *Target: lp_1173 in WCFS1 annotated as UDP-N-acetylglucosamine 2-epimerase

ST5.2. Description of samples and barcodes used. Note that each two barcodes correspond to one biological replicate except of those marked with * which are technical duplicates.

Type of sample	Temperature	Medium	Time point	-PMA	+PMA
STATIC BIOFILM	20°C	BHIG	24h	cctatgg ccattc	ccttca cgaaca
			48h	tactct tactac	cttctt cttcgc
			72h	ctcgcg ctcgac	cgtcgg cgtcac
		BHIMnG	24h	ccgaca ccgac	ccttgc cctcgt
			48h	tacgta tacgcc	cttcaa cttata
			72h	ctccgt ctcatt	cgtagc cgtagc
		BHIMnGHK	24h	ccgcac ccgcct	atgcaa atgatt
			48h	taccga taccat	cttact ctgtga
			72h	ctcacc ctcagg	cggttc cggtgg
	25°C	BHIG	24h	ccgcgg ccgcta	atgacc atgaag
			48h	tacatc tacagt	ctgtcc ctggcg
			72h	ctatta ctatat	cggtca cggtat
		BHIMnG	24h	ccggaa ccggcc	atcttc atctgg
			48h	taagtt taatta	ctggca ctgctc
			72h	ctaggc ctagct	cggttc cggcgc
	30°C	BHIG	24h	ccgtcg ccgtgt	atcggt atcgag
			48h	taatgc taatag	ctgcag ctgacg
			72h	ctagaa ctaccg	cggccg cggcaa
		BHIMnG	24h	cctaag cctacc	atccga atccat
			48h	taagaa taactc	ctgaac ctcttg
			72h	cgttcg cgttaa	cggata cggaqt
	37°C	BHIG	24h	cctaga cctgcg	atcagc atcacg
			48h	taacgt taacca	ctctgc ctctct
			72h	ctaadc ctaaga	cggaacc cggaag
		BHIMnG	24h	cctgta ccttat	atatgc atagca
			48h	ctgtgc cttggt	ctctaa ctcgta
			72h	cgtgca cgtagag	cgctta cgctag

ST5.2. Continued

Type of sample	Temperature	Medium	Time point	-PMA	+PMA
BIOFILM IN DYNAMIC FLOW CONDITIONS	20°C	BHIG	24h	cgacgt cgagcg	cgcgga cgcgtc
			48h	cgcgat cgccac	cgcact cgcatg
			72h	cgagta cgatcc	cgatga cgattg
		BHIMnG	24h	cgacag cgaagg	aggctt aggtag
			48h	agtaca aggtga	agtcag agtcga
			72h	agtgcg agtgtc	agtgaa agtcct
		BHIMnGHK	24h	cattgg ccaacg	cattct ccaagt
			48h	ccacaa ccactg	ccaata ccagcg
			72h	ccatct ccagtt	ccagca ccagac
PLANKTONIC STATIC	20°C	BHIG	24h	aggcta aggcac	agagtg agaggc
			48h	acctgt acctcc	aatcca aatcat
			72h	attaat attagg	cacgaa caccta
		BHIMnG	24h	aggatg aggagc	actgtg actgcc
			48h	accgga accgcg	aagggt aagtat
			72h	attatc attcac	cagtta cataca
		BHIMnGHK	24h	aggaat agctgc	actcgg actcaa
			48h	accata accagg	aacgct aacggc
			72h	attcct attctg	catagt catccg
	25°C	BHIG	24h	agctca agctat	agacgg agacca
			48h	accaat acatca	aatcgc aatatt
			72h	attgga caacac	cactcc cacgtt
		BHIMnG	24h	agcgta agcgcc	actagt actacg
			48h	acatac acagtc	aactaa aagagg
			72h	caacga caactt	catctc catgat
	30°C	BHIG	24h	agccgt agcctg	agaact acttga
			48h	acagct acagag	aataag aagtgc
			72h	caagag caagggt	cagaat cactgt
		BHIMnG	24h	agccaa agcatt	actaac acgttc
			48h	acactt acacga	aacatg aaccac
			72h	caagtc caataa	catgcc catgga

ST5.2. Continued

Type of sample	Temperature	Medium	Time point	-PMA	+PMA		
PLANKTONIC STATIC	37°C	BHIG	24h	agcaga agcaac	acggca acggac		
			48h	acaatg acaacc	aagcgt aagtcg		
			72h	caatcg cacaac	cagatg cagagc		
		BHIMnG	24h	agatta agatcg	acgtaa acgtgg		
			48h	aatgta aatggt	aacctt aaccgg		
			72h	cacacg cacaga	cattac catgtg		
		PLANKTONIC SHAKING	20°C	BHIG	24h	agttcc agttgt	agagat agactc
					48h	acgcgc acgccg	aatgac aataga
					72h	atgcgg atggat	caccgc caccag
BHIMnG	24h			atacag atacgt	acttct acttag		
	48h			acgcat acgaga	aaggag aaggcc		
	72h			atggcg atggtc	cagcca caggac		
BHIMnGHK	24h			atacta atagac	actgat actctc		
	48h			acgact accttg	aagatc aacacc		
	72h			atgtgt atgttg	caggcg cagtag		
Mixture of the 12 strains				MRS		aacacc aacatg*	
11 strains + WCFS1 10x diluted						aaccac* aaccgg*	
11 strains + WCFS1 100x diluted						aacctt* aacgct*	
11 strains + WCFS1 1000x diluted						aacggc* aactaa*	
11 strains + WCFS1 10000x diluted						aagagg* aagatc*	
11 strains + WCFS1 killed with isopropanol					aagcgt	aagtcg	
11 strains + WCFS1 killed with isopropanol 10x diluted		aaggag	aagtgc				
11 strains + WCFS1 killed with isopropanol 100x diluted		aaggcc	aataag				
11 strains + WCFS1 killed with isopropanol 1000x diluted		aaggtt	aataga				
11 strains + WCFS1 killed with isopropanol 10000x diluted		aagtat	aatatt				

ST5.3. Relative *L. plantarum* strain abundance of 8 independent replicates.

	ATCC 14917	CIP 104440	CIP 104448	FBR1	FBR4	FBR6	NC8	NCTH 19-2	SF2A 35B	FBR2-5
¹⁾ Mix.0	12.42	11.66	3.10	9.45	8.95	5.64	15.49	3.25	3.19	21.17
Mix.1	13.37	12.32	3.28	10.05	9.51	6.42	16.14	3.67	3.36	21.20
Mix.2	13.58	12.11	3.03	10.16	9.17	6.22	16.55	3.39	3.56	22.01
Mix.3	13.72	12.29	3.10	10.45	9.45	5.99	16.04	3.27	3.24	22.28
Mix.4	13.46	12.17	3.29	10.23	9.29	6.02	16.20	3.44	3.52	22.19
^{*)} Dead.0	12.82	11.08	3.92	9.59	8.76	5.73	15.76	3.78	3.72	20.49
Dead.1	14.14	11.75	3.88	10.13	9.00	5.69	16.29	3.69	3.54	21.33
Dead.2	13.91	11.76	3.83	10.38	9.07	5.86	16.65	3.89	3.44	20.99
Dead.3	13.97	11.83	4.09	10.15	9.23	5.81	16.37	3.62	3.47	21.27
Dead.4	13.94	11.89	3.96	10.15	9.13	5.82	16.28	3.70	3.44	21.54
^{*)} PMA.0	14.06	12.41	3.09	10.00	9.61	5.91	16.59	3.35	3.15	21.64
PMA.1	14.12	12.81	2.85	10.18	10.40	6.13	16.86	3.11	2.51	20.86
PMA.2	13.25	12.69	4.16	9.72	11.03	6.83	16.08	3.42	2.51	20.16
PMA.3	13.60	12.44	3.44	10.11	10.58	6.58	16.63	3.38	2.77	20.33
PMA.4	13.31	12.64	3.25	10.43	9.97	6.46	15.94	3.21	2.90	21.72
Average	13.58	12.12	3.48	10.08	9.54	6.07	16.26	3.48	3.22	21.28
Standard deviation	0.49	0.46	0.44	0.29	0.66	0.36	0.37	0.23	0.38	0.64
CV	0.04	0.04	0.13	0.03	0.07	0.06	0.02	0.07	0.12	0.03

The variable amount of strain WCCFS1 was a dilution series and was subtracted from the other strains.1) Mix: mixture of the 12 strains after overnight growth; *WCF51 dilution series were prepared with cells killed with isopropanol, prior to DNA extractions PMA was not added to Dead and added to PMA.

ST5.4. Heat map showing the $\log_2(\text{PMA}/+\text{PMA})$ signifying the relative contribution of individual strains to the fraction of dead cells and/or extracellular DNA in planktonic phase. The scale ranges from blue (lowest values) to red (highest values).

Culture Type	Temperature	Medium	Time Point (h)	FBR4	CIP104448	NCTH19-2	CIP104440	NC8	ATCC14917	FBR1	FBR6	SF2A35B	WCFS1	FBR2-5
Planktonic static	20°C	BHIG	24	0.28	1.25	0.62	0.15	0.10	0.19	2.98	0.35	0.42	0.41	-0.16
			48	0.73	1.61	0.85	0.95	1.01	1.80	5.08	1.35	0.65	0.84	0.73
			72	1.59	2.87	2.17	1.54	1.66	2.62	4.53	2.75	1.63	2.02	1.27
		BHI MnG	24	0.55	0.32	0.16	0.01	0.11	0.87	2.59	0.17	0.82	0.32	0.18
			48	-0.31	0.13	-0.04	-0.54	-0.59	0.47	1.36	-0.51	0.96	-0.25	-0.53
			72	0.16	0.54	-0.21	-0.51	-0.63	0.30	1.86	-0.78	1.28	0.15	-0.22
		BHIMn GHK	24	0.33	0.48	0.56	0.13	0.14	0.67	2.92	0.72	0.78	0.45	0.21
			48	-0.73	-1.85	-1.11	-0.87	-0.64	1.63	1.68	-0.50	-1.06	-0.91	-0.76
			72	-1.52	-0.99	-1.63	-1.79	-1.65	0.30	1.06	-1.06	-1.14	-1.41	-1.68
	25°C	BHIG	24	-0.15	0.46	0.23	0.28	0.42	1.65	3.16	0.64	-0.04	0.01	-0.16
			48	1.72	2.88	2.38	2.20	2.05	3.68	3.99	2.95	3.19	2.05	1.82
			72	2.06	3.47	2.83	2.66	1.90	3.69	4.31	3.30	3.69	2.65	1.76
		BHI MnG	24	0.19	0.18	0.09	0.29	0.05	1.62	1.08	0.25	0.17	-0.32	0.21
			48	0.83	0.63	-0.32	0.13	0.34	1.86	1.30	-0.70	-0.04	0.12	0.58
			72	-0.12	0.56	-0.41	-0.83	-0.79	-0.99	-0.43	0.08	0.94	-0.34	-0.13
		BHIG	24	0.41	1.28	0.97	0.85	1.18	3.21	3.20	1.25	0.82	0.25	0.38
			48	1.69	3.11	2.37	2.41	2.14	4.13	4.75	2.68	4.56	1.71	1.49
			72	2.23	4.04	3.46	3.09	2.27	3.98	5.12	2.96	6.31	3.08	2.08
	30°C	BHI MnG	24	0.34	-0.24	0.11	0.06	0.33	1.75	1.31	0.50	0.36	-0.09	0.35
			48	-0.69	-0.81	-1.75	-1.49	-1.02	1.20	-0.03	-2.12	-1.69	-1.79	-0.79
			72	1.42	2.17	0.50	0.99	1.30	3.64	2.58	0.67	0.54	1.42	1.42
		BHIG	24	0.94	0.89	1.50	0.23	0.37	3.28	5.49	2.45	3.07	2.08	0.47
			48	2.93	2.41	2.53	2.08	2.20	5.81	6.48	3.30	6.96	1.05	1.87
			72	4.84	2.73	4.11	3.14	2.73	6.95	7.33	5.28	8.04	1.69	2.63
		BHI MnG	24	1.39	1.14	-0.09	1.01	1.24	3.57	5.15	0.61	-0.06	0.36	1.20
			48	0.52	1.54	1.23	-0.11	-0.08	-0.12	3.09	0.76	1.29	0.67	0.22
			72	2.31	3.37	3.70	1.96	1.60	1.34	4.08	1.36	3.71	3.33	2.19
	37°C	BHIG	24	0.37	1.06	0.56	0.49	0.22	0.36	2.15	0.51	-0.51	0.11	-0.36
			48	1.59	3.33	1.77	2.43	1.53	2.10	4.70	2.36	1.65	1.57	1.40
			72	2.63	4.52	2.88	3.64	2.23	2.81	5.05	3.26	2.11	2.11	1.84
Planktonic shaking	20°C	BHI MnG	24	0.22	0.61	0.37	0.00	-0.12	0.31	2.19	0.65	0.30	0.50	-0.18
			48	0.10	0.60	0.23	-0.33	-0.39	-0.17	1.54	0.49	1.34	0.25	-0.47
			72	-0.44	-0.99	-1.40	-0.84	-0.90	1.14	2.26	-1.00	-0.05	-0.84	-0.92
		BHIMn GHK	24	1.03	0.71	0.43	0.99	1.13	1.47	3.97	0.68	0.35	0.86	0.82
			48	-0.13	-0.01	-0.63	0.07	-0.14	0.18	1.30	0.08	-0.84	-0.02	-0.46
			72	-1.90	-0.58	-2.08	-2.12	-2.39	-1.42	1.02	-0.33	-1.15	-1.03	-2.28

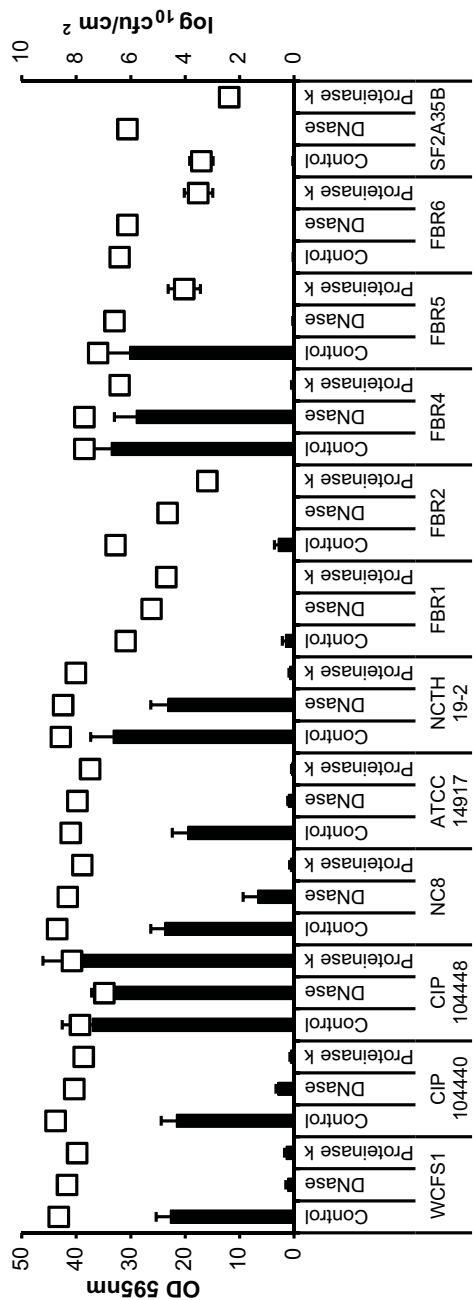
ST5.5. Genes flanking the invertase genes in CIP104448, NCTH19-2 and FBR4. The invertase genes are marked in grey.

