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ROLE OF EXOPOLYMERIC SUBSTANCES IN BIOFILM FORMATION ON
DAIRY SEPARATION MEMBRANES

BY

NURIA GARCIA-FERNANDEZ

A dissertation submitted in partial fulfillment of the requirements for the

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Major in Biological Sciences

Specialization in Dairy Science

South Dakota State University

2016

ROLE OF EXOPOLYMERIC SUBSTANCES IN BIOFILM FORMATION ON DAIRY
SEPARATION MEMBRANES

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Biological Science degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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Date

To my family.

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ABSTRACT

ROLE OF EXOPOLYMERIC SUBSTANCES IN BIOFILM FORMATION ON DAIRY
SEPARATION MEMBRANES

NURIA GARCIA-FERNANDEZ

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In the concentration of cheese whey by membrane filtration processes, fouling is a major limiting factor that leads to flux decline and affects product quality. The growth of biofilms on membranes contributes to biofouling. Biofilms are very difficult to eliminate, and will serve as the seed for additional biofilm development and product contamination. The biofilm matrix is composed mainly of microbial exopolymeric substances (EPS), and therefore EPS is thought to help bacteria to adhere and form biofilms by acting as a cementing agent.

The first research project under this study was conducted with the aim of elucidating the role of the EPS produced by lactic acid starter cultures on reverse osmosis (RO) membranes. A second study was designed to investigate the role of EPS produced by spore forming bacteria on RO membrane biofilms. A third study was conducted to evaluate the application of Catalase (Cat) on spores and biofilms formed by spore forming bacteria that produce EPS. Finally, the fourth study evaluated the application of natural depolymerases from bacteriophages and bacteria to disrupt biofilms.

In the first research project we observed that the EPS of *Streptococcus thermophilus* strain 3534 enhanced biofilm formation but the EPS of *Lactococcus lactis* ssp. *cremoris* strain JFR inhibited the attachment and biofilm formation to RO membranes. This was associated to the cell-surface hydrophobicity of the strains, which

was affected by the EPS production. In the second study a greater biofilm formation was observed for a *Bacillus* strain that produced poly glutamic acid, and it was established that hydrophobicity of the EPS, regardless of whether it is composed of polysaccharides or poly-amino acids, played an important role in biofilm formation on RO membranes. Food grade Cat preparations significantly reduced the number of spores in milk and the viable counts of EPS producing *Bacillus* cells on RO biofilms, showing a great potential in antimicrobial cleaning formulations in the dairy industry. In our final study the application of crude enzymes from different EPS degrading microorganisms although resulted in poor reductions, when they were applied to biofilm formed by single resistant species, revealed the importance of choosing compatible hydrolytic enzymes due to strong specificity for greater effectiveness.

Chapter 1

INTRODUCTION AND BACKGROUND INFORMATION

1. Dairy processing membranes

The use of membrane separation processes allows concentration and fractionation of whey components to obtain valuable ingredients for food and other applications. Manufacturers employ a number of different membrane separation techniques including ultrafiltration (UF), microfiltration (MF), nanofiltration (NF), and reverse osmosis (RO), to separate out the different components of whey and create whey products to the exact specifications of the end user (Kumar et al., 2013). Different whey products such as whey powder, whey protein concentrate (WPC), whey protein isolate (WPI), whey protein fractions, whey protein hydrolysate, reduced-lactose whey, and demineralized or reduced-mineral whey, are achieved by varying protein, mineral, and lactose levels, as well as various functional properties (Mollea et al., 2013). The product's value increases with protein concentration. The main difference between MF, UF, NF and RO membranes is their selectivity. The retention factor (R_f) is a measure of the membrane's ability to separate components of the feed solution as it reflects the membrane selectivity (Tamime, 2012). The pore size (determined by the structure of the membrane material) will define which molecules are retained and which molecules flow right through. As the molecular cut-off is lower the operating pressure needed is greater. The membranes consist of semi-permeable polymeric or ceramic materials. Inorganic ceramic tubular membranes have great pore size uniformity and good selectivity, and are very resistant to temperature and chemicals (Tamime, 2012). They are also easy to clean, and their lifetime is over 10 years, however, their cost is 3-10 times more than organic spiral-

wound polymeric membranes, come in a limited range of pore sizes, and are normally only used for microfiltration and in some cases ultrafiltration processes (GEA, 2012). Spiral-wound polymeric membranes consist of several layers of membrane materials rolled around a permeate tube and is one of the most common materials because of larger surface area, limited floor space, high packing density, high permeate flows and lower replacement cost. Some of the disadvantages are limited life, susceptible to fouling, difficult cleaning, extensive pretreatment required, less suitable for viscous feed solutions, limited pH and temperature tolerances for cleaning and operating (Tamime, 2012).

The ideal separation conditions would be continuous flow of the liquid at the applied pressure. However, membrane performance is compromised by fouling or the buildup of adsorbed macromolecules, gels and deposited particles on or in the membrane surface, which cause concentration polarization, and affects membrane selectivity. To control fouling, and reduce the problem of pore blocking, filtration is run at increased cross-flow velocities and low trans membrane pressure (Tamime, 2012). Fouling is one critical issue in membrane filtration technology, especially in polymeric membranes that are relatively hydrophobic and facilitate protein adhesion when compared with ceramic membranes (Baruah et al., 2006). Fouling in general has a significantly detrimental effect on the efficiency of the separation processes causing flux decline, pressure drop, increased pumping energy required (and associated cost), reduced membrane lifetime, inconsistent product composition and quality.

Whey is the liquid by-product of cheese making and casein manufacturing processes and it is composed mainly by water (93g/100g), lactose and whey proteins.

Water concentration by reverse osmosis (RO) is frequently used to reduce volumes and increase solids content prior to transportation or further processing. RO consists of a pressure-driven membrane separation technique in which a membrane which has pore sizes less than 0.001 micron is employed to separate different components in a fluid mixture. Biofouling, or the buildup of suspended particles is one of the critical issues in membrane filtration technology (Peinemann, 2010), as it has a significantly detrimental effect on the efficiency of the separation processes causing flux decline, pressure drop, reduced membrane lifetime, and inconsistent product composition and quality. Biofilms play a major role in biofouling, but in the dairy environment, the main area of concern is the proliferation of pathogenic bacteria in biofilms, which contribute to product contamination with potentially pathogenic bacteria and possible threat to public health.

The composition and texture of the membrane surface is going to have an impact on bacterial initial attachment. Factors that can affect the development of biofilms are a source of utilizable carbon, temperature, production of exopolysaccharides, inefficient cleaning and disinfection, and the hydrodynamics of the fluid distribution system (Florjanic and Kristl, 2011).

2. The problem of biofilms on filtration membranes

Through the nature, bacteria thrive predominantly in multicellular communities or biofilms attached to surfaces and embedded in a self-produced matrix of extracellular polymeric substances (EPS) (Characklis and Marshall, 2001). This is a non-trivial lifestyle choice and it has great consequences for cell physiology and survival (Romeo, 2008). In many industrial and domestic domains biofilms are detrimental, causing corrosion, pipe blockages, equipment failures, unpleasant appearance and odors, product

spoilage and infections, resulting in heavy costs of cleaning and maintenance (Garrett et al., 2008). Biofilms play a major role in membrane biofouling and contribute to product contamination with potentially pathogenic bacteria and possible threat to public health. To restore flux membrane processes have to be stopped after being running continuously for at least 20 hours, and cleaning in place (CIP) is applied.