CIP104448	Function
CIP104448_2924	Site-specific recombinase, DNA invertase Pinrelated protein
CIP104448_2925	Universal stress protein family
CIP104448_2926	Manganese transport protein MntH
CIP104448_2927	Mn-dependent transcriptional regulator MntR
CIP104448_2928	hypothetical protein
CIP104448_2929	hypothetical protein
CIP104448_2930	Site-specific recombinase, DNA invertase Pinrelated protein
CIP104448_2931	hypothetical protein
CIP104448_2932	hypothetical protein

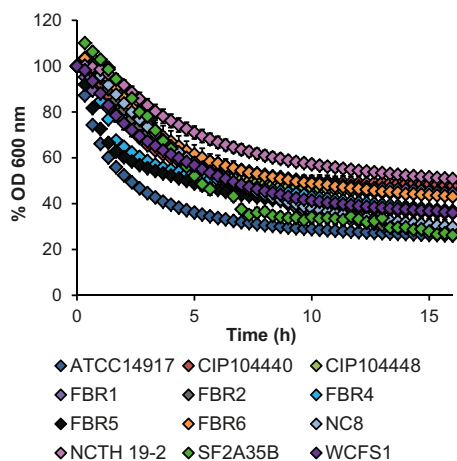
NCTH19-2	Function
NCTH19-2_2551	Mobile element protein
NCTH19-2_2552	hypothetical protein
NCTH19-2_2553	Site-specific recombinase, DNA invertase Pinrelated protein
NCTH19-2_2554	transposase, fragment (putative)
NCTH19-2_2555	Ribonucleotide reductase of class Ib (aerobic),beta subunit (EC 1.17.4.1)
NCTH19-2_2556	Ribonucleotide reductase of class Ib (aerobic),beta subunit (EC 1.17.4.1)
NCTH19-2_2557	Ribonucleotide reductase of class Ib (aerobic),alpha subunit (EC 1.17.4.1)
NCTH19-2_2558	Ribonucleotide reduction protein NrdI

NCTH19-2	Function
NCTH19-2_2600	Ribonucleotide reduction protein NrdI
NCTH19-2_2601	Ribonucleotide reductase of class Ib (aerobic),alpha subunit (EC 1.17.4.1)
NCTH19-2_2602	Ribonucleotide reductase of class Ib (aerobic),beta subunit (EC 1.17.4.1)
NCTH19-2_2603	Ribonucleotide reductase of class Ib (aerobic),beta subunit (EC 1.17.4.1)
NCTH19-2_2604	transposase, fragment (putative)
NCTH19-2_2605	Site-specific recombinase, DNA invertase Pinrelated protein
NCTH19-2_2606	hypothetical protein
NCTH19-2_2607	Mobile element protein

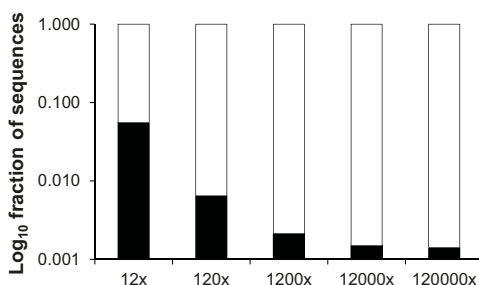
FBR4	Function
FBR4_2644	hypothetical protein
FBR4_2645	hypothetical protein
FBR4_2569	Site-specific recombinase, DNA invertase Pinrelated protein
FBR4_2570	hypothetical protein
FBR4_2577	Na(+)/H(+) antiporter
FBR4_2578	Voltage-gated chloride channel family protein



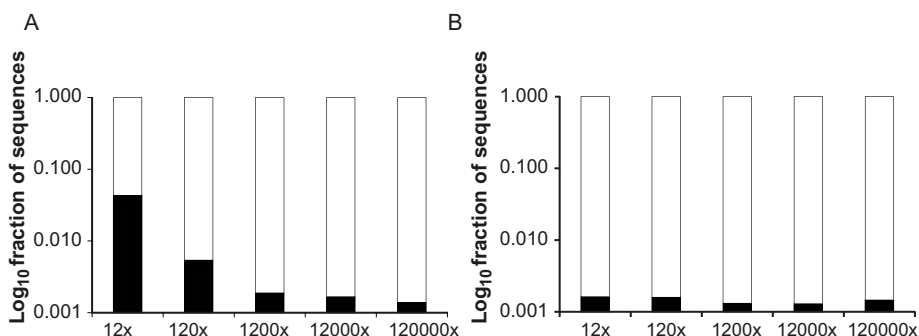
S5.1. Effect of DNase and Proteinase K treatment on mature biofilms formed on polystyrene. Biofilms were quantified by both CV (bars) and enumeration of cell counts (squares). The graph represents the average of three biological replicates of mature biofilms treated for 1h at 30 °C with either PBS (control), 10µg/ml Proteinase K or 100µg/ml DNase I.



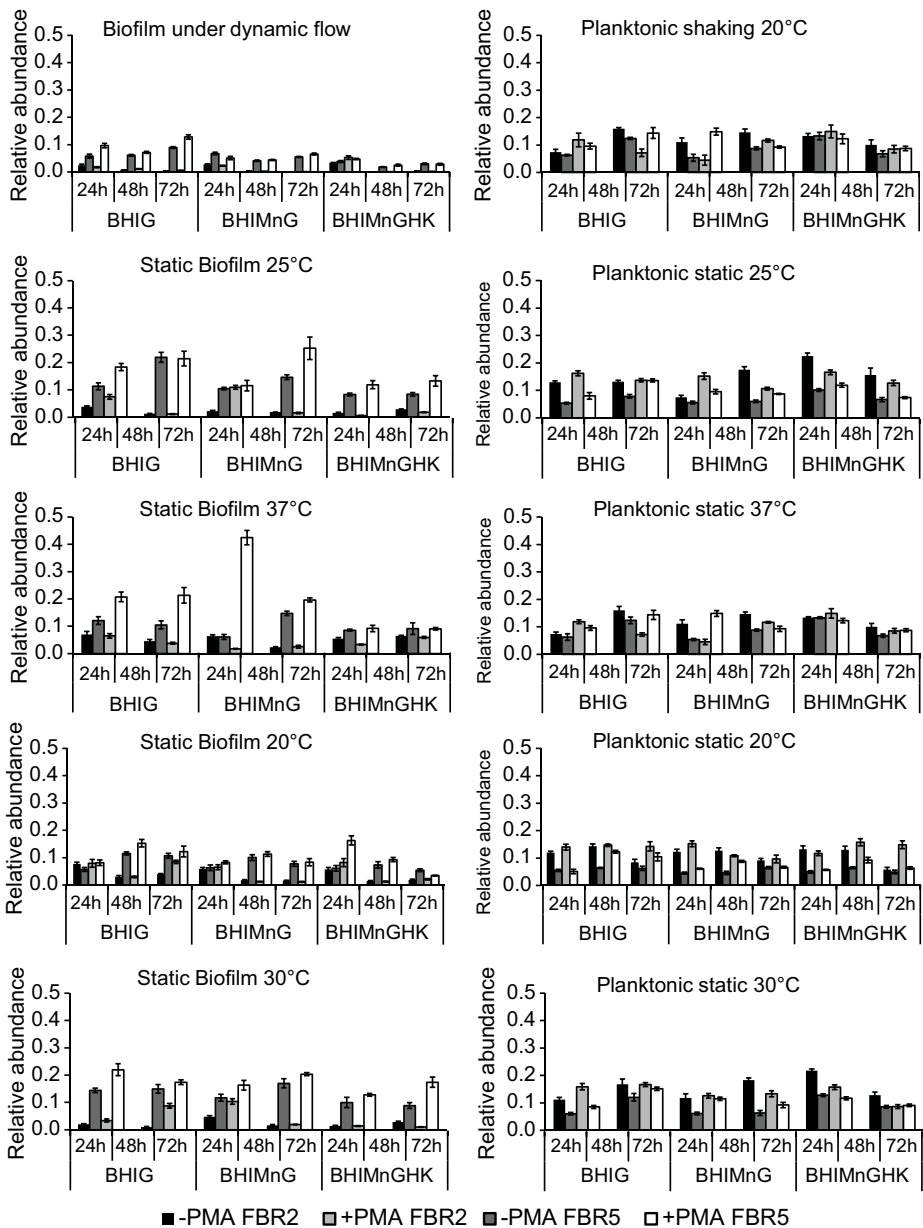
S5.2. Triton X-100 (0.05 %)–induced autolysis of *L. plantarum* strains. The data represent the average and the standard deviation of three biological replicates of residual OD 600 (%) after treating exponentially growing cells of each strain with 0.05% Triton X-100.



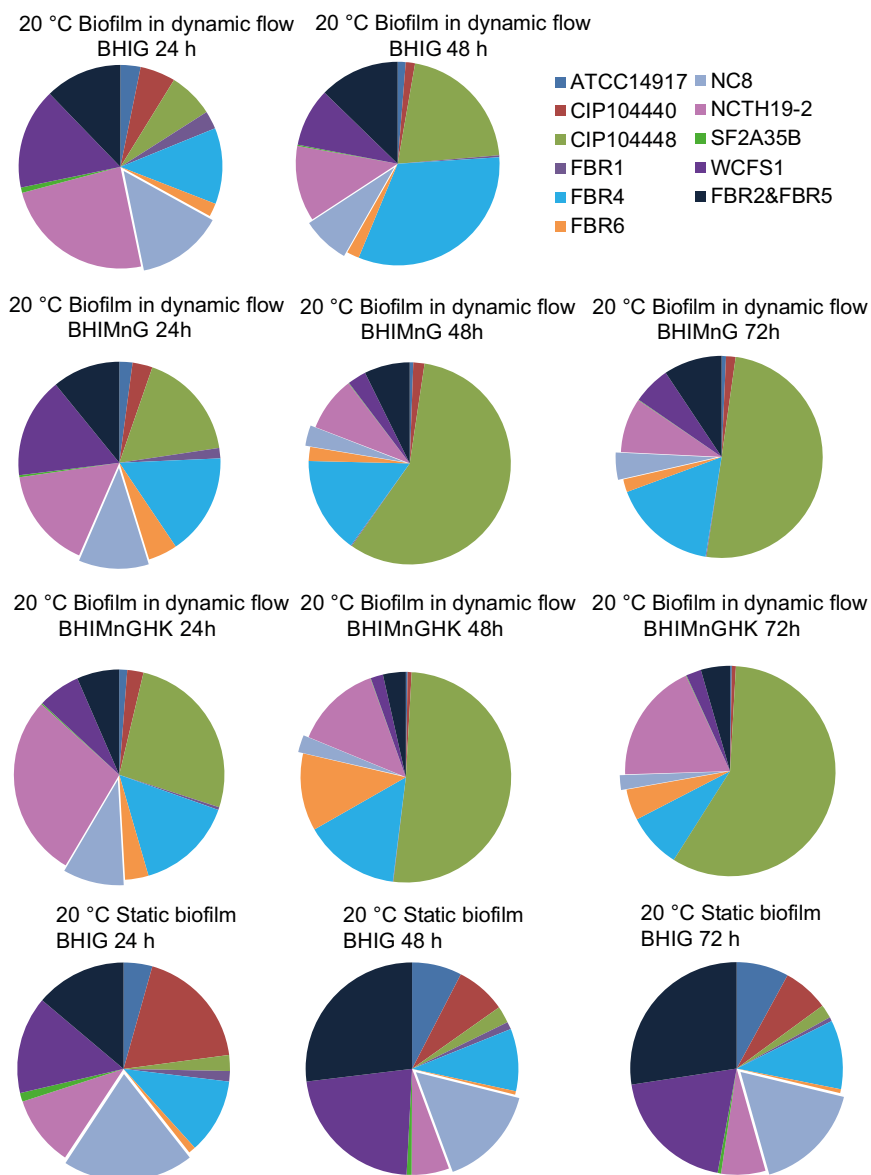
S5.3. Relative abundance of ten-fold dilutions of *L. plantarum* WCFS1 mixed with 11 strains. The relative number of sequences for WCFS1 are depicted (black bars) and the 11 undiluted strains are represented (white bars). The total number of sequences per sample is set as 1. Two independent replicates were made per mixture.