Biofilms are well known for resistance to cleaning agents and biocides. Within biofilms, the bacteria cells that are located in the deeper layers are protected from toxic substances and have a better access to nutrients. The pervasiveness and importance of biofilms have driven considerable research efforts in trying to develop materials that prevent bacterial adhesion in addition to new cleaning agents or strategies to remove the existing persistent biofilms.

Our knowledge on the structure of biofilms and their formation has extended remarkably in the last decade, but even after all the molecular and genetic mechanisms and mathematical models that have been studied in detail (Blaschek et al., 2007), many industries struggle with the eradication of this problem. In dairy filtration systems, some questions remained to be addressed: (i) whether using starter cultures that produce exopolysaccharides during cheese making, would enhance the formation of biofilms in the whey processing membranes due to the presence of these polymers that would act as a “glue”, (ii) whether the exopolymeric substances (EPS) produced by the organisms that are typically living in those membranes would have an effect on the initial attachment of the cells to the membrane or rather they support the cell-cell cohesion or both, (iii) whether there could be a strategy to reduce biofilm formation on dairy filtration membranes targeting the bacterial EPS that holds cells together.

The structure of a biofilm is not uniform in time or space (Characklis and Marshall, 2001). It is important to study the interactions between bacteria and the surfaces in a specific food-processing environment to provide more effective measures for prevention of biofilm formation and for its removal (Wong, 1998). Understanding the mechanism of bacterial attachment is fundamental in the development of antifouling technologies for membrane systems.

3. Mechanisms of bacterial adhesion

The conditioning layer is the foundation on which a biofilm grows. Attachment of a cell to a substrate is termed adhesion, and cell-to-cell attachment is termed cohesion. The mechanisms behind these forms of attachment, ultimately determine the adhesive and cohesive properties a biofilm will exhibit (Garrett et al., 2008). Adhesion refers to the state in which two dissimilar bodies are held together by intimate interfacial contact such that mechanical force or work can be transferred across the interface (Wu, 1982). The interfacial forces holding the two phases together can be van der Waals forces, chemical bonding or electrostatic attraction.

Biofilms are initiated when bacterial cells attach to a surface and they develop into different stages of maturation as bacteria grow (Ridgway et al., 1999). The first phase of bacterial adhesion involves physicochemical interactions, after which molecular and cellular interactions take place (Vadillo-Rodriguez et al., 2005). Multiple factors influence the adhesion of cells to a substratum including environmental factors such as nutrients availability, flow conditions or surface characteristics of microorganisms and substratum (Palmer et al., 2007).

3.1 Cell / substrate surface characteristics

In the initial attachment of bacterial cells to surfaces certain physicochemical interactions between their surfaces and the substratum occur, which are described by the extended Derjaguin, Landau, Verwey, and Overbeek theory (van Loosdrecht et al., 1989). Such forces include Lifshitz-van der Waal's, acid/base, and electrostatic interactions.

It has been observed that hydrophobic cells adhere to a greater extent than hydrophilic cells to polystyrene disks on a rotary shaker (van Loosdrecht et al., 1987). It was first noted that the extent of microbial colonization appears to increase as the surface roughness increases, because on rough surfaces the surface area is greater and the attached cells are protected from the shear forces, having more time for the initial attachment (Characklis, 1990). For biomaterials consideration should be given to basic surface characterization, such as surface roughness, physical configuration, hydrophobicity, or even chemical composition (An and Friedman, 1997). There are evidences that show that microbial adhesion strongly depends on the hydrophobic-hydrophilic properties of the interacting surfaces (Liu et al., 2004). Now it is commonly recognized that membranes with smooth, hydrophilic, and electrically neutral surfaces are less prone to biofouling than rough, hydrophobic, and charged surfaces (Mansouri et al., 2010). Membrane manufacturers apply coatings of hydrophilic materials to the surface of membranes to reduce biofouling. Polyamide RO membrane has a contact angle of 55 ± 1.8 at neutral pH (Hurwitz et al., 2010) and is coated to increase the surface hydrophilicity, however, Hassan et al. observed that less than 50% of the surface of a new membrane was covered by this hydrophilic layer (Hassan et al., 2010).

The physical appendages of some bacteria (flagella, fimbriae and pili) may stimulate irreversible adhesion to a surface (De Weger et al., 1987). Other non-piliated

bacteria express adhesins on the cell wall which play a role in host-microbe colonization (Kline et al., 2009).

3.2 Biopolymers as adhesives

Some Gram-positive and Gram-negative bacteria can produce biopolymers as capsules and slime layers, which form the glycocalyx. Capsule is a well-defined polysaccharide layer attached to the cells, while slime layer is an unorganized layer of polysaccharides located outside the bacteria cells. The presence of these two structures is not exclusive (Mesnage et al., 1998).

Biofilms are composed primarily of microbial cells and EPS. After the first attachment, a strengthening of the bonds occurs often involving the formation of polymer bridges between the organism and the surface. These sticky polymers form the matrix network, providing mechanical stability to the biofilm (Garrett et al., 2008). EPS account for 50-90% of the total organic carbon of biofilms (Flemming et al., 2000). Scientists have found that the EPS in biofilms are composed by exopolysaccharides, glycoproteins, glycolipids and e-DNA (Flemming et al., 2007). Seven categories of EPS have been proposed: structural, sorptive, surface-active, active, informative, redox-active and nutritive EPS (Flemming et al., 2007). Structural EPS are neutral polysaccharides that act as architectural component in the matrix, facilitates retention of water and nutrients, and provides protection. Sorptive EPS are charged polymers that attach to other charged molecules involved in cell-surface interactions. Surface-active EPS are molecules with an amphiphilic behavior that have different chemical structures and surface properties. These molecules are involved in biofilm formation and some of them have antibacterial or antifungal properties. The active EPS group includes extracellular proteins and

enzymes required for biofilm formation and architecture.

Adhesives are substances that act as bridges between surfaces and help them to stick together. For a strong adhesive bond the establishment of intimate molecular contact is necessary. An ideal adhesive will have a contact angle of zero or near zero, low viscosity (a few centipoises) and is able to spread over the surface and assist in the displacement of trapped air and other particles that may be present (Kinloch, 1987). Adhesive secretions are produced in aquatic and terrestrial live organisms with different functions. The large diversity of these adhesive biopolymers and their significant complexity makes their characterization a difficult task, and often a combination of classical chemical and molecular techniques are needed (Hennebert et al., 2015).