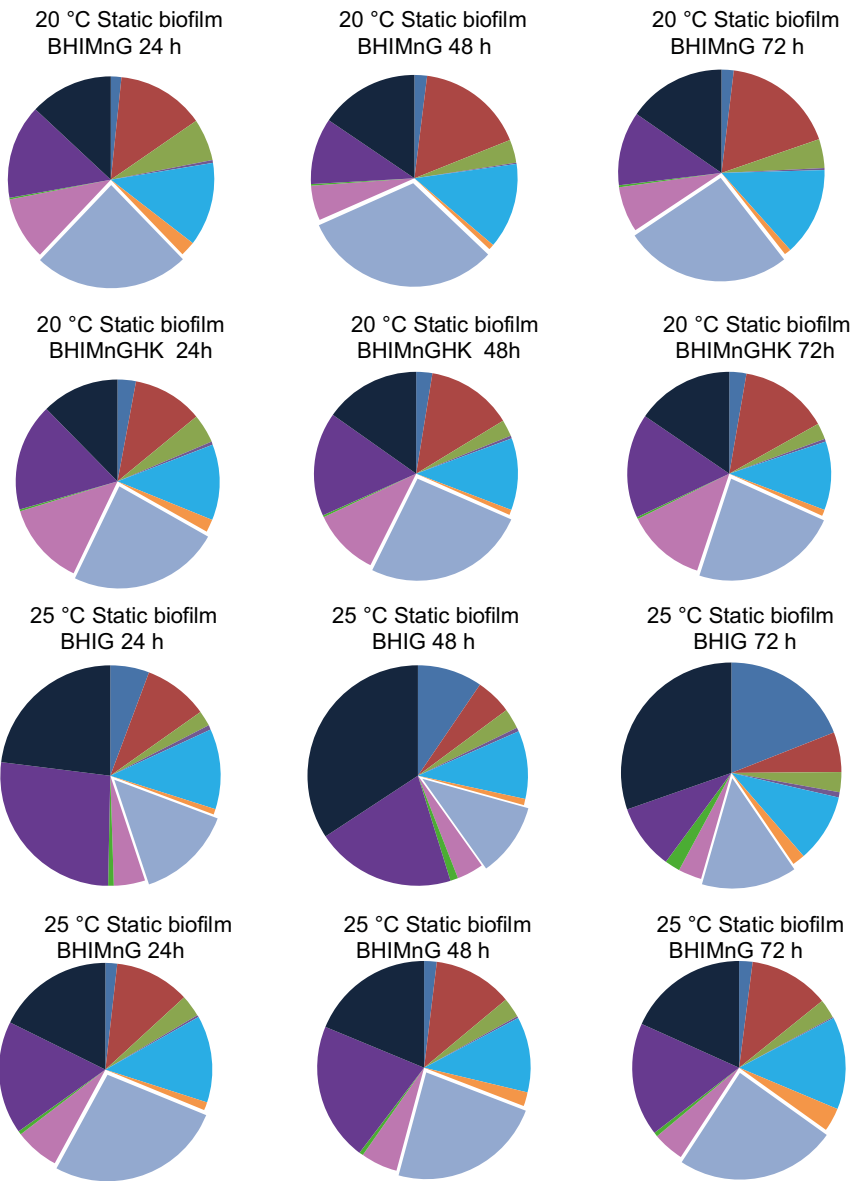
S5.4. Relative abundance of ten-fold dilutions of *L. plantarum* WCFS1 dead cells mixed with 11 strains. The quantification was done without (A) or with propidium monoazide (B). WCFS1 was treated with 70 % propanol to permeate the cells and compromise the cell membrane. The relative number of sequences for WCFS1 are depicted (black bars) and the 11 undiluted strains are represented (white bars). The total number of sequences per sample is set as 1. Two independent replicates were made per mixture. Each mixture was prepared independently.



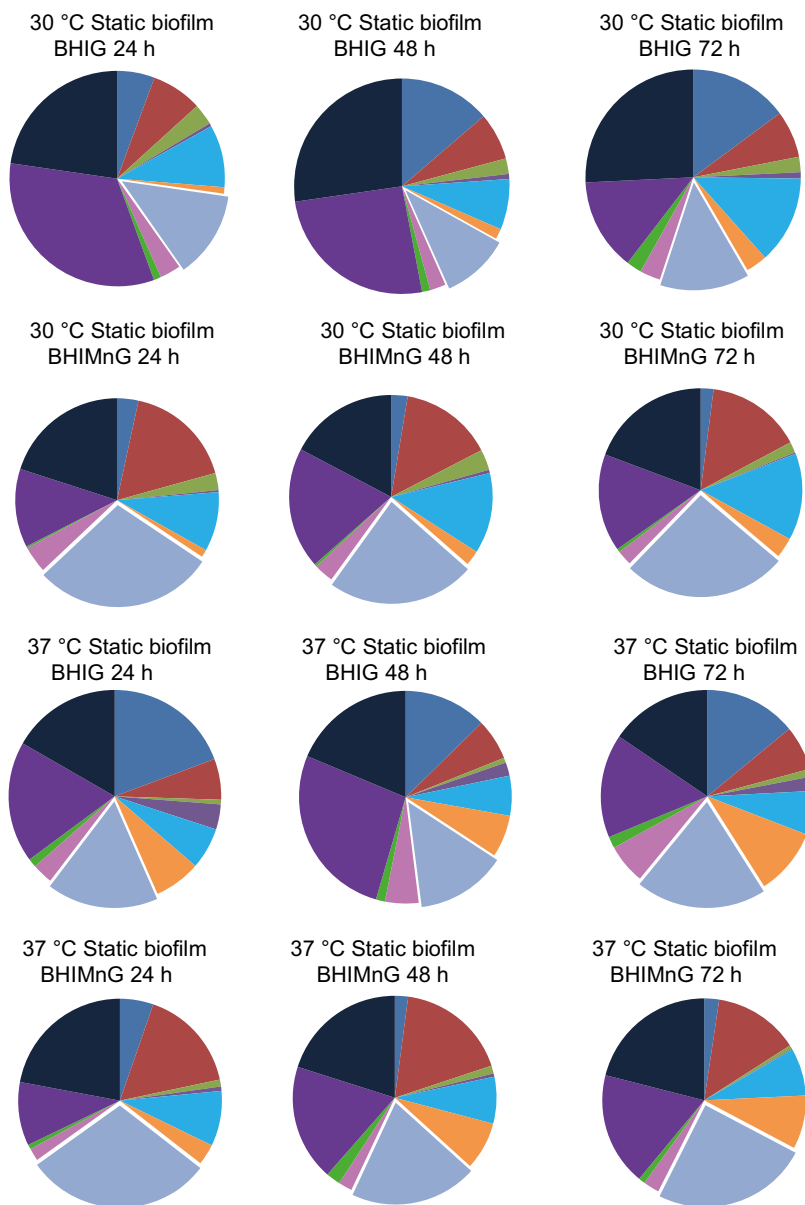
S5.5. qPCR quantification for the discrimination of strains FBR2 and FBR5 in biofilm and planktonic growth. The DNA concentration from all samples was determined with the specific FBR2 and FBR5 primers, the fractions were calculated from the total DNA concentrations obtained by the quantification with primers *groEL*.



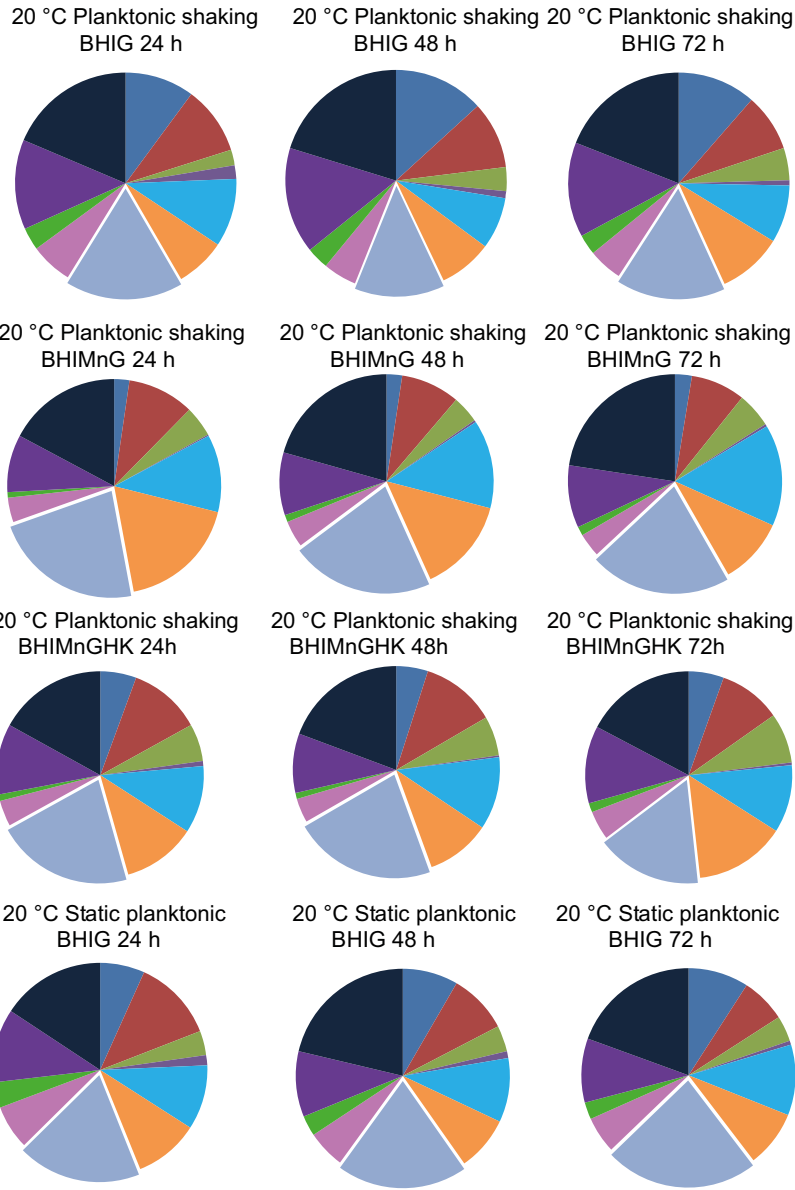
S5.6. Strain specific relative abundance in a biofilm detected by next generation sequencing MiSeq. The samples represent the average of two biological replicates of biofilms grown under dynamic flow and static conditions in BHIG, BHIMnG or BHIMnGHK at 20, 25, 30 or 37°C for 24, 48 or 72h.



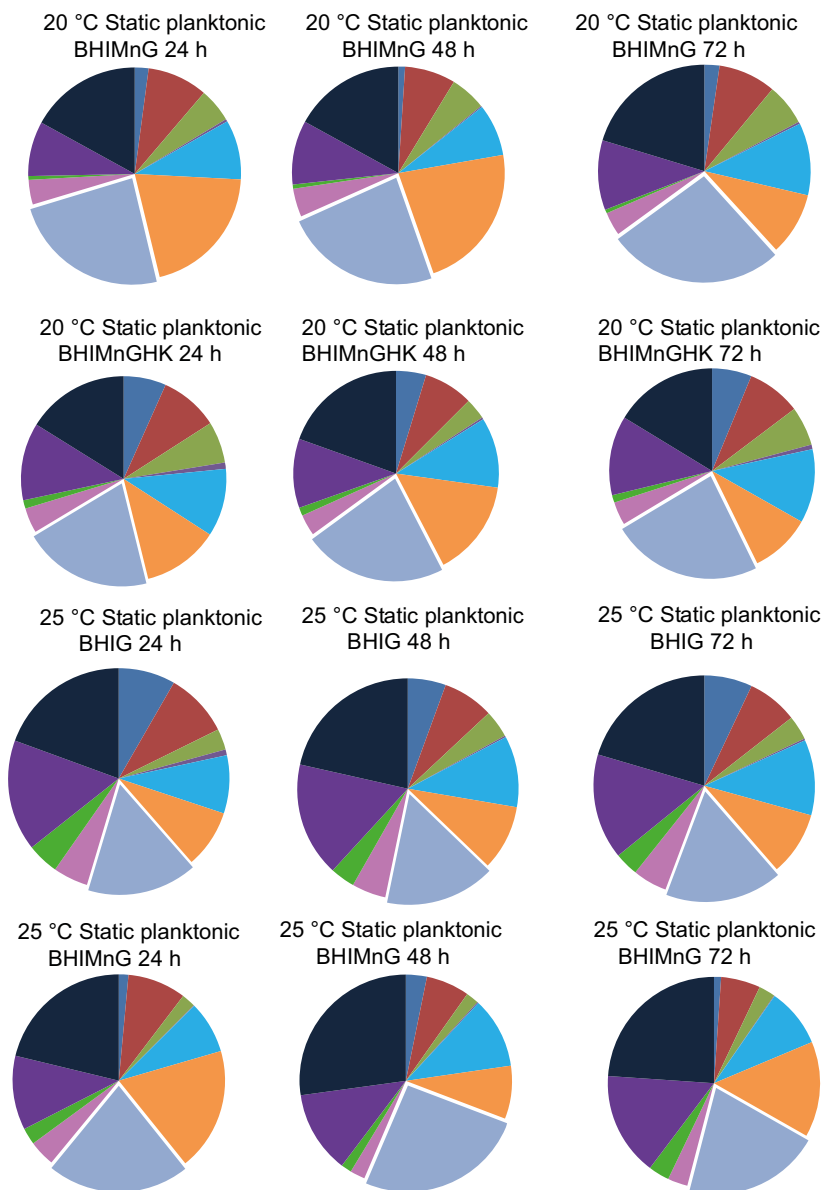
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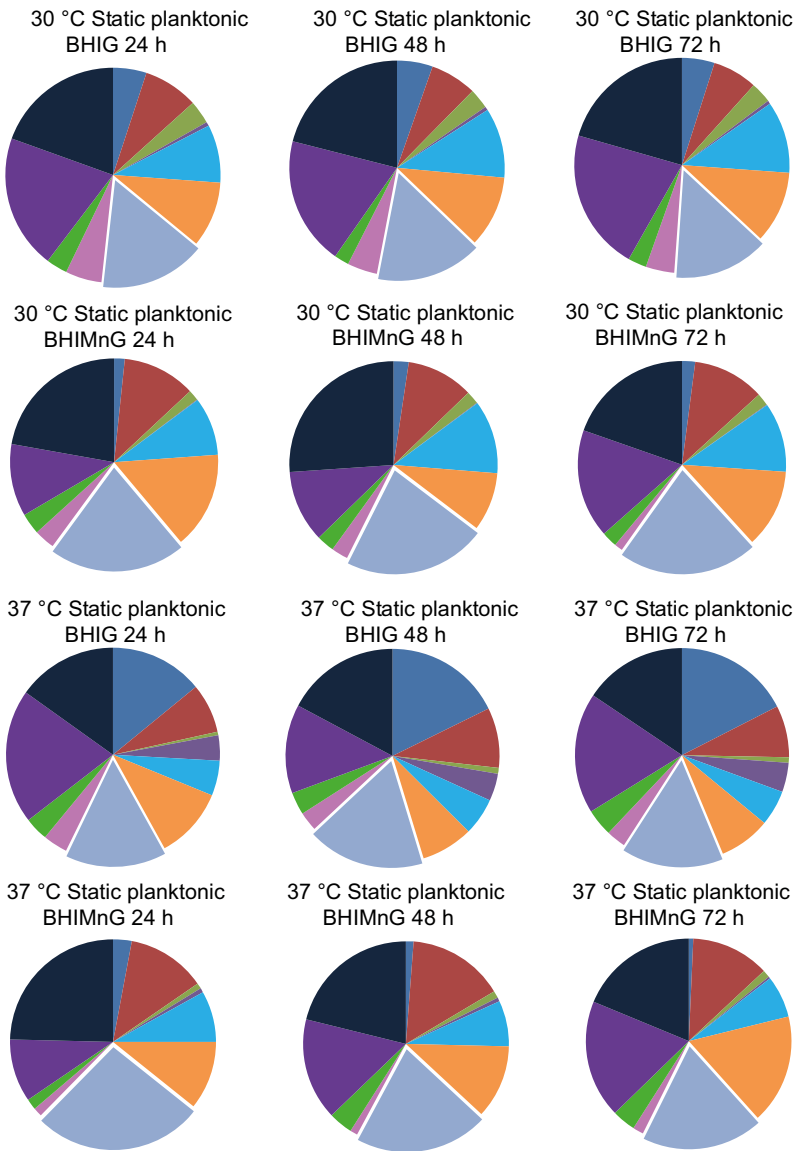
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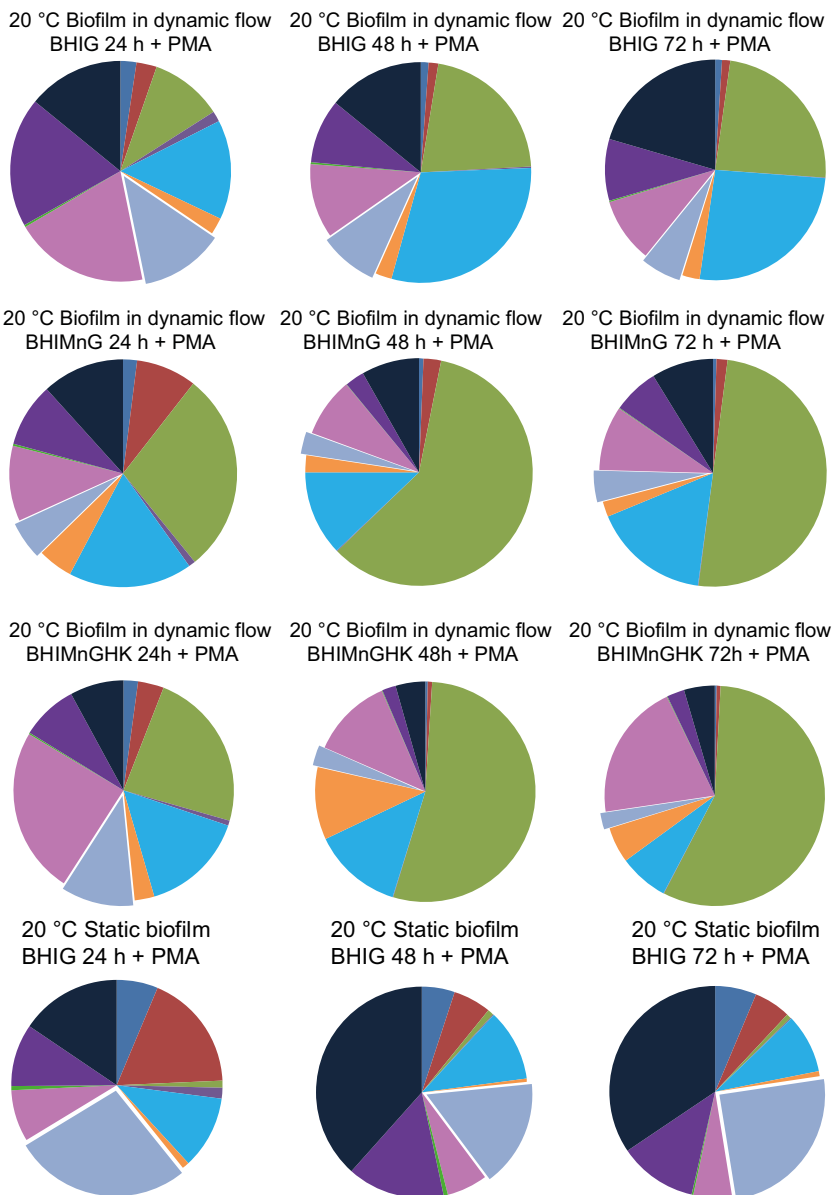
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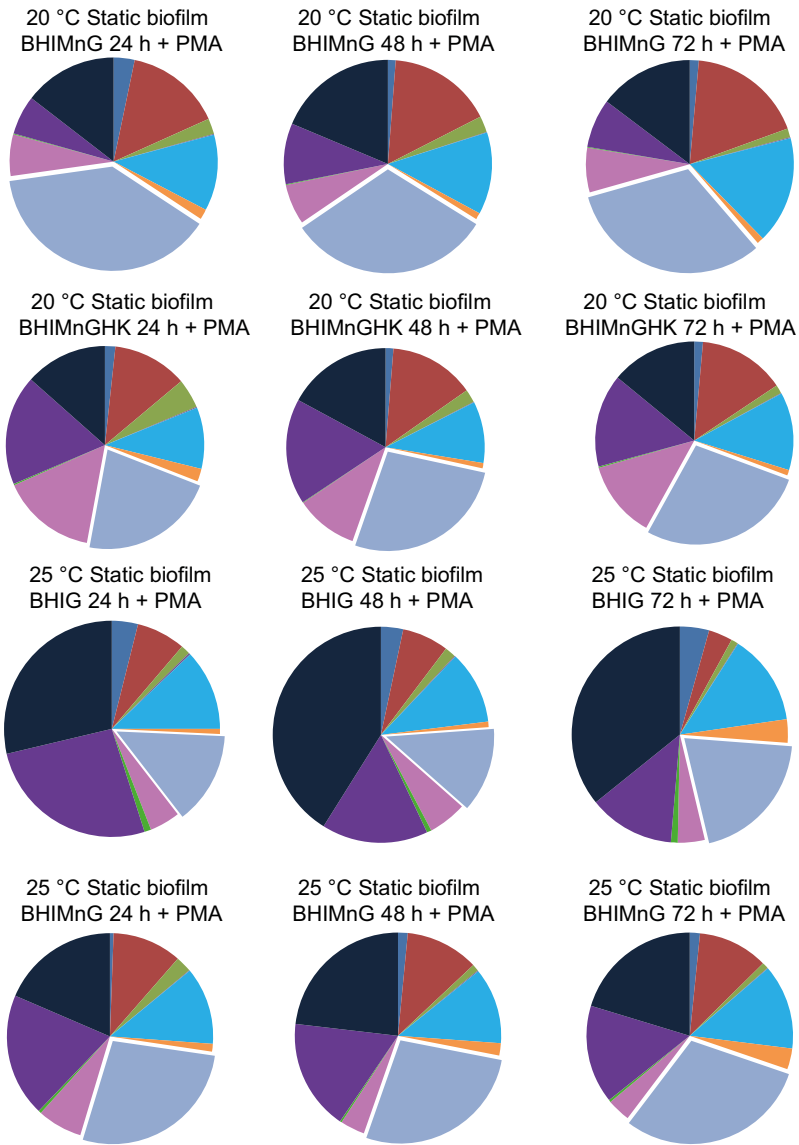
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S5.6. Continuation

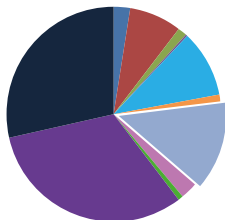


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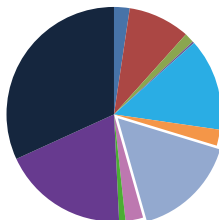


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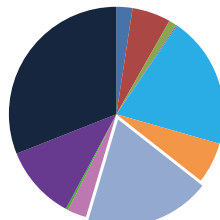
30 °C Static biofilm
BHIG 24 h + PMA



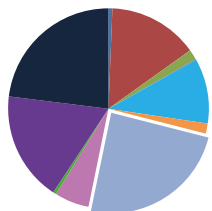
30 °C Static biofilm
BHIG 48 h + PMA



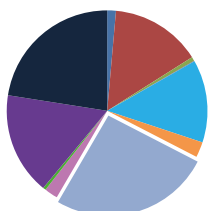
30 °C Static biofilm
BHIG 72 h + PMA



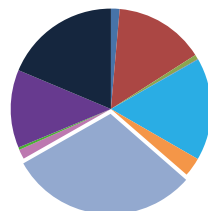
30 °C Static biofilm
BHIMnG 24 h + PMA



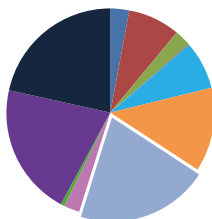
30 °C Static biofilm
BHIMnG 48 h + PMA



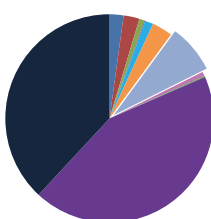
30 °C Static biofilm
BHIMnG 72 h + PMA



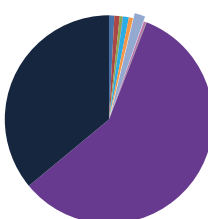
37 °C Static biofilm
BHIG 24 h + PMA



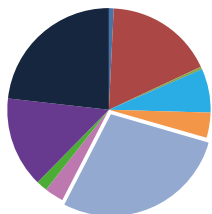
37 °C Static biofilm
BHIG 48 h + PMA



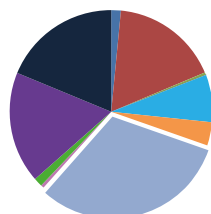
37 °C Static biofilm
BHIG 72 h + PMA



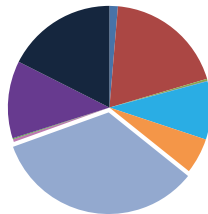
37 °C Static biofilm
BHIMnG 24 h + PMA



37 °C Static biofilm
BHIMnG 48 h + PMA

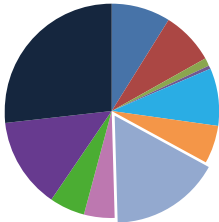


37 °C Static biofilm
BHIMnG 72 h + PMA

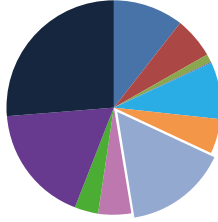


S5.6. Continuation

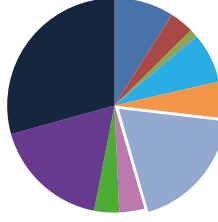
20 °C Planktonic shaking
BHIG 24 h + PMA



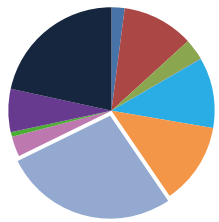
20 °C Planktonic shaking
BHIG 48 h + PMA



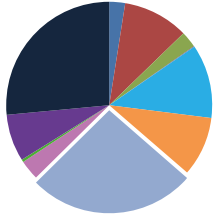
20 °C Planktonic shaking
BHIG 72 h + PMA



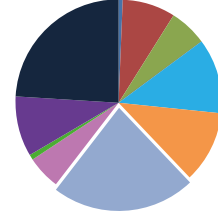
20 °C Planktonic shaking
BHIMnG 24 h + PMA



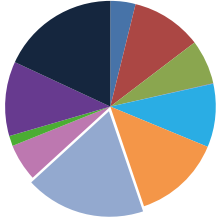
20 °C Planktonic shaking
BHIMnG 48 h + PMA



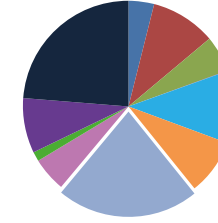
20 °C Planktonic shaking
BHIMnG 72 h + PMA



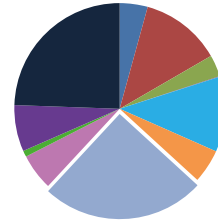
20 °C Planktonic shaking
BHIMnGHK 24 h + PMA



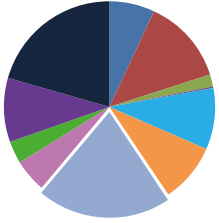
20 °C Planktonic shaking
BHIMnGHK 48 h + PMA



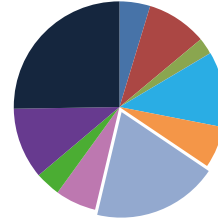
20 °C Planktonic shaking
BHIMnGHK 72 h + PMA



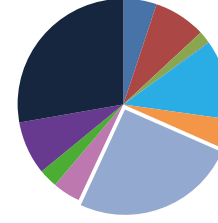
20 °C Static planktonic
BHIG 24 h + PMA



20 °C Static planktonic
BHIG 48 h + PMA

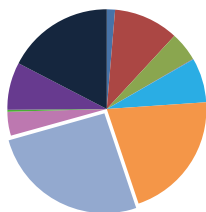


20 °C Static planktonic
BHIG 72 h + PMA

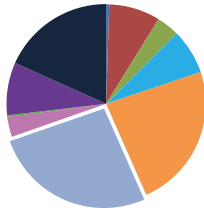


S5.6. Continuation

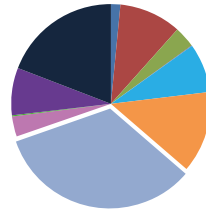
20 °C Static planktonic
BHIMnG 24 h + PMA