4. EPS producing cultures in the dairy industry

The first chapter of this dissertation involves the study of biofilm formation ability of different EPS producing lactic acid bacteria (LAB) that are relevant for the dairy industry. Fermented milks produced with ropy starters cultures have been traditionally used in northern Europe. These products have a characteristic viscous consistency and ropiness that is stable when stored in a cool room for weeks or even months (Fuquay et al., 2011). Nowadays the use of ropy cultures is popular especially for the manufacturing of low fat products. The EPS of most mesophilic and thermophilic LAB consist of a polysaccharide slime material that is produced in abundance during the late exponential and early stationary phases of growth, and modify the physical properties of fermented milk by increasing viscosity, reducing syneresis (due to its water holding capacity), and modifying texture in general by interference with protein-protein interactions. These properties together improve smoothness and mouthfeel (Hassan,

2008).

The majority of EPS produced by LAB that are used in the dairy industry are heteropolysaccharides consisting of a backbone of repeated subunits of sugars (e.g. glucose, galactose, rhamnose, N-acetyl-galactosamine, N-acetyl-glucosamine), and with a wide variation in structure (De Vuyst et al., 2001, Vaningelgem et al., 2004).

Over the last few years significant work has been done to relate production of exopolymeric substances by bacteria to biofilm formation, however, the participation of exopolysaccharides produced by LAB used as starters on biofilm formation in the dairy industry is still unknown. Since EPS is thought to be important for biofilm formation the hypothesis is that when EPS producing LAB are used in the manufacturing of cheese, some EPS will be released in the whey, and this will increase the risk of biofilm formation on the dairy separation membranes used for the processing of whey. There is a need for evidence towards whether bacterial exopolysaccharides produced by LAB have a role in cell adhesion and biofilms formed on dairy separation membranes using different substrate mediums for bacterial growth and to elucidate physical and chemical interactions between cells, and membrane solids. This type of characterization of the current EPS producing cultures is of interest for the dairy industry and has been a focus of discussion between dairy manufacturers and culture companies in the last decade.

5. Slime-producing endospore forming bacilli in the dairy industry

Bacteria from genus *Bacillus* spp. are Gram-positive rods that produce heat-resistant endospores and are among the most predominant species isolated from biofilms of commercial dairy plants (Sharma and Anand, 2002). Endospores are best verified by testing the culture survival after a temperature of 70-80°C for 10 minutes followed by

cultivation under suitable conditions (Holt, 1994). Spore forming bacteria are known to produce food spoilage. Their spores survive extreme heat and other stresses, such as radiation, desiccation and disinfectants, making them troublesome contaminants in the food industry challenging preservation of food. The majority of *Bacillus* species has little or no pathogenic potential and is rarely associated with diseases in humans or animals. Some exceptions are *Bacillus anthracis* (anthrax), *Bacillus cereus* (food poisoning due to various toxins produced and opportunistic infections), and *Bacillus thuringiensis* (pathogenic to invertebrates) (Vos, 2011).

Spores are formed by two membranes that surround the forespore, the inner and outer forespore membranes. These membranes derive from the mother cell membrane, and eventually, the inner membrane will constitute the cytoplasmic membrane once the spore germinates. The spore coats take up to 50 % of the spore volume, and are made of protein and some complex carbohydrates, lipid and phosphorous (Block, 2001). Not all strains will readily form spores when grown in a routine growth medium if stored in the incubator or on the laboratory bench, but most of them will sporulate on a routine or nutritionally weaker solid medium supplemented with 5mg/L manganese sulfate incubated for a few days. After most of the cells have sporulated the culture can be store refrigerated for many years (Vos, 2011).

Different exopolysaccharides have been reported in *B. subtilis* strains such as strain FT-3 in which are known to be comprised of glucose, galactose, fucose, glucuronic acid and O-acetyl groups in an approximate molar ratio of 2:2: 1:1:1.5 (Morita et al., 1979), although specific roles for these polysaccharides have not been elucidated. *Bacillus* capsules have been studied and its composition analyzed. *B. licheniformis* and *B.*

subtilis may synthesize a water-soluble viscous slime containing non polysaccharides but D and L-glutamic acid residues polymerized via amide linkages between the alpha-amino and gamma-carboxylic groups. This polyglutamic acid (PGA) is the main responsible of the characteristic texture of the traditional Japanese food “Itohiki-natto”, a dish prepared with fermented soybeans using *B. subtilis*, but it is also produced by the pathogen *B. anthracis*, and is particularly non-immunogenic contributing to virulence as help to escape host recognition (Hanby and Rydon, 1946). PGA can be thermal depolymerized above 200°C by an unzipping mechanism with generation of pyroglutamic acid (Portilla-Arias et al., 2007, Portilla, 2008). In *B. subtilis*, a robust pellicle in liquid culture and colonies on plates with web-like structures (i.e. bundled structures) has been described in EPS producing wild-strains (Branda et al., 2001).

Despite an extensive study of the EPS genes and their roles, the structure and function of the EPS produced by *Bacillus* resulting from the expression of these genes remain unexplored (Marvasi et al., 2010). After observing differences in the hydrophobicity of EPS producing LAB that affected their biofilm formation ability, we decided to pursue the study of biofilm formation by slime-producing sporeforming species which differ on their surface hydrophobicity properties (described in the Chapter 3 of this dissertation). One of the targeted organisms is a strongly hydrophobic slime-producing *Bacillus mojavensis* (strain Bc) which is phenotypically virtually indistinguishable from *Bacillus subtilis* and *Bacillus vallismortis*, and the practical distinction of this species are questionable (Vos, 2011). Perfect spherical water drops were observed over agar cultures of this strain in our lab, indicating contact angles as high as Teflon. Another slime-producing strain used in our work is *B. licheniformis*

(strain K1), which displayed hydrophilic contact angles, and is one of the most common spore forming species isolated from dairy powders in the Midwest and across the US (Buehner et al., 2014, Buehner et al., 2015).

6. Characterization of microbial exopolymeric substances (EPS)

Most lactic acid bacteria produce EPS consisting of complex carbohydrates of high molecular weight composed of polymers of monosaccharides with glycosidic bonds. The majority of polysaccharides from microbial origin are heteropolysaccharides composed of two to five different types of monosaccharides (Sutherland, 1990). There is a wide range of possible structures and differences in the properties of EPSs due to the many possible linkages and configurations. Each hexose can be alpha or beta-linked (in the pyranose or furanose form), and the links can be through the 2, 3, 4, or 6 positions. Most of the reported EPS are formed by two or three sugars and various acyl substituents (esters or amidas) (Dumitriu, 1998). Having this in mind, one can decide on what methodology is the most appropriate to quantify and elucidate the structure of EPS.