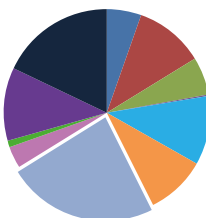
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BHIMnG 48 h + PMA



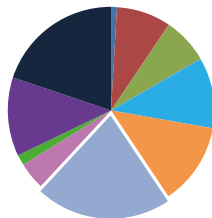
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BHIMnG 72 h + PMA



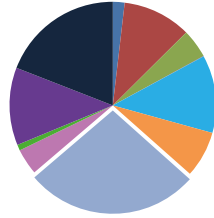
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BHIMnGHK 24 h + PMA



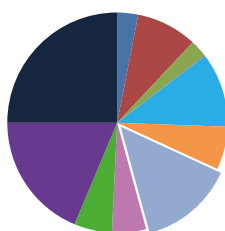
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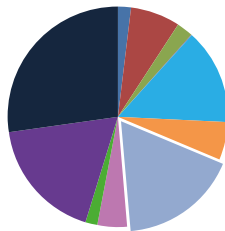
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BHIMnGHK 72 h + PMA



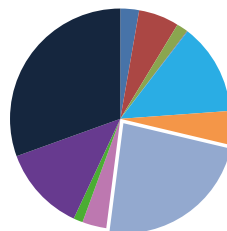
25 °C Static planktonic
BHIG 24 h + PMA



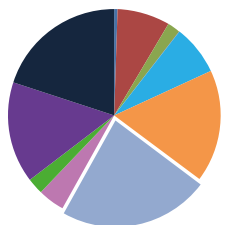
25 °C Static planktonic
BHIG 48 h + PMA



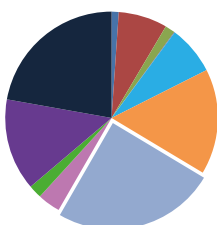
25 °C Static planktonic
BHIG 72 h + PMA



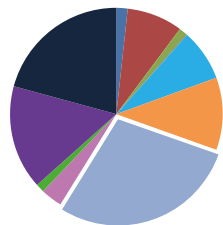
25 °C Static planktonic
BHIMnG 24 h + PMA



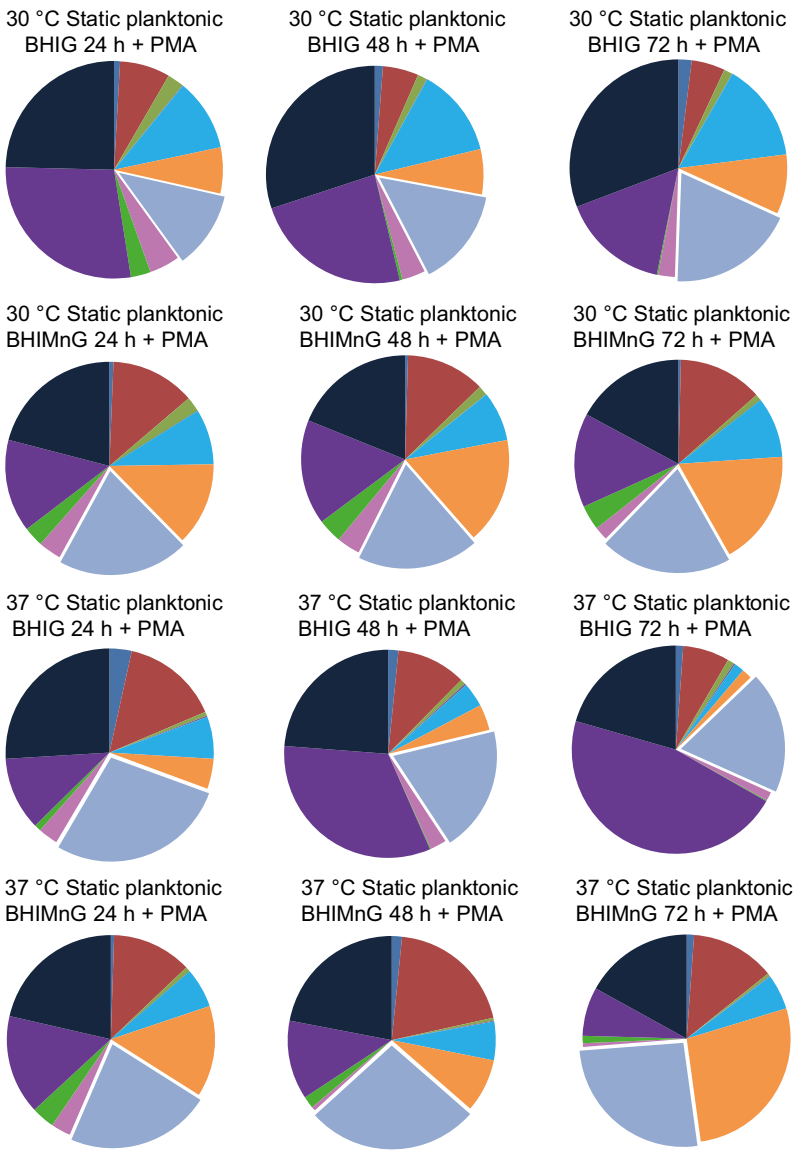
25 °C Static planktonic
BHIMnG 48 h + PMA



25 °C Static planktonic
BHIMnG 72 h + PMA



S5.6. Continuation



S5.6. Continuation

CHAPTER 6

General discussion, conclusions and future perspectives

Lactobacillus plantarum displays large genotypic and phenotypic diversity which allows this species to occupy diverse ecological niches (Siezen and van Hylckama Vlieg, 2011; Siezen et al., 2010) including (acidic) food products. The presence and growth of this species can cause economical losses and undesired product loss. *L. plantarum* can withstand various food preservation stresses such as low pH, weak acids and high salt concentrations (Sanders et al., 2015; van de Guchte et al., 2002). An additional trait of *L. plantarum* that allows survival of harsh conditions is the fact that it can form biofilms. The polymeric matrix of the biofilm protects *L. plantarum* from preservatives and disinfectant used in food processing. Once established, biofilms are difficult to remove from processing lines and can be a source of contamination or recontamination of food products.

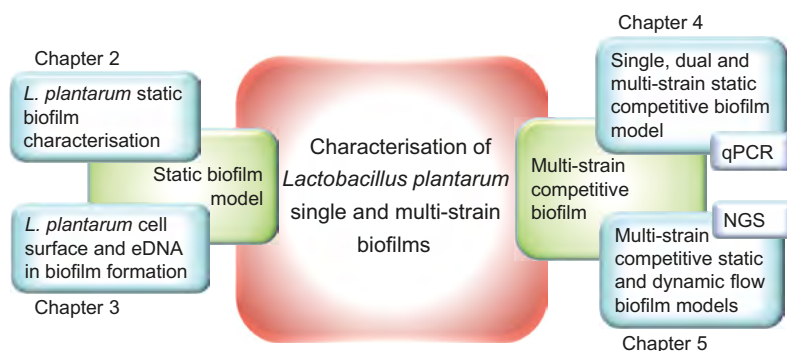


Figure 6.1. Overview of the research topics covered in this thesis. qPCR: quantitative PCR; NGS: next generation sequencing

Figure 6.1 offers an overview of the research topics discussed in this thesis. The aim of this research was to characterize biofilm formation of *L. plantarum* WCFS1 and selected *L. plantarum* strains isolated from a range of environments including faeces, fermented foods and spoiled foods to cover diversity within this species. Additionally, the inclusion of *L. plantarum* WCFS1 derived mutants targeted in cell surface protein composition (*srtA* mutant), capsular polysaccharide (CPS) composition (*cps* mutants) and lysis behaviour (*lys2*, *lytA* and *acm2* mutants) allowed assessment of the role of Sortase A (SrtA) dependent proteins (SDPs), CPS and cell lysis on adhesion and static biofilm formation. Based on the findings in chapters 2 and 3, a model for static *L. plantarum* biofilm formation is presented.

Biofilm formation of *L. plantarum* was further studied in competitive multi-strain static and dynamic flow biofilm models. In chapter 4, performance of six food spoilage strains and WCFS1 was studied in competitive dual and multi-strain static biofilm models using strain-specific quantitative PCR (qPCR). As a follow up, in chapter 5, the population dynamics of a mixture of 12 *L. plantarum* isolates of different origins was studied in competitive planktonic and biofilm growth models as a function of different parameters, namely maturation time, temperature and medium composition (BHIGlucose containing low or high Mn(II), and without and with added haem and vitamin K2 (menaquinone) using a next generation sequencing approach (NGS).

Static biofilm formation

L. plantarum biofilm formation was studied for the model strain WCFS1 and six food spoilage isolates originating from salad dressings (FBR1, FBR2, FBR3 and FBR5), cheese with garlic (FBR4), and onion ketchup (FBR6). All strains displayed a comparable growth rate both in MRS medium and Brain Heart Infusion (BHI) supplemented with 0.005% MnSO_4 and 2% glucose (BHIMnG), but showed diverse biofilm forming capacity. BHIMnG medium resulted in highest biofilm formation of six food spoilage related *L. plantarum* strains and WCFS1 in comparison to other media tested (MRS, mTSB, AOAC) (Chapter 2) and was selected for further biofilm studies. All tested strains were able to attach not only to polystyrene but also to stainless steel, a material that is typically used in food processing. Crystal violet (CV) staining and cell enumeration were used to quantify *L. plantarum* biofilms. Findings in this thesis (Chapter 2) show the importance of using complementary techniques for total biofilm quantification and numbers of culturable cells in the biofilm, especially since the latter parameter is an important indicator of biofilm-associated (re)contamination capacity and spoilage risk. As an example, *L. plantarum* FBR1 showed poor biofilm formation (30°C, 48 h, BHIMnG) based on CV staining (below detection limit) but based on cell enumeration approximately 10^7 cells per cm^2 were attached to the surface. Chapter 2 showed that for all strains the CV stained material increased as the incubation time increased and a same effect was observed with increasing temperature. Nevertheless, the cell numbers in the biofilm decreased considerably and this shows that in addition to CV staining, cell enumeration is necessary as a complementary method to assess (re)contamination potential. Furthermore, high cell numbers (FBR6, 24h: 6 log cfu/ cm^2) were found to be attached to the surface even when the CV measured was below the detection limit.

L. plantarum biofilm matrix composition

Cell death and eDNA release

In Chapter 2 it was shown that the number of culturable cells in the biofilms decreased at elevated incubation temperatures (37°C) and incubation times (72 h) with concomitant increase in CV-stainable biofilm conceivably correlated with the presence of eDNA in *L. plantarum* biofilms. The presence of eDNA in the biofilm matrix was also suggested by experiments that showed dispersal of biofilms after treatment with DNase (Chapter 2). Next, in Chapter 3 it was demonstrated that strains with a higher lysis capacity and conceivably higher release of DNA and consequently higher eDNA levels, can form high CV stainable biofilms. This was further supported by studies with ΔlytA and Δacm2 mutants of WCFS1, which revealed that a reduced lytic capacity and consequently low levels of eDNA, result in a reduction of total biofilm formation measured by CV staining. These data are in line with previous reports that provided evidence that autolysins contribute to cell

lysis and consequently eDNA release into the biofilm matrix of for example enterococci and staphylococci (Bayles, 2007; Frese et al., 2013; Guiton et al., 2009; Qin et al., 2007; Rice et al., 2007; Thomas et al., 2008). Notably, the *L. plantarum* $\Delta acm2$ mutant strain displayed extensive cell chaining, as the major hydrolase Acm2 was shown responsible for cell separation and the introduction of peptidoglycan breaks (Mercier et al., 2002; Rolain et al., 2012). The addition of low levels of lysozyme prevented cell-chain formation by exogenously introducing peptidoglycan breaks (Chapter 3), and in these conditions, the number of cells in the biofilm was restored almost to the WT levels. Nevertheless, the reduced autolytic capacity of mutant strain $\Delta acm2$ resulted in lower eDNA release, thus in lower total quantified biofilm. Additionally, it was shown that single and multiple $\Delta cps1$ mutants affected in the production of CPS, showed enhanced lysis resulting in higher total biofilm formation measured by CV staining. The underlying mechanisms remain to be elucidated including a possible role for autolysins in this phenomenon.

Proteins

Next to eDNA, a role of proteinaceous compounds in the *L. plantarum* biofilm matrix was suggested in this thesis, as Proteinase K treatment prevented biofilm formation and eradicated matured *L. plantarum* biofilms. A role of proteins in *L. plantarum* biofilms was further shown using a sortase A (*srtA*) mutant in Chapter 3, that showed severely reduced adhesion to polystyrene and subsequent biofilm formation. Similar findings were reported previously for a different *L. plantarum*, strain CMPG5300 (Malik et al., 2013), and the sortase was shown to be 20 times more abundant in biofilm cells in comparison to planktonic cells (De Angelis et al., 2015).

For strain *L. plantarum* WCFS1, 27 SDPs are predicted (Boekhorst et al., 2005; Kleerebezem et al., 2010) and these are interesting candidates for analysis of functionality in biofilm formation. A role for three of these SDPs has been tested in this PhD thesis project: *lp_1229* (*msa*) and *lp_0373* (Pretzer et al., 2005) and *lp_2940* (Bron et al., 2007) (the latter two being cell surface protein precursors) using targeted mutants, but biofilm formation measured as CV-stainable material and the number of culturable cells in the biofilm were not affected in these mutants. Whether one or more of the other 24 proteins play a role in biofilm formation remains to be determined. A subset of these SDPs, *lp_0800*, *lp_1124*, *lp_1447*, *lp_2925*, *lp_2940*, and *lp_3074* (all coding for cell surface protein precursors with the exception of *lp_1124* that codes for cell surface hydrolase) were previously shown to be induced *in vivo* in the gastrointestinal tract of mice and humans (Marco et al., 2010) and it may be interesting to test surface adhesion to polystyrene and subsequent biofilm formation with *L. plantarum* WCFS1 mutants targeted in the indicated genes. Since it cannot be excluded that covalent attachment of multiple SDPs to peptidoglycan is required for biofilm formation, next to single gene deletions, also the impact of multiple gene deletions should be assessed. Roles of specific cell wall attached proteins have

been described in *Staphylococcus aureus*, including clumping factors A and B (ClfA/B) and fibronectin-binding proteins A/B (FnbpA/B) acting as ligands in cell-matrix interactions (Hobley et al., 2015).

Capsular polysaccharides

Four different gene clusters encoding CPS biosynthesis are located in the *L. plantarum* WCFS1 genome and the role of these cell surface polysaccharides in probiotic functionality were studied in deletion mutants lacking either individual or multiple *cps* gene clusters (Remus et al., 2012). Deletion of the individual CPS clusters *cps2*, *cps3* or *cps4* did not affect biofilm formation of *L. plantarum* WCFS1 but the *cps1*, *cps1-3* and *cps1-4* mutants showed increased total CV-stainable biofilms compared to the WT. Initial attachment of *L. plantarum* WCFS1 *cps* mutants was not affected which rules out that inability to produce polysaccharides enhances the attachment to polystyrene surface as previously described by Lebeer et al. (2009) for *welE* mutants of *L. rhamnosus* GG. The higher eDNA content in the *cps1* single and combined mutants (*cps1-3*, *cps1-4*) observed with fluorescence microscopy combined with the increased Triton-induced lysis suggest that the higher CV stainable biofilm may result from increased autolysis of the *cps1* mutants.

Production of CPS1 appears to be a unique feature of *L. plantarum* WCFS1 as corresponding genes are not found in genomes of the FBR1-6 food isolates. The gene context of the *cps2* and *cps3* clusters varies considerably in size, composition, sequence similarity and gene order in the genome of the FBR1-6 strains. Only the CPS4 cluster was present in all the FBR1-6 strains and WCFS1. It was previously reported for WCFS1, that *cps2* was upregulated when *lamA*, the regulatory gene of the *agr*-like two component system, was deleted and it was hypothesized that higher CPS2 levels caused shielding of adhesion factors (Sturme et al., 2005). Other polysaccharides either encoded by the other three CPS clusters or by other genes or gene clusters in *L. plantarum* that affect biofilm formation remain to be identified.

Model for static *L. plantarum* biofilm formation

Biofilms have been studied mostly for motile species where flagella play a role in the active movement of cells to the surface. A model for non-motile microorganisms was previously proposed by Lemon et al. (2008) for static biofilm formation. This model is based on a sedimentation step instead of active movement of cells to a surface. Using this model as a basis, a modified version based on the results from Chapters 2 and 3 is proposed for *L. plantarum* biofilm formation (Figure 6.2) offering more detail in the cell-cell interaction and biofilm maturation steps.

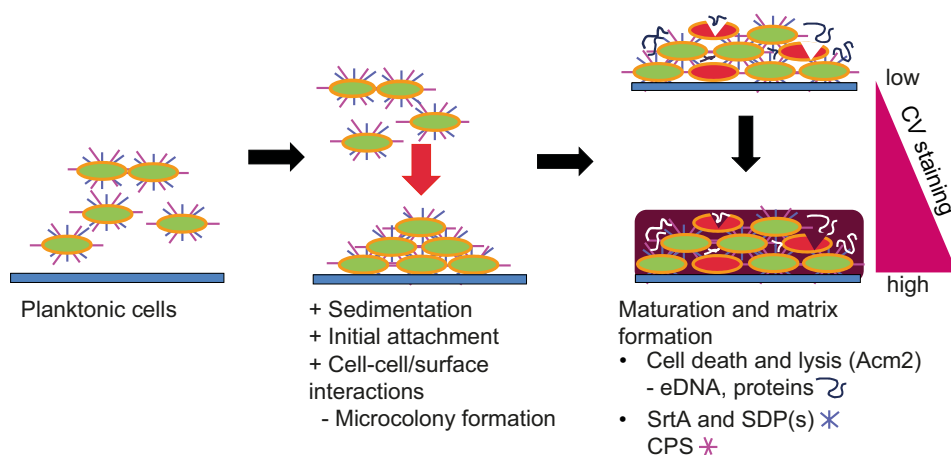


Figure 6.2. Model for *L. plantarum* static biofilm formation. This representation was based on the results in Chapters 2 and 3. SDPs: sortase A dependent proteins; CPS: capsular polysaccharides; eDNA: extracellular DNA; Acm2: major autolysin.

Cell-cell and cell-surface interactions

L. plantarum is non motile and consequently the first contact with a surface depends on passive processes which include sedimentation and electrostatic interactions and SDPs which favour cell-cell and cell-surface interactions rather than active movement driven by the presence of flagella as is the case for motile species (Lemon et al., 2008). In addition to sedimentation, it was previously shown that aggregation of *L. plantarum* CPMG5300 promoted adhesion to vaginal epithelial cells and biofilm formation on polystyrene (37°C, MRS) (Malik et al., 2013). Interestingly, the aggregating phenotype was abolished in the *L. plantarum* CPMG5300 *srtA* mutant derivative which also resulted in impaired attachment and biofilm formation indicating a role of SDPs in aggregation and initial attachment (Malik et al., 2013). The relevance of aggregation for initial attachment was also shown for *Lactococcus lactis* IL1403 (Oxaran et al., 2012). For this strain the overexpression of the *pil* operon was required to cause aggregation and subsequent adhesion and biofilm formation to polystyrene (30°C, M17) (Oxaran et al., 2012). In the present research, only strain CIP104448 (Chapter 5) showed an aggregative phenotype, however this was not the only strain showing high CV staining and a high number of viable cells in the biofilm, and therefore the specific contribution, if any, of aggregation to enhanced adhesion to abiotic surfaces and *L. plantarum* biofilm formation remains to be elucidated.

The role of flagella has been extensively studied (Davey and O'Toole, 2000; Simões et al., 2010; van Houdt and Michiels, 2010). A role for passive rather than active process in the initial attachment of *L. plantarum* is also suggested by the fact that initial attachment was not affected by contact materials such as polystyrene and stainless steel for the six FBR strains and WCFS1 (data not shown). The comparable initial attachment suggested that

observed differences in biofilm formation between strains result from later stages of the biofilm formation.

Strain FBR1 is unique as it is the only *L. plantarum* strain known to carry genes required for pili synthesis encoded on its genome (Sanders et al.). For several microorganisms such as *L. lactis* and *L. rhamnosus* GG, a role for pili in the attachment and biofilm development on polystyrene has been reported (Lebeer et al., 2009; Oxaran et al., 2012). An advantage of pili in biofilm formation was not observed under the conditions tested since strain FBR1 was a very poor biofilm former. However, it cannot be excluded that the pili were not expressed in standard laboratory conditions used. Previously, expression of pili-encoding gene clusters was not observed for several *L. lactis* strains grown in M17 at 30°C (Oxaran et al., 2012) whereas *L. rhamnosus* grown in AOAC or MRS at 37 °C could express SpaCBA pili but not *spaDEF*-encoded pili (Lebeer et al., 2012; Reunanen et al., 2012). Further studies on expression regulation of pili-encoding genes in *L. plantarum* FBR1 are required, and if differential expression is observed in specific conditions, preferably in combination with targeted FBR1 pili mutants, their role in adhesion to abiotic surfaces and subsequent biofilm formation can be assessed.

Cell-cell and cell-surface interactions are required for the irreversible attachment and for the continuation of biofilm development. Chapter 3 focused on the role of the SDPs and CPS. Whether extracellular polysaccharides have a role as structural components in *L. plantarum* biofilms remains to be determined. Polysaccharides have been studied extensively as major components of the biofilm matrix (Branda et al., 2005; Christensen, 1989; Sutherland, 2001). For *Salmonella* and *Escherichia coli*, cellulose is an important component of the biofilm matrix (Zogaj et al., 2001). For staphylococci, polysaccharide intercellular adhesins and poly-N-acetyl glucosamine serve as adhesins (Mack et al., 1994; Maira-Litrán et al., 2002). *L. plantarum* SF2A35B (used in chapter 5) and FBR2 showed a ropy phenotype in liquid cultures and on agar plates but displayed low biofilm formation (both CV and number of culturable cells). The poor biofilm formation could be associated with the production of putative polysaccharides responsible for the ropy phenotype, resulting in shielding of cell surface components with a role in the colonisation of abiotic surfaces. In *L. rhamnosus* GG, the production of a galactose rich polysaccharide was shown responsible for low biofilm formation capacity on abiotic surfaces (Lebeer et al., 2009).

L. plantarum strains FBR3 and FBR6 are highly similar based on genome content, and differences are mostly found in the presence of 4 large plasmid fragments in FBR6 in the range of 5 kb up to 21 kb which are not present in strain FBR3. These FBR6 plasmid fragments include for example genes with predicted function in extracellular polysaccharides (EP) synthesis and heavy metal transport (Fernández Ramírez et al., 2016). Notably, FBR3 is a good biofilm former whereas FBR6 is a poor biofilm former (based on CV staining and viable cell numbers). Since initial surface adhesion capacity is similar for FBR3 and FBR6,

it is conceivable that plasmid-encoded functions affect biofilm development of FBR6. The plasmid carried by FBR6 could therefore be introduced in FBR3 to study the impact on adhesion and biofilm formation. Alternatively, strain FBR6 could be cured of the plasmid, and tested for adhesion and biofilm formation. Such a combined approach may reveal additional insights in the putative functions of these plasmid-encoded genes in biofilm formation.

Matrix formation

Biofilm related proteins

Besides the role of SDPs described in Chapter 3, other extracellular proteins may be involved. An *in silico* analysis of extracellular proteins (Siezen and Eibrink, unpublished results) of the six food isolates and compared to *L. plantarum* strains P8, ATCC14917, ZJ316, UCMA3037, STIII, NC8, Lp16, JDM1, and WCFS1, identified seven new secretome proteins from which two are on the chromosome of strain FBR4 (extracellular lysozyme-like protein and cell surface collagen-binding protein) and five are on putative plasmids, one in FBR4 and four in FBR1 (mucus binding protein and pili related proteins, respectively). Strain FBR2 lacks four secretome proteins that are present in all five other *L. plantarum* spoilage strains and the reference strains mentioned above, and include a cell surface LPXTG anchored protein (also absent in strain NC8), an extracellular Asp-rich lipoprotein precursor, a transpeptidase (penicillin binding protein 2B) and a serine-type D-Ala-D-Ala carboxypeptidase. In contrast to FBR4, the FBR1 and FBR2 strain showed poor biofilm forming capacity. Secreted proteins, including enzymes, adhesins, carbohydrate binding proteins, biofilm associated proteins, lectins and pili/flagella have been described previously as relevant matrix components (Branda et al., 2005; Flemming and Wingender, 2010). Further studies are required to elucidate the role of the *L. plantarum* secretome in attachment and biofilm formation on abiotic surfaces.

L. plantarum growth in BHIMnG medium results in acidification due to the production of lactic acid during fermentation. It was found that the presence of excess glucose in BHIMnG medium resulted in an additional decrease in pH during biofilm formation compared to BHI without glucose favouring cell lysis. In *Staphylococcus aureus*, cytoplasmic proteins released upon cell lysis, were also found to be part of the biofilm matrix (Foulston et al., 2014). It is conceivable that lysis of *L. plantarum* also results in the release of cell wall-associated and cytoplasmic proteins that are subsequently trapped in the biofilm matrix.

***L. plantarum* static biofilm morphology**

The morphology of *L. plantarum* biofilms including that of *L. plantarum* WCFS1 has been recently described by Aoudia et al. (2016). Using confocal laser scanning microscopy

(CLSM), static biofilms of *L. plantarum* WCFS1 grown in MRS at 30°C were shown to be composed of multiple layers of cells with voids conceivably containing eDNA (Aoudia et al., 2016). Since in our studies biofilms retained after several washing steps are studied, we used CLSM and scanning electron microscopy (SEM) to study this type of biofilm (Figure 6.3). CLSM shows live cells (with intact membranes) distributed throughout the biofilm, whereas increased propidium iodide stained material, indicating the presence of dead cells and eDNA at the biofilm surface (Fig. 6.3AB). Notably, voids were not observed, which is conceivably due to the washing protocol which removes part of the loosely bound biofilm material. The aerial image obtained by SEM (Fig. 6.3C) shows the presence of crevices, as the sample treatment includes dehydration it is possible that biofilm matrix components such as proteins or eDNA are lost during preparation. To study the distribution of biofilm matrix components in more detail, CLSM can be performed using a range of fluorescent stains next to live/dead stains, that target the different components including lipids, proteins, and polysaccharides (Chen et al., 2007; Oniciuc et al., 2016).

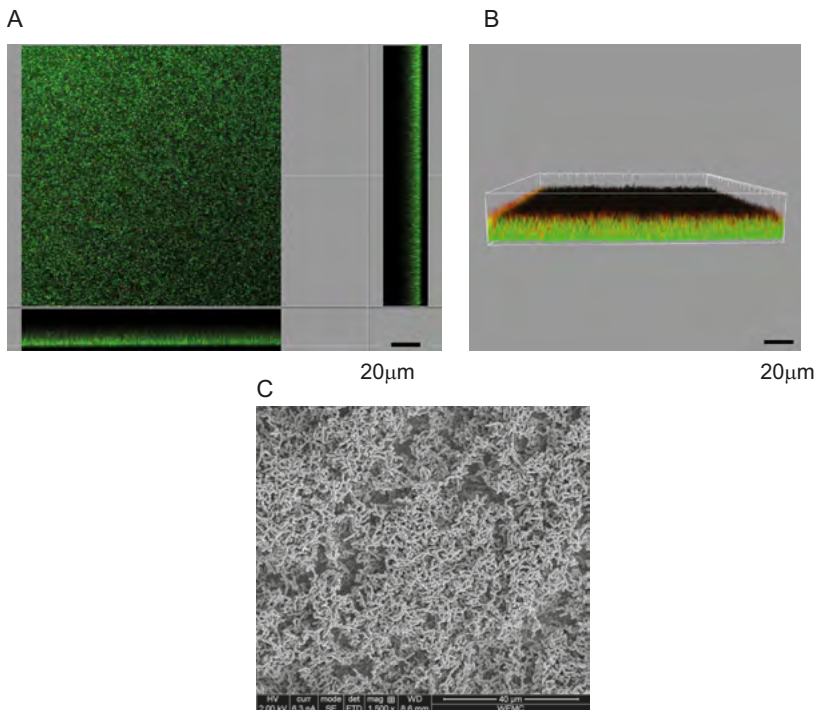


Figure 6.3. Visualisation of static biofilms formed by *L. plantarum* WCFS1. (A) CLSM section view and (B) 3D projection constructed with Imaris software of biofilms stained with Syto 9 and propidium iodide and (C) SEM imaging. The biofilms were grown for 72h and incubated at 30°C in BHIMnG and subsequently washed according to the used standard protocol. Unpublished CLSM data were obtained in cooperation with de group of Dr R. Briandet (INRA and AgroParisTech, UMR1319 Micalis, France).