6.1 Methods of isolation and purification of bacterial EPS

Any characterization method requires a preceding isolation method. The amount of EPS detected depends on the method of extraction, but often, the analyst finds the problem of compromise between sufficient yield and adequate purity. Purification methods often require precipitation of the polymeric material with a solvent, and separation by centrifugation, dialysis against water to remove small molecules from the fermentation medium, and freeze-drying (Dumitriu, 1998). The separation of microbial cells from EPS in the viscous culture broth is a limiting step, which also depends on the degree of association of the EPS to the microbial cells (as slime or capsule). EPS (and

capsular EPS after a dissociation step) can be recovered by solvent precipitation (ethanol or acetone) of the culture supernatant. The precipitate is harvested by centrifugation, filtration, or settling. Proteins and salts of the medium are also precipitated along with the EPS, and therefore deproteinization with proteinase treatment (Xu, 2007) and dialysis with a molecular mass cutoff of 3,500 Da (Vaningelgem et al., 2004) may be needed for pure exopolysaccharides. This is of special importance for the recovery of EPS produced by dairy starters using milk or whey as the growth medium. These mediums are rich in casein and whey proteins that must be removed prior to EPS isolation, which involves culture dilution, digestion with proteolytic enzyme for 16-24h at 37-40°C at pH 7.5 in the presence of a growth inhibitor, followed heat treatment at 100°C to inactivate the enzyme (Dumitriu, 1998). The residual peptides are removed by precipitation with 10% Trichloroacetic acid (TCA) followed by centrifugation at 6,000-16,000 xg for 20-30 min at 4°C. Then the supernatant is concentrated by vacuum evaporation or ultrafiltration, and EPS is precipitated by adding 3-4 volumes of cold ethanol, storing overnight at 4°C, and centrifugation at 6,000-20,000 xg for 30 minutes at 4°C. Pellet is dissolved in distilled water and dialyzed for at least 24 h at 4°C to remove residual sugars and small molecules. This dialysate is then freeze-dried and stored at 4°C (Dumitriu, 1998). This method, although allows for the extraction of highly pure EPS, have nonetheless some important drawbacks. One of them is the need for TCA treatment, which will precipitate up to 50% of EPS along with proteins, reducing considerably the yield. The second is, that the dilution of the medium will increase the amount of solvent needed raising significantly the cost of extraction. When the EPS is suspected to be of protein nature, TCA precipitation and protein digestion are skipped.

6.2 Methods of analysis of bacterial EPS

Here we describe some of the recommended techniques to analyze and characterize pure EPS. The quantification of total EPS produced by a culture is usually achieved by extracting and purifying the EPS from the fermentation medium and determining the total carbohydrate and total protein content in the purified sample.

The structural characterization of exopolysaccharides is a very complex process, due to the diversity in sugar monomers, linkages, and unique structures present in the carbohydrate fraction (Flemming et al., 2007). A structural investigation of a polysaccharide often starts with sugar analysis. Not only is the identity of the carbohydrate components in a polysaccharide important for its interaction with other molecules in a given system but also its three-dimensional structure. Once the identities of the monosaccharide units have been revealed, next step is to determine their relative amounts, their connectivity, if the chain is branched etc. Unfortunately, there is no single method that will give complete information about monomer composition, sequence and linkage. Contrarily, a combination of complex physical methods (high resolution nuclear magnetic resonance, mass spectrometry, polarimetry, ultraviolet and infrared spectroscopy, X-Ray) and separation techniques (gel filtration and affinity chromatography, ion exchange, high performance liquid chromatography, gas chromatography, electrophoresis) are necessary, and the output can be time consuming and costly.

6.2.1 Analysis of total carbohydrate content and total protein content of EPS

To quantify EPS in a sample the total carbohydrate content is generally analyzed

by colorimetric techniques. The most utilized and probably most reliable method for determination of sugars and related substances is the Phenol–Sulfuric Acid method described by Dubois (1951).

Total protein can be determined by the universal method of Kjedahl to determine nitrogen content (FAO, 2003). Nitrogen content has to be multiplied by a factor to arrive at protein content, which depends on the sample nature. The Lowry method (Lowry et al., 1951) is best used for cell fractions with protein concentrations of 0.01–1.0 mg/mL, as the Bradford method (Bradford, 1976), but the later must be used with caution, especially in homopolyamino acids as the dye has poor affinity for some amino acids.

6.2.2 Monosaccharide analysis

A standard method used to analyze exopolysaccharides is gas chromatography in tandem with mass spectrometry (GC-MS), which will offer information about monosaccharide composition. Peaks from the gas chromatogram are assigned a tentative identity based on standards and these identities are confirmed with MS analysis (Pottier, 2012). For linkage analysis, the EPS is first permethylated and the methylated-polysaccharide is later hydrolyzed by treatment with 2 M TFA (120°C for 2 h) and the methylated monosaccharides converted to their corresponding methylated alditol acetates. The identity of the variously methylated alditol acetates is determined by GC-MS and by analysis of the individual fragmentation patterns observed in the MS. Because it is coupled with mass spectrometer, the molecular mass of the compounds that are separated with the GC chromatographer can be measured and therefore the compounds are identified. If appropriate standards are included, a quantification of the monomers can be achieved. One of the disadvantages of this method is that it requires a pre-treatment

consisting of digestion of the EPS sample with an acid at high temperatures to obtain single monomers that will be later separated. Also, volatility of sample is a requirement for GC analysis, which can be achieved by derivatization to modify the analyte's functionality in order to enable chromatographic separations and will render highly polar materials to be sufficiently volatile so that they can be eluted at reasonable temperatures without thermal decomposition or molecular re-arrangement. A suitable way to derivatize a variety of functional groups prior to GC analysis is through Trimethylsilyl (TMS) ethers. One reagent commonly used that reacts quickly and quantitatively with alcohols, acids, and amines is bis (trimethylsilyl) trifluoroacetamide (BSTFA), giving by-products that are volatile and therefore can be injected directly into the GC without extraction. Pyridine, a basic catalyst, is added with the aim of speeding the reaction with sterically hindered groups (Sessions, 2009).

Once the separate monosaccharide units have been assigned, the substitution pattern within the repeating unit is established using Nuclear Magnetic Resonance (NMR) (Jonsson, 2010). NMR is the most powerful and non-destructive tool for use in structural determination (Jacobsen, 2007). It can be applied to hydrated samples of polysaccharides that gives rise to conformational stabilization, which is reflected in spectra by narrowing and splitting of resonance lines, or dry samples with solid-state NMR spectroscopy that preserves the 3D structure of the analyzed polymer (Saito et al., 1989). One-Dimensional (1-D) experiments can offer quick insight into the presence or absence of carbohydrate material, presence of certain non-sugar moieties, chemical modifications to the polysaccharides, and alpha or beta anomeric configurations. For a more comprehensive picture of the structure of a complex carbohydrate, 2- dimensional

(2-D) NMR spectroscopy can be implemented. Samples are exchanged thrice with deuterium oxide (99.9% D₂O) in order to deuterate all free hydroxyl groups thus eliminating these signals from the resulting spectra. The exchange is performed by dissolving the polysaccharide sample (usually 1-3 mg) in a few drops of D₂O and freeze-drying. Once fully exchanged, the sample is dissolved in 590 μL of D₂O and ready for analysis. Trimethylsilyl-tetradepro sodium propionate (TSP) is used as an external standard for both proton and carbon experiments.