Biofilms formed under dynamic flow conditions

In this thesis, it has been shown for the first time that besides static biofilms, *L. plantarum* is capable of forming biofilms under dynamic flow conditions. All tested *L. plantarum* strains were able to form high cell density biofilms (approximately 11 log cfu/cm²) under dynamic flow conditions. These types of biofilms can be encountered in pipelines during food processing. Dynamic biofilms are different from static biofilms in the fact that they encounter a constant supply of fresh medium containing high levels of nutrients and oxygen that may evoke oxidative stress in the biofilm cells. Thus, oxidative stress mechanisms can also be expected to influence biofilm formation under dynamic flow conditions. Along this line, manganese and thioredoxin reductase, factors known to play an important role in oxidative stress response of *L. plantarum* (Archibald and Duong, 1984; Archibald and Fridovich, 1981; Serrano et al., 2007), may contribute to biofilm formation in dynamic flow biofilm models.

A comparative proteome analysis between of *L. plantarum* DB200 planktonic cells and cells originated from static biofilms, revealed thioredoxin reductase to be 20 times more abundant in the latter type of cells (De Angelis et al., 2015). It would therefore be interesting to evaluate the static and dynamic flow biofilm forming capacity of *L. plantarum* WCFS1 mutants described by Nierop Groot et al. (2005), lacking Mn(II) transporters i.e., *mntH2*, *mtsA* or *mntA* and the double mutant strain $\Delta mtsA \Delta mntH2$, in the presence and absence of Mn(II) (Nierop Groot et al., 2005). The over-expression of *trxB*, encoding thioredoxin reductase, resulted in upregulation of *mntH2* and other stress related genes (Serrano et al., 2007) which could contribute to biofilm development in flowing conditions. Therefore, inclusion of mutants affected in thioredoxin expression $\Delta trxB$ (deletion mutant) and *trxB*⁺⁺ (overexpression of thioredoxin) in dynamic flow biofilm experiments may reveal whether factors playing roles in oxidative stress response contribute to surface colonisation. Alternatively, biofilm formation in dynamic flow conditions can be studied using anaerobic or low level oxygenated media. In conclusion, more studies on *L. plantarum* dynamic flow biofilm formation and underlying mechanisms are required.

Multi-strain biofilm formation

In nature, multiple strains of the same species can share similar niches. The environment can influence the population dynamics of such multi-strain communities. Chapters 4 and 5 show that in a multi-strain setting, a linear relation exists between the abundance of viable cells in static planktonic and static biofilm growth modes. The determination of relative abundance was conducted by two different techniques: quantitative PCR (qPCR) and next generation sequencing (NGS). qPCR was chosen for dual and multi-strain biofilm

formation of *L. plantarum* spoilage related strains and WCFS1 (Chapter 4). Moreover, the use of propidium monoazide (PMA) allowed the quantification of viable cells (with non-compromised membranes), which can pose a risk for contamination and recontamination of food products.

In dual and multi-strain competition consisting of five food *L. plantarum* isolates and WCFS1, strain FBR5 dominated in planktonic and in static biofilm growth. Notably, strain FBR5 has the highest *D*-values at 55 and 58°C compared to 20 other *L. plantarum* strains including the spoilage isolates studied in this thesis and WCFS1 (Aryani et al., 2016). The robustness of strain FBR5 might pose a higher risk for premature spoilage when it dominates the biofilm. Strain FBR5 was also used in a competition setting along with 11 other strains including food isolates ATCC14917, SF2A35B and NCTH19-2 (Chapter 5) where it also formed part of the group of dominant strains along with NC8, WCFS1 and CIP104440 in static biofilms.

To study *L. plantarum* multi-strain biofilm formation in more detail, a more complex population consisting of 12 strains was studied and quantified by a modified version of the NGS approach previously developed by (van Bokhorst-van de Veen, van Swam, et al., 2012) to follow *in vivo* GI-tract persistence. The impact of environmental conditions such as temperature and Mn(II) concentration was studied on population dynamics in planktonic phase (static or shaking) and in static and dynamic flow biofilms.

The determination of relative abundance by NGS allows strain-specific tracking in complex ecosystems such as for example biofilms. By using a unique 6nt barcode for the reverse primer designed to the target region for strain tracking, individual experiments (different temperatures, media, biofilm models etc.) can be pooled and analysed in a single NGS run. The number of strains that can be included in one study is dictated by the sequence variety of the selected target region. The technique is not limited to the lp_1173 region used in this thesis but can be optimized for each strain set by selecting hypervariable sequence regions.

Results obtained in the multi-strain competition model of 12 *L. plantarum* strains showed that the four dominant strains in static biofilm formation (NC8, CIP104440, WCFS1 and FBR5) belong to a subgroup of strains that showed highest viable cell numbers in single strain biofilms, indicating that single strain performance can predict behaviour in a competitive static biofilm model. In static conditions high cell numbers in the culture are key to prominent contributions in static biofilms in multi-strain competition models. This may offer an explanation for the fact that comparative genome analysis of the dominant strains in static biofilms compared to the remaining strains did not reveal unique shared features.

Data obtained without and with PMA proved to be useful for the determination of the strains which contributed to eDNA release in multi-strain competition models. Three strains showed enhanced lysis during multi-strain biofilm formation: ATCC14917, SF2A35B and FBR1. The early release of eDNA can help the other cells to attach and possibly to aggregate in the biofilm. The early eDNA release showed to be more relevant during static biofilm formation and no evident effect was observed during biofilm formation under dynamic flow conditions.

It is remarkable that the strains which were DNase resistant and had higher individual CV stainable biofilms (CIP104448, NCTH19-2 and FBR4) dominated in the multi-strain biofilm grown under dynamic flow conditions. Since the 12 individual *L. plantarum* strains showed no significant difference in biofilm formation capacity in dynamic flow conditions, it is conceivable that specific features contributed to the success of the three dominant strains in the multi-strain model. The gene content analysis pointed to a DNA invertase Pin-related protein as a candidate gene that supports biofilm formation in dynamic flow conditions. Further research is needed to elucidate its exact function. In addition, gene presence or absence might not be able to explain the biofilm forming capacity of the different strains and it is therefore likely that differential gene expression levels of conserved genes result in a different behaviour.

Multi-strain biofilm formation can result in the competition for space and nutrients between the different strains. The mechanisms for multi-strain biofilm formation might differ from single strain biofilm formation as more factors are involved. Some strains can act as the initial colonisers and they could have either a cooperative effect in which they promote co-aggregation or they could inhibit the adhesion of other strains (Burmølle et al., 2014; Elias and Banin, 2012; Moons et al., 2009; Rendueles and Ghigo, 2012). Additionally, it has been reported that microorganisms can produce compounds that inhibit surface adhesion and/or biofilm formation of competitors, for example *Lactobacillus acidophilus* produces a biosurfactant which inhibits initial adhesion and biofilm development of *S. aureus* (Walencka et al., 2008). Another strategy includes the production of an extracellular DNase by *B. licheniformis*, which is able to disperse biofilms of other Gram positive and Gram negative bacteria (Nijland et al., 2010). Also the production of antimicrobial peptides (bacteriocins), which can inhibit the growth of target bacteria, can play a role in the formation of biofilms composed of multiple species and/or strains. Comparative analysis of gene contents of WCFS1 and selected spoilage isolates and strains obtained from other origins, however, did not reveal the presence of additional gene clusters encoding bacteriocins.

Visualization of multi-strain biofilms at different developmental stages may provide relevant information about the spatial distribution of the strains in such multi-strain biofilms. In this way it would be possible to know which strains are the initial colonizers

and if they positively or negatively affect other strains. Moreover, it would also be valuable to determine if synergistic effects take place in multi-strain communities by determining total biofilm formation using CV staining or by determination of the biovolume using CLSM, and to identify and quantify matrix components formed, since these could add additional protection of biofilm cells to disinfectants. Such combined information is required for optimization of cleaning and disinfecting regimes discussed below.

***L. plantarum* biofilm control strategies**

Both eDNA and proteins/proteinaceous material are important components of the *L. plantarum* biofilm matrix. This suggests that DNase and/or proteinase enzyme-mediated strategies may aid in intervention and prevention of biofilm formation as part of cleaning and disinfection strategies. Cell history and the physiological state can play a role in the resistance to disinfectants and affect (re)contamination potential of cells originated from biofilms. The response to cleaning and disinfect agents including for example quaternary ammonium compounds such as benzalkonium chloride (BKC) or oxidative agents such as peracetic acid (PAA) may be influenced by these factors. Moreover, survival capacity in mixed species biofilms, can for example be different from that in single strain conditions (Jahid et al., 2015; van der Veen and Abee, 2011) thereby possibly affecting the (re) contamination potential of spoilage and pathogenic bacteria.

The (re)contamination potential of cells originated from biofilms formed in processing environments is relevant for the food industry. Cells in a biofilm can have different phenotypes compared to their planktonic counterpart; including increased resistance to antimicrobials (Bridier et al., 2011; Mah and O'Toole, 2001; van Houdt and Michiels, 2010). When the biofilm is damaged or detached, free cells pose a higher risk for contamination if they are able to survive outside the biofilm. The increased resistance of cells in a biofilm to antimicrobials can be the result of different mechanisms including diffusion limitation caused by the biofilm matrix, decreased growth rate, adaptive stress responses or gene transfer and mutation (Bridier et al., 2011; Jahid and Ha, 2012; Lewis, 2001; Mah and O'Toole, 2001). In the case of *L. plantarum* JCM 1149, Kubota et al. (2008) showed that cells within the biofilm are more resistant to organic acids commonly used for food preservation. Moreover, also cells dispersed from biofilms appeared to be more resistant to acetic acid (Kubota et al., 2009).

The efficacy of widely used disinfectants PAA and BKC against *L. plantarum* cells within the biofilm and cells originated from resuspended biofilms was tested using a static biofilm model. When the cells within biofilms of the three selected strains (WCFS1, FBR2 and FBR4) were treated with BKC, the cells within the biofilm had a comparable resistance

as the planktonic cells. Nevertheless, when biofilms of the three selected strains were resuspended and the disinfectant was applied, the cells appeared to be very sensitive and within 3 min culturable cells could no longer be detected (Figure 6.4.I). The exposure to PAA revealed higher resistance of the cells in the biofilm in comparison to the planktonic cells for strains WCFS1 and FBR2. Notably, the cells treated after biofilm resuspension were found to be more sensitive (Figure 6.4.II A and B). On the other hand, strain FBR4, showed not only increased resistance to PAA of cells within the biofilm but also the corresponding cells originated from resuspended biofilms were found to be as sensitive as the planktonic cells (Figure 6.4.II C).

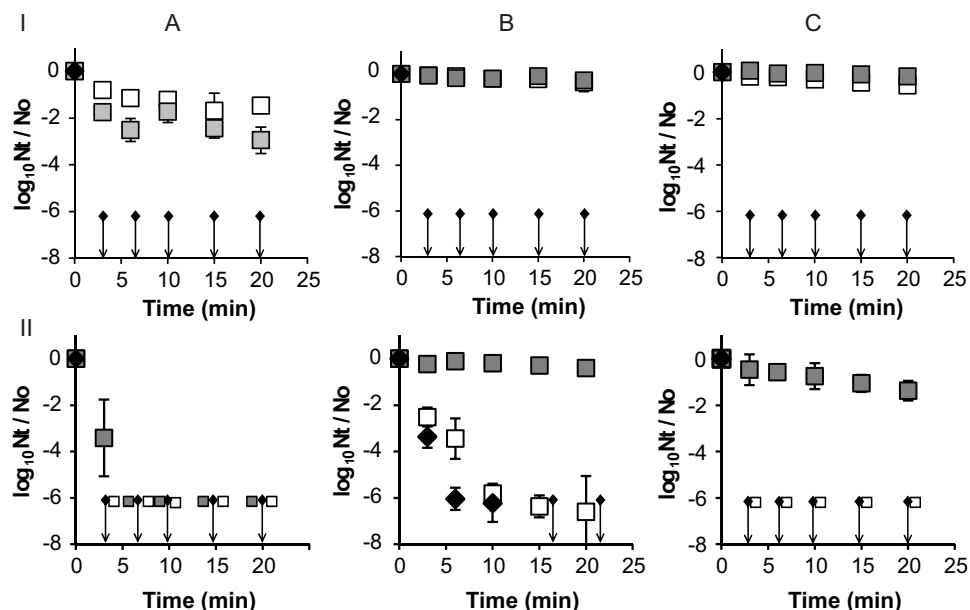


Figure 6.4. Disinfection treatment of planktonic cells, cells within the biofilm and cells originated from resuspended biofilms of a selection of *L. plantarum* strains. The data show the average three biological independent inactivation experiments of planktonic cells (open squares), cells within the biofilm (grey squares) and cells originated from resuspended biofilms (black diamonds) grown for 48 h at 30°C in 12-wells polystyrene plates. The strains tested were (A) FBR2, (B) FBR4 and (C) WCFS1. The disinfectants used were (I) 100 µg/ml benzalkonium chloride and (II) 20 µg/ml peracetic acid. The detection limit is indicated with an arrow and respective code.

The results obtained indicate that cells in the biofilm appear sensitized to disinfectants, i.e., cells originated from dispersed biofilms are less resistant than planktonic cells, but that they are somehow protected in the biofilm conceivably due to diffusion limitation of the used antimicrobials. The results suggest that loosening up of biofilm structure and/or dispersal of cells can enhance disinfectant efficacy.

The use of enzymes for biofilm dispersal has been previously reported. Lequette et al. (2010) showed that serine proteases successfully removed *B. mycoides*, *B. cereus* and *Pseudomonas fluorescens* biofilms. For more effective results in biofilm removal, it was suggested to use a mixture containing next to proteases, for example also anionic surfactants and dispersing agents (Lequette et al., 2010). Furthermore, the presence and relevance of eDNA in the biofilm matrix of several microorganisms has been extensively studied (Das et al., 2013; Flemming and Wingender, 2010; Jakubovics et al., 2013) and it has also been suggested to use DNase I for biofilm prevention and removal (Das et al., 2013; Okshevsky et al., 2015). Based on the results presented in Chapters 2 and 3, such a combination of enzymes can be beneficial also for the control of *L. plantarum* biofilms. Out of 12 strains *L. plantarum* strains tested (Chapter 5) only two strains, FBR4 and CIP104448, were resistant to DNase I treatment. The resistance could be due to matrix components interacting with the eDNA as described by (Okshevsky et al., 2015). Nevertheless, strain CIP104448 was found to be resistant also to proteinase K and further studies are needed to reveal the cause behind the robustness of its biofilm matrix. The described enzyme mixtures may show higher capacity for biofilm control, but obviously, their application should be cost effective (Okshevsky et al., 2015).

Concluding remarks and future perspectives

The results presented in this thesis contribute to a better understanding of the mechanisms underlying *L. plantarum* biofilm formation, resulting in the design of a more detailed model for static biofilm formation on abiotic surfaces. This model was built based on the analysis of biofilm mass (including matrix components) and culturable biofilm cells retained on the surface after multiple washing steps. Combining all observations, it is proposed that sedimentation of planktonic cells to surface is not only required for the initial attachment, but also for biofilm development. Both, eDNA and protein-protein interactions and/or proteinaceous material contribute to cell-cell/cell-surface interactions and biofilm matrix. Based on the model, a role for DNase and/or proteinase enzyme-mediated prevention of biofilm formation and dispersal has been suggested. In addition, the newly obtained insights on the population dynamics of *L. plantarum* isolates obtained from different origins in competitive static and dynamic flow biofilm models, provide further leads for research into underlying mechanisms involved and may further add to development of tools and strategies to prevent (re)contamination from different types of biofilms in food processing environments.

Further work should focus on mechanistic approaches to identify genes responsible for dominance in dynamic or static biofilm formation. NGS can be used to determine transcriptome-phenotype matching and to identify the role of conserved genes

responsible for biofilm formation. Transcriptome-phenotype matching has already been used to build a fermentation platform (FermDB) for *L. plantarum* (Bron et al., 2012), this platform aides with the identification of transcriptome signatures underlying physiological variations resulting from diverse fermentation conditions (Bron et al., 2012) and it also contributed to identification of genes responsible for GI tract persistence (van Bokhorst-van de Veen, Lee, et al., 2012). A similar approach can be used to build a data base to extend the knowledge on *L. plantarum* biofilm formation.

It is not only relevant to study biofilms composed of multiple strains but also those composed of different species. In particular, the interaction of food spoilage microorganisms and pathogens is very relevant (Sanchez-Vizuet et al., 2015), since spoilage bacteria such as *L. plantarum* can contribute to survival of pathogenic microorganisms in mixed biofilms by shielding against disinfectants and cleaning agents, and by inducing adaptive stress responses that offer protection against a range of environmental challenges (van der Veen and Abee, 2011). Therefore, the understanding of the mechanisms of biofilm formation of spoilage bacteria such as *L. plantarum* can help indirectly to the control of pathogenic microorganisms. Insights obtained on *L. plantarum* adhesion and biofilm formation on abiotic surfaces may also support the identification of factors that contribute to relevant probiotic features such as prevention of pathogen binding to epithelial cells in the host.

Overall, the study of single strains provides clues in the biofilm formation mechanisms and is a good basis for the study of multi-strain/species biofilms. Nevertheless, single strain behaviour can change in a mixed community and further efforts are needed for a better understanding of the individual strain performance as part of a community. Moreover, further research on biofilms formed under dynamic flow conditions will be valuable to build a biofilm formation model and to identify fitness and robustness parameters that determine the success of dominant strains in competitive biofilm models.

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Summary

Acknowledgments

About the author

List of publications

Overview of training activities

Summary

Research described in this thesis provides new insights in single and multi-strain biofilm formation of *Lactobacillus plantarum* WCFS1 and *L. plantarum* strains isolated from different environments including fermented and spoiled foods. Studies on biofilms in relation to *L. plantarum* strain diversity and mechanisms of biofilm formation are highly relevant for the development of biofilm control and prevention strategies.

Chapter 1 describes an overview of the role of *L. plantarum* in food spoilage in relation to its nutrient requirements and colonisation of abiotic surfaces. Additionally, general concepts of biofilm formation with single and multiple microbial species are provided, including a description of tools used to study biofilm formation and a description of cellular factors that influence *L. plantarum* biofilm development.

Chapter 2 describes the analysis of the biofilm forming capacity of *L. plantarum* WCFS1 and six *L. plantarum* food spoilage isolates. Biofilm formation as quantified by crystal violet (CV) staining and colony forming units was largely affected by the medium composition, growth temperature and maturation time as well as by strain specific features. All strains showed highest biofilm formation in Brain Heart Infusion medium supplemented with manganese and glucose. For *L. plantarum* biofilms, the CV assay, that is routinely used to quantify total biofilm formation, correlates poorly with the number of culturable cells in the biofilm. This can in part be explained by cell death and lysis resulting in CV stainable material, conceivably extracellular DNA (eDNA), contributing to the extracellular matrix. For example, increasing biofilm maturation times from 24 h up to 72 h, and increasing temperature from 20 to 37°C, resulted in decreased culturable cell numbers with a concomitant increase of CV staining. The strain to strain variation may in part be explained by differences in release of eDNA, likely as result of differences in lysis behaviour. In line with this, biofilms of all strains tested, except for one spoilage isolate, were sensitive to DNase treatment. In addition, biofilms were highly sensitive to treatment with Proteinase K suggesting a role for proteins and/or proteinaceous material in surface colonisation. This study shows the impact of a range of environmental factors and enzyme treatments on biofilm formation capacity for selected *L. plantarum* isolates associated with food spoilage, and may provide clues for disinfection strategies in food industry.

Chapter 3 provides new insights into biofilm development by *L. plantarum* WCFS1 through comparative analysis of wild type and mutants affected in cell surface composition, including a mutant deficient in the production of Sortase A involved in the covalent attachment of 27 predicted surface proteins to the cell wall peptidoglycan (Δ *srtA*) and mutants deficient in the production of capsular polysaccharides (CPS1-4, (Δ *cps1-4*). Surface adhesion and biofilm formation studies revealed none of the imposed cell surface modifications to affect the initial attachment of cells to polystyrene while CV-stainable

biofilm was severely reduced in the Δ *srtA* mutant and significantly increased in mutants lacking the *cps1* cluster, compared to the wild-type strain. Fluorescence microscopy analysis of biofilm samples pointed to a higher presence of eDNA in *cps1* mutants and this corresponded with increased autolysis activity. Subsequent studies using Δ *acm2* and Δ *lytA* mutants, deficient for specific peptidoglycan hydrolases and affected in lytic behaviour, revealed reduced CV staining of biofilms, confirming the relevance of lysis for the build-up of the biofilm matrix with eDNA.

Additionally, performance of *L. plantarum* strains obtained from different origins was determined in single and in competitive multi-strain biofilm formation models. Two different approaches were used to monitor the population dynamics of multi-strain biofilm formation: quantitative PCR (qPCR) (**Chapter 4**) and next generation sequencing (NGS) (**Chapter 5**). In **Chapter 4**, six spoilage related *L. plantarum* strains (FBR1-FBR6) and *L. plantarum* WCFS1 were characterised in single and multiple strain competition models. A quantitative PCR approach was used with added propidium monoazide (PMA) enabling quantification of cells in the biofilm without membrane damage, representing the viable cell fraction that determines the food spoilage risk. The results show that the performance of individual strains in multi-strain cultures generally correlates with their performance in pure culture, and relative strain abundance in multi-strain biofilms positively correlated with the relative strain abundance in suspended (planktonic) cultures. The total biofilm quantified by CV staining of the multi-strain biofilms showed a positive correlation with CV values of the dominant strain obtained in single strain studies. However, the combination of FBR5 and WCFS1 showed significantly higher CV values compared to the individual performances indicating that total biofilm formation was higher in this specific condition. Notably, *L. plantarum* FBR5 was able to outcompete all other strains and showed the highest relative abundance in multi-strain biofilms. All the multi-strain biofilms contained a considerable number of viable cells, representing a potential source of contamination.

As a follow up, in **Chapter 5**, the population dynamics of a mixture of 12 *L. plantarum* isolates of different origins was studied in competitive planktonic and static and dynamic flow biofilm growth models as a function of different parameters, namely maturation time, temperature and medium composition (BHIGlucose containing low or high Mn(II), in presence and absence of haem and vitamin K2). A NGS approach based on detection of strain specific alleles was used to determine the relative abundance of each strain in the different conditions. Data were obtained in the presence and absence of PMA, thus allowing for identification and quantification of relative contributions of each individual *L. plantarum* strain to the fraction of viable cells in planktonic and biofilm phase and the fraction of dead cells (compromised membrane) and levels of eDNA in the biofilm matrix, respectively. This approach revealed that the relative abundance of each strain in the static biofilm positively correlates with its performance in static planktonic conditions.

The genome content of the two groups of strains that dominated the static and dynamic flow biofilms was explored to identify genetic factors that potentially contribute to biofilm forming capacity under static and dynamic flow conditions, respectively.

The results presented in this thesis contribute to the understanding of mechanisms of biofilm formation and matrix composition of *L. plantarum* resulting in the design of a more detailed model for static biofilm formation on abiotic surfaces (**Chapter 6**). This model was built based on the analysis of biofilm mass (including matrix components) and culturable cells retained on the surface after multiple washing steps. Combining all observations, it is proposed that sedimentation of planktonic cells to surface is not only required for the initial attachment, but also for biofilm development. Both, eDNA and protein-protein interactions and/or proteinaceous material contribute to cell-cell/cell-surface interactions and biofilm matrix. Based on the model, a role for DNase and/or proteinase enzyme-mediated prevention of biofilm formation and dispersal has been suggested. The newly obtained insights on the population dynamics of multi-strain *L. plantarum* isolates obtained from different origins in static and dynamic flow biofilm models, provide further leads for research into underlying mechanisms involved and may further add to development of tools and strategies to prevent (re)contamination from biofilms in food processing environments.

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“Feeling gratitude and not expressing it is like wrapping a present and not giving it.”

William Arthur Ward

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Mónica

About the author

Mónica Dolores Fernández Ramírez was born on March 3, 1984 in Mexico City, Mexico. In 2003 she started studying the Diplom in Food Chemistry at the National Autonomous University of Mexico (Universidad Nacional Autónoma de Mexico (UNAM)) where she graduated with honours. In 2009 she received the CONACyT scholarship for the MSc program Food Technology with the specialization in Food biotechnology at Wageningen University in 2009. Her major thesis was conducted at the Laboratory of Food Microbiology and focused on the identification of new genes responsible for biofilm formation in *Listeria monocytogenes* EGD-e. As part of the MSc program she did an internship at Corbion, where she was working on the development of antimicrobial formulations based on organic acids and bacteriocin combinations. After graduating in 2011 she started her PhD research entitled: Characterisation of *Lactobacillus plantarum* single and multi-strain biofilms at the Laboratory of Food Microbiology of Wageningen University. The results of her project are described in this thesis.

List of publications

Fernández Ramírez, M.D., Boekhorst, J., de Jong, A., Kuipers, O.P., Abee, T., Nierop Groot, M.N., 2016. Draft whole-genome sequences of three *Lactobacillus plantarum* food isolates. Genome Announcements 4.

Fernández Ramírez, M.D., Smid, E.J., Abee, T., Nierop Groot, M.N., 2015. Characterisation of biofilms formed by *Lactobacillus plantarum* WCFS1 and food spoilage isolates. International Journal of Food Microbiology 207, 23-29.

Overview of completed training activities

Courses

Advanced visualisation, integration and biological interpretation of -omics data	VLAG, Wageningen	2011
Food fermentation	VLAG, Wageningen	2012
Hands-on BiolT	TIFN/CMBI, Nijmegen	2012
Management of Microbiological Hazards in Foods	VLAG, Wageningen	2013
Pheno Link	TIFN/CMBI, Nijmegen	2013
CLSM training	WUR, Wageningen	2013
Fungi identification workshop	WUR, Wageningen	2013
Genetics and physiology of food-associated microorganisms	VLAG, Wageningen	2013
CLSM training and image analysis	INRA, France	2014

Conferences

Biofilm 5 (Poster presentation)	Paris, France	2012
Biofilm 6 (Poster presentation)	Vienna, Austria	2014
LAB conference (Poster presentation)	Egmond an Zee, The Netherlands	2014

General courses

Philosophy and Ethics of Food Science and Technology	VLAG, Wageningen	2012
Project and time management	WGS, Wageningen	2012
PhD Week	VLAG, Wageningen	2012
TIFN IP	TIFN, Wageningen	2012
Advanced Course Guide to Scientific Artwork	WUR, Wageningen	2013
Scientific Writing (SWR)	WGS, Wageningen	2014

Optional courses

Preparation of research proposal	WUR, Wageningen	2011
PhD trip Japan	FHM, Wageningen	2012
Organization of the PhD trip Japan	FHM, Wageningen	2012
PhD trip Ireland	FHM, Wageningen	2014
FHM department meetings	FHM, Wageningen	2011-2015
TIFN expert and biofilm meetings	TIFN, Wageningen	2011-2015

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