Nuclear magnetic resonance (NMR) gives information about the number and types of atoms in a molecule, for example, hydrogen atoms in ¹H-NMR, or carbon atoms using ¹³C-NMR, phosphorous atoms using ³¹P-NMR. NMR signals are in the form of peaks, which can be split into doublet, triplet, quartet, multiplet, etc, by the influence of neighboring nonequivalent hydrogens. NMR spectrum is of help in determining linkage of monosaccharides within the polysaccharide chain. ¹H, ¹H-NOESY NMR experiments, allows connecting protons close in space, and a ¹H, ¹³C-HMBC NMR experiment, shows long-range couplings over the glycosidic linkage (Jonsson, 2010). Polysaccharides are different from other biological polymers such as proteins, nucleic acids, glycoproteins and glycolipids because they contain repetitive structural features (Aspinall, 1983). As a result of these repetitive structures, NMR spectrums are extremely hard to interpret, since there are multiple signals that overlap in the same region of the spectrum. By comparing the chemical shifts of the carbons in the EPS with the respective carbons in the unique monosaccharides the glycosylation shifts are revealed. They are usually in the range of 2-10 ppm (region of spectra) and indicate the connection points (Jonsson, 2010). NMR presents a big challenge to EPS researchers as most polysaccharide NMR spectra suffer

from peak overlapping in the ring region (δ H 3.1-4.4) due to the similarity of the carbonyl groups that exist in the different monosaccharide molecules, and therefore the NMR spectra of EPS are extremely difficult to interpret. To overcome this, multiple experiments with 2D NMR techniques are often applied and the interpretation by an expert with advanced knowledge in stereochemistry is required.

6.2.3 Amino acid analysis

Not all EPS produced by microorganisms of interest in dairy biofilms are polysaccharides. For instance, *Bacillus* species have been reported to produce a variety of polymers such as polyamino acids, polylactic acid, polyhydroxyalcanoates (Sansinenea, 2012). Polyamino acids, which have been related to biofilm formation, are linear polymers of amino acids connected by peptide bonds. To analyze the composition of proteins and of polyamino acid polymers the amino acids are first separated and then detected and quantified. Amino acid (acid) hydrolysis using HCl is the basic method of choice to separate amino acids (Stein and Moore, 1950, Kambourova et al., 2001). One classical technique used was paper chromatography. Hydrophilic interaction liquid chromatography (HILIC) provides an effective method to separate amino acids over other chromatographic methods for its suitability to analyze compounds that always elute near the void, and offers good solubility in the aqueous mobile phase and it can be conveniently coupled to mass spectrometry (MS). MS is a more efficient (requires very small amounts of sample material) and accurate technology used in the amino acid analysis and is currently applied in protein sequencing (Bork, 2000, Rutherford and Gilani, 2009). Concentrations of amino acids are calculated using an external calibration curve and expressed in weight percentage.

7. Control strategies for biofilm formation of RO membranes

The spiral wound membrane elements are the most common membrane design used in dairy and other beverage industries due to their attractive low investment required, and to the fact that they allow the installation of multiple elements in a bundle in parallel or in series in a small floor space. This configuration however is very difficult to clean and prone to biofilm formation and once the flux is irreversibly compromised they require a replacement of the complete hardware of the membrane module (Wagner, 2001). The dairy industry continues to invest in efforts to develop improved methods to control biofilms in dairy filtration membranes, but in order to create a good disruptive technique, the elements that hold the microbial cells together need to be fully understood.

7.1. Cleaning-in-place procedures (CIP)

Membrane systems require periodical cleaning to ensure optimal operation, and the cleaning protocol has to be carefully designed to obtain maximum fouling removal with the minimum cost of chemicals and avoiding adverse effects on the life of the membrane surface and on the food product. The compact membrane units can be cleaned without having to dismantle them thanks to CIP procedures. The cleaning regime will vary considering the type of membrane (Tamime, 2008). RO membranes have the tightest pores (less than 0.002 μm) of all membrane processes in dairy filtration, and only water can pass through, while the rest of suspended materials are rejected in the retentate. The cleaning process is an important part of the production cost and involves time, energy, chemicals and water consumption (Anand et al., 2014). RO permeate can be used for flushing the product until the retentate appears clean, and even for rinsing and cleaning reducing cost of cleaning. A CIP procedure used to clean RO dairy membranes consists

of a minimum of five stages, which include an alkaline wash, an acid wash, a alkaline wash, an enzyme wash and a final antimicrobial or membrane preservative wash (Tamime, 2008). The membrane is rinsed with water in between stages with the objective of removing gross residual dairy products (proteins and fats) and biofilm debris. The actual composition of commercial cleaning solutions is unknown however it has been reported that favorable cleaning conditions of RO membranes include a metal chelating agent (EDTA) to help reduce calcium-induced foulant–foulant interactions (Ang et al., 2006) and the inclusion of surfactants to help dissolve food residues by emulsification of fats, reduction of the surface tension, and protein denaturation (Mosteller and Bishop, 1993, Maukonen et al., 2003). The effectiveness of cleaning and sanitation of the membrane system depends on temperature, mechanical design, turbulence, and nature of various chemicals. A combination of chemical, mechanical and enzymatic methods have been developed to detach the bacterial aggregates from the surface, however the efficacy of these methods relies basically on the power on the cleaning agents due to access restriction for mechanical cleaning of the compact membrane modules. Mechanical forces can be induced by a turbulent flow that can be achieved through modification of velocity of circulation of the fluids and pressure (Tamime, 2012). Due to the limited resistance of the organic polymeric materials, special consideration is needed when designing a cleaning protocol, as high temperature and corrosive chemicals considerably reduce the lifetime of the membranes. Use of adequate temperatures and time, flow and chemical strength are the basic features that operators can modify to improve cleaning efficiency. However, microorganism can always recolonize the membrane surfaces and sometimes plants run for longer periods before cleaning, which allows time to grow and

form stronger biofilms that are more resistant to cleaning agents.

7.2. Antimicrobial approach to inactivate the embedded cells

Antimicrobials or disinfecting agents are substances that control, prevent or destroy harmful microorganisms. A biocide (bactericide, virucide or sporicide) refers to a chemical agent that kills microorganism, as opposed to bacteriostatics, virostatics and sporostatics, which merely inhibit growth or prevent multiplication (Dvorak, 2005). Sanitizers are formulations that reduce the number of microbial contamination to levels considered safe for public health (Dvorak, 2005). Biofilms are difficult to eradicate, and require oxidative cleaners and sanitizing chemicals for their removal (Folsom and Frank, 2006). After many attempts to control biofilm development in different industries from medical to food industries, investigators have realized that the use of antimicrobials and biocides (which are very effective in planktonic cells) are not effective against biofilms given that only the exposed areas of surface-attached cells are susceptible to biocidal activity (Bower and Daeschel, 1999). This is why the application of an effective disinfectant at the appropriate level can only achieve a decrease in viable bacterial counts of biofilms that remain after the cleaning procedures have been applied (Spreer, 1998, Walstra et al., 2006). However it has been recognized that a periodic treatment with sanitizers should be sufficient to control early stages of biofilms, as more mature and well-established biofilms would be more difficult to remove (Anwar et al., 1992). A range of chemicals is known to produce bacterial injury and decrease viability of cells in biofilms. These biocides are classified generally in two major groups: oxidants and non-oxidants (Wiencek and Chapman, 1999). Some of the oxidizing agents commonly used in disinfection of membrane systems include sodium hypochlorite, hydrogen peroxide and

peracetic acid (Zeman and Zydney, 1996). The disadvantage of using these chemicals in organic polymeric membranes is that they reduce their life due to their corrosive action. In example, chlorine can remove the sensitive polyamide layer from polysulphone RO and NF (Tamime, 2008). However, non-oxidizing biocides such as quaternary ammonium compounds or formaldehyde are often less used due to the lower cost of the oxidizing agents such as chlorine.

7.3. Enzymatic degradation of the biofilm matrix

The use of chemical cleaning agents can have a big environmental impact, and food and dairy sectors are in need to find alternative solutions that are mostly organic and biodegradable. Enzymes have several advantages over chemicals including specificity, high efficiency and compatibility with the environment (Schäfer et al., 2005). Also, since they are not corrosive, they can be used in RO and NF membrane systems. The actual choice of enzymatic cleaners depends of the nature of the soiling matter to be removed (Tamime, 2008). The biofilm matrix is composed of a mixture of polymers that provide mechanical stability to the biofilm (Garrett et al., 2008). Therefore, the specific degradation of the main polymers that support the structure of biofilms should render microorganisms free, helping in removal of biofilms. Enzymatic cleaners have been used mostly to remove proteinaceous fouling in membranes and they consist of proteolytic enzymes (proteases) (Tamime, 2012). Proteases are a group of enzymes that hydrolyze the peptide bonds of proteins, releasing polypeptides or free amino acids (Casas-Godoy and Sandoval, 2015). Besides, typical enzymatic cleaners contain amylases used to cleave glucose linkages in large polysaccharide macromolecules releasing smaller soluble polysaccharides that can be easily removed (Zeman and Zydney, 1996). To make the use

of these enzymes economically feasible, most enzymatic cleaners are produced by microorganisms that are genetically modified to produce large quantities of the enzyme of interest. An effective enzymatic formulation for whey filtration membranes must be able to degrade the proteinaceous whey residue and the exopolymeric substances produced by starter cultures and spoiling bacteria. During this research work, several attempts have been done to isolate enzymes from natural sources (starter cultures, spoiling bacteria, and bacteriophages) with the objective of degrading specifically the EPS produced by microorganisms of importance in biofilms formed on whey processing membranes. Enzymes involved in polymer degradation such as hydrolases (and less frequently other enzymes such as lyases) are abundant in biofilms, producing low-molecular weight products that can be potential carbon and energy sources for bacteria within the biofilms (Wingender et al., 1999). The specificity of these enzymes makes them very attractive as biofilm removal agents.

Chapter 2

Role of exopolysaccharides produced by dairy starter cultures in formation of whey concentration membrane biofilms

ABSTRACT

EPS producing starters used in cheese making intensify biofouling and are thought to increase the risk of biofilm formation on whey separation membranes due to the cementing action of EPS. Two different EPS(+) starters and their isogenic EPS(-) variants were used to study attachment of bacterial cells in the absence of growth (at 4°C) and biofilm formation (at 37°C) on Reverse Osmosis (RO) membranes. M17 broth and a 10% solution of whey protein concentrate 35 were used as growth media for biofilm formation under static conditions. *Streptococcus thermophilus* ST3534 EPS(+) showed significantly greater counts (cfu/cm²) of ST3534 cells in biofilm than those of ST5842 EPS(-) (P<0.05) whilst counts of cells attached to the membrane in the absence of growth did not differ between the isogenic pair. Interestingly, *Lactococcus lactis* ssp. *cremoris* JFR(+) EPS(+) counts were significantly lower than those of its EPS- mutant (JFR-) under all conditions. These findings indicate that EPS produced by ST3534 may play a role building up the three dimensional structure of the biofilm rather than assisting in the attachment to the membrane at the initial steps of biofilm formation while EPS produced by JFR(+) interfere mainly with the initial attachment. While no differences were observed in cells surface charge between the EPS producing cultures, surface hydrophobicity could be associated to the different adhesive properties of these microorganisms. Furthermore, modification of cell surface hydrophobicity by surfactants had an impact on attachment and biofilm formation. In conclusion, EPS produced by

starter cultures vary in their role in bacterial attachment and biofilm formation due to variations in their molecular characteristics, and hydrophobicity is the leading feature that dictates the adhesive behavior to RO membranes.

INTRODUCTION

The EPS producing lactic acid bacteria (LAB) are relevant for the dairy industry and have been used as starters for the manufacturing of low fat products. The EPS modify the physical properties of fermented milk as they increase viscosity and water holding capacity, reduce syneresis, and interfere with protein-protein interactions. These properties together improve smoothness and mouthfeel (Hassan, 2008). Over the last several years significant work has been done to relate production of exopolymers by bacteria to biofilm formation, however, the participation of exopolysaccharides produced by LAB used as starters on biofilm formation in the dairy industry is still unknown. Whey, the liquid by-product of cheese making and casein manufacturing processes that is composed mainly of water (93g/100g), lactose and whey proteins, is frequently concentrated by reverse osmosis (RO) to reduce volumes and increase solids content prior to transportation or further processing. Biofouling, or the buildup of suspended particles is one of the critical issues in membrane filtration technology (Field, 2010), as it has a significantly detrimental effect on the efficiency of the separation processes causing flux decline, pressure drop, reduced membrane lifetime, and inconsistent product composition and quality. Biofilms play a major role in biofouling and contribute to product contamination with potentially pathogenic bacteria and possible threat to public health.

Biofilms are initiated when bacterial cells attach to a surface and they develop

into different stages of maturation as bacteria grow (Ridgway et al., 1999). The first phase of bacterial adhesion involves physicochemical interactions, after which molecular and cellular interactions take place (Vadillo-Rodriguez et al., 2005). Multiple factors influence the adhesion of cells to a substratum including environmental factors, surface characteristics of microorganisms and substratum (De Weger et al., 1987, An and Friedman, 1997, Palmer et al., 2007, Kline et al., 2009). There are evidences that show that microbial adhesion strongly depends on the hydrophobic-hydrophilic properties of the interacting surfaces (Liu et al., 2004). After the first attachment, a strengthening of the bonds occurs often involving the formation of polymer bridges between the organism and the surface. These sticky polymers form the matrix network, providing mechanical stability to the biofilm (Liu et al., 2004). Scientists have found that the extracellular polymeric substances in biofilms are composed by exopolysaccharides (EPS), glycoproteins, glycolipids and e-DNA (Flemming et al., 2007).

Since EPS is thought to be important for biofilm formation there is a concern that when EPS producing LAB are used as cheese starters in the manufacturing of cheese, some EPS will be released in the whey, and this will increase the risk of biofilm formation on the dairy separation membranes used for the processing of whey. We hypothesized that EPS production affects cell surface characteristics and the ability of bacteria to attach and form biofilm; therefore EPS producing LAB will form greater biofilm than their respective EPS(-) mutants, which are genetically identical except for the EPS phenotype. To test this hypothesis two EPS producing LAB with different rheological properties and their respective EPS(-) mutants were selected to study their adhesion behavior and biofilm forming abilities. The extracellular matrix has been

described as an essential part of biofilm formation by many different organisms, but there is a significant variety in the composition of exopolymeric substances produced by microorganisms (Vu et al., 2009). For that reason, two different ropy organisms were selected for this study. The objective of this research was to provide new insights on the role of bacterial exopolysaccharides produced by LAB on cell adhesion and biofilms formed on RO membranes using different substrate mediums for bacterial growth and to elucidate physical and chemical interactions between cells, and membrane solids. The ultimate goal of the findings of this project is to help developing biofilm-free membrane separation processing systems.

MATERIALS AND METHODS

Source of EPS producing bacteria and other cultures

Streptococcus thermophilus strain ST3534 [EPS(+)] was provided by Chr Hansen (Hoersholm, Denmark). *Lactococcus lactis ssp. cremoris* strain JFR(+) [EPS(+)] was isolated from a retail ropy buttermilk. *Bacillus* spp. strain 10/1 was isolated from a biofilm on a 10 month used dairy RO membrane from a local dairy plant. This strain was selected for being beta-hemolytic and showing low attachment and biofilm ability to RO membranes.

Selection of the EPS(-) mutants

The EPS(-) mutant *Streptococcus thermophilus* strain ST5842 [EPS(-)] was provided by Chr Hansen.

A spontaneous mutant *Lactococcus lactis ssp. cremoris* strain JFR(-) [EPS(-)] was isolated in our laboratory by serial passages in non-selective medium M17 (Bergman, 2011). Three replicate clones of strain JFR were inoculated into tubes with 10

mL of M17 broth and incubated to stationary phase at 30°C under static conditions. One loop full of the top layer of the liquid cultures was collected and aseptically transferred to another tube with 10 mL of fresh medium, which was incubated under the same conditions. This was repeated during a period of 10 days and the cultures were checked for colony morphology daily. Eventually, colonies of the same morphology but lacking ropiness, were observed and isolated. This mutant lost the ability to ferment lactose [Lac(-)].

The loss of plasmid of this strain was validated by plasmid profile comparison (Figure 1) between JFR(+) and JFR(-). Plasmid DNA was extracted using a rapid mini-prep procedure recommended for lactococci (O'Sullivan D and Klaenhammer, 1993), and digested with 1µl of Eco RI in 15 µl of total volume. Fifteen µl of DNA was loaded onto a 0.7% agarose gel and an electrophoresis was conducted for 2 h at 90 mV using a horizontal electrophoresis system (Bio-Rad, Hercules, CA). A MALDITOF analysis conducted at the Animal Disease Research and Diagnostic Laboratory of South Dakota State University (Brookings, SD) confirmed the strain identity. Culture samples from the EPS- culture were stored frozen (-80°C in 5% glycerol).

Growth curves were performed using M17 with 5% lactose [or with 2.5% glucose for JFR(-)] and no significant differences were observed between the EPS(+) and negative mutants. TEM images of EPS(+) and negative mutants were obtained as described below to detect presence of protruding filamentous structures in their surface that could potentially affect their adhesion properties, including capsule, fimbria or flagella.

Preparation of RO membranes for in vitro biofilm studies

Five pieces of a Reverse Osmosis (RO) membrane (Toray membranes, Poway, CA) were used in our experiments. This membrane was made of cross-linked aromatic polyamide, had a negative net charge and was hydrophilic. Each piece, consisting of 2 membrane squares (2 x 2 cm) glued together to expose the retentate side was pretreated by soaking in sterile distilled water for 5 days with daily water changes and then sanitized with hydrogen peroxide (0.5% solution of H₂O₂), rinsed in sterile distilled water three times and placed in a sterile petri dish.

Study of attachment and biofilm formation

Fifty mL of M17 with 5% lactose or 10% solution of WPC 35 were inoculated with 1 mL of the culture in late exponential phase containing 10⁸ cfu/mL of the test strain, and added to the petri dish containing the membrane pieces as described above, which was then incubated for 24 h under static conditions at 30 °C [(JFR(+)/JFR(-)] or 35 °C (ST3534/ST5842) for biofilm formation experiments. Fifty mL of M17 with 5% lactose containing 10⁸ cfu/mL of the test strain was incubated for 24 h under static conditions at 4 °C to study attachment in the absence of growth. M17 or WPC containing 2.5 % glucose were used as growth media for JFR(-) [EPS(-), Lac(-)]. No significant differences were found in the growth rate between JFR(+) and its genetic variant in all media used (not shown). For the quantification of viable bacteria attached to RO membranes, the membrane pieces were rinsed three times in distilled water to remove loosely attached cells (Hinsa-Leasure et al., 2013) and embedded cells were detached from membranes by stomaching at 230 rpm for 2 min. Counts of attached viable cells were determined by enumeration of microorganisms on M17 agar after incubation for 24 h at the respective growth temperatures.

Microscopic observations

Structures of the EPS(+)/EPS(-) cultures surface associated with attachment (pilli, fimbriae, flagella and capsule) were examined microscopically. In addition, biofilm structure of 3 days old and 1 month old membranes were observed.

Transmission Electron Microscopy (TEM) observation of bacterial cell surface structures. The EPS(+) and EPS(-) cultures were inoculated in separated sterile tubes with 10 mL of M17 broth and incubated overnight at their respective growth temperatures. Then 0.1 mL of each culture was suspended in 1 mL of fresh broth in separated sterile cryovials and sent overnight for analysis of cell surface by TEM to the University of Georgia (Athens, GA). Samples were prepared as follows. A drop containing the bacterial cells were placed on Formvar coated 100 mesh copper grids (Electron Microscopy Sciences, Hatfield PA) and left to settle for 5 min. The excess liquid and any unbound cells were wicked off with a #1 Whatman filter wedge. A drop of 1 % aqueous uranyl acetate was placed on each grid and grids were air dried before imaging on a FEI Tecnai20 TEM (FEI, Inc, Hillsboro OR) operating at 200 KeV and images were taken digitally with an Advanced Microscopy Techniques (AMT) camera (AMT, Woburn MA).

Confocal Laser Scanning Microscopy (CLSM) observation of bacterial capsules. Overnight individual cultures separately grown in M17 broth were suspended at 10 % (vol/vol) in reconstituted skim milk that was heat treated at 95°C for 15 min and one drop of each milk suspension was deposited in a glass slide and observed with an Olympus Fluoview FV300 CSLM (Olympus Corporation of the Americas, Center Valley PA) in its reflectance mode as previously described (Hassan et al., 1995). Strains with no capsules

are not visible in the milk suspensions since caseins and cells are reflective, but encapsulated cells are revealed by observing a black area around the cells due to the lack of light reflection of capsules.

Scanning Electron Microscopy (SEM) observations of biofilms on RO membranes.

Three days old, and one month old biofilms were developed on RO membrane pieces as described above, by inoculating individual cultures of ST3534, ST ST5842, JFR EPS(+) and EPS- mutants in M17 broth with 5 % lactose as the growth medium. In the case of JFR(-) [EPS(-) lac(-)] mutant, the lactose was substituted by 2.5 % glucose. Membrane pieces were incubated under static conditions at 36°C (ST cultures) or 30 °C (JFR cultures) in aerobiosis under static conditions. For SEM observations of membrane biofilms, each membrane piece with 3 days old or 1 month old biofilm was rinsed 3 times with water, and air dried at 22°C under laminar air flow for 24 h to minimize possible structural damage to the biofilm (Hassan et al., 2010). After dehydration, membranes were sputter coated with a 5 nm layer of gold using a CRC-150 sputtering system (Plasma Science, Inc. Lorton, VA). Specimens were observed using Hitachi scanning electron microscope model S-3400N (Hitachi Sci. Systems Ltd., Tokio, Japan) at 5 to 10 kv.

Cell surface hydrophobicity

Microbial adhesion to hydrocarbons (MATH) was performed to determine cell surface hydrophobicity (Rosenberg, 1984). One mL of xylene was added to 4 mL of cells of overnight cultures suspended in PBS, vortexed for 2 min and equilibrated at 37°C for 30 min. OD600 nm before and after treatment with xylene of a portion of the aqueous lower layer was determined using a Cary 50 Bio spectrophotometer (Varian, Palo alto,

CA). Hydrophobicity was calculated as follows: MATH values (%) = (OD600 before treatment - OD600 after treatment) x100 /OD600 before treatment.

Absolute cell surface hydrophobicity was determined by measuring contact angle on intact colonies grown on M17 agar plates by the sessile drop technique (Woodward, 1999). A lawn of cells was created by plating 1 mL of an overnight culture and incubating the plate for 12 h at the appropriate growth temperature. Then a cross section of 75 by 25 mm of the agar was cut with a scalpel and placed on a glass slide. A drop of 10 μ l of sterile distilled water was placed on the surface of the agar plate and an image was taken with a camera angle of 0 °C so that it looked exactly horizontally at the drop using a Canon EOS Rebel T1i camera with a EF-S 18-200mm f/3.5-5.6 IS Standard Zoom Lens (Tokio, Japon). The static contact angle was measured using ImageJ software by manual points selection. If the contact angle was lower than 90°, the cells surface was considered hydrophilic, if the contact angle is wider than 90°, the cells surface is named hydrophobic. Reported values are the averages of triplicate contact angle measurements.

Zeta-potential of bacterial cells

The four cultures were grown in M17 incubated at 35°C (ST cultures) or 30°C (JFR cultures). Media contained 0.5% lactose for ST3534 and ST5842, 5% lactose for JFR (+) and 2.5% glucose for JFR(-). Cells were washed in PBS twice. Last suspension was prepared right before the measurement. Surface charges at different ionic strengths were measured for cells obtained from overnight cultures in M17 (10^7 cfu/mL) suspended in 1, 5 or 10 mM solutions of PBS at pH 7.4 (Hassan and Frank, 2004). Eight hundred μ L of the cell suspension freshly prepared was pipetted into a disposable capillarity cell. Zeta potentials were measured at 25°C using Zetasizer Nano series instrument (Malvern

Instruments Ltd, Worcestershire, UK).

Influence of EPS producing cultures on biofilm formation by potential food pathogens

Biofilm formation by a β -hemolytic *Bacillus* spp. strain 10/1 (isolated from a used whey RO membrane) co-cultured with JFR(+) or JFR(-) was studied. Membranes pieces were incubated with sterilized milk containing 10^8 cfu/mL of each of *Bacillus* and JFR(+) or JFR(-) cultures at 30 °C for 24 h under shaking conditions. The pH was maintained between 6.5 and 6.7 by changing the milk medium; once pH dropped to 6.5, membrane pieces were rinsed in sterile distilled water and transferred to fresh medium. Membranes were rinsed in distilled water and stomached at 230 rpm for 2 min. Viable cell counts of JFR(+) and JFR(-) were determined in M17 agar incubated under anaerobic conditions while *Bacillus* spp. was counted on M17 containing Bromocresol green as a pH indicator (pK 4.6) under aerobic conditions (McDonald et al., 1987).

Effects of surfactants on cell attachment and biofilm formation

The study of effect of surfactants on cell adhesion was inspired by our observations of biofilm formation and cell surface hydrophobicity. It has been demonstrated that anionic surfactants are adsorbed to the surface of bacteria cells (Noda and Kanemasa, 1986) and can modify the cell surface tension parameters including Lifshitz-van der Waals ($\gamma(LW)$), electron-donor ($\gamma(-)$) and electron-acceptor ($\gamma(+)$) and thereby the bacterial cell hydrophobicity, depending on the surfactant type and concentration and the bacterial surface characteristics (Feng et al., 2013). Therefore, we studied the effect of two anionic surfactants, one Tween 20 that has high HLB and Span 80 with low HLB on cell adhesion of the two genetic pairs of LAB under different

temperature conditions: a) attachment in absence of growth, b) biofilm formation, and c) biofilm formation after pre-attachment. For attachment in absence of growth (a) cells were suspended in a conical tube containing 20 mL of cold M17 broth that was previously stored at 4°C with 5% lactose (or 2.5% glucose for JFR-) at a final concentration of 10^8 cells/mL. An excess of the surfactant (0.1 %) was added to the bacterial suspension and the tube was agitated by mechanical stirring in a vortex for 30 s and let stand for 1 min. Then the suspension was transferred to a sterile Petri dish containing 3 RO pretreated membrane pieces (2x2x2cm) and the plate was incubated for 24 h at 4°C in static conditions. For biofilm formation (b) cells were prepared in a similar manner and plates incubated at the right growth temperature for each culture. In addition, cells were allowed to form biofilm after a previous incubation at 4°C for pre-attachment to the membrane (c) in the presence of the surfactant so that cells form biofilm on a membrane that is covered with cells to evaluate whether the ability of EPS to help in biofilm or not, was due to a cell-membrane or cell-cell interaction. A control without surfactant was used in every experimental condition. The number of viable cells attached to the membranes was determined as described above.

Statistical analysis

All experiments were performed in three replicates. Statistical analysis was completed using computer program SAS 9.3 TS LEVEL 1M0 W32_7PRO platform (SAS Institute Inc., Cary, NC). The pair-wise comparisons between treatments using a Student's T-test were performed to examine whether biofilm formed by cheese starters was significantly different among EPS producing/non-EPS groups. ANOVA test was performed to determine significant differences between means. The significance level

was set at 5%.

RESULTS

Study of attachment and biofilm formation

The exopolysaccharide-producing strain of *S. thermophilus* (S3534) formed greater biofilm than its EPS(-) mutant. Contrarily, JFR(+) formed less biofilm than its mutant lacking EPS production (Table 1). The growth medium did not affect the adherence behaviors observed for the two isogenic pairs although the differences between each EPS(+) and EPS(-) strains within species were less dramatic in WPC compared with M17 broth. This could be explained by our previous observation that whey and WPC sterilized by autoclaving or filtration support less growth than broth media. When only attachment of bacterial cells to RO membranes was studied in absence of growth, no significant difference ($P<0.05$) was observed for *S. thermophilus* (Table 2), indicating that the EPS produced by this strain influenced cell-cell cohesion rather than cell-membrane adhesion. Interestingly, EPS produced by JFR(+) seem to interfere with attachment, as we observed a significantly greater number of cells of JFR(-) attached to the RO membrane (Table 2), what suggest that the EPS produced by this strain interferes with both, cell-surface adhesion and cell-cell cohesion.

Cell surface hydrophobicity and charge

Because the physicochemical characteristics of the bacterial cell surfaces are critical features that will determine cell adhesion to a particular surface, we studied the cell surface charge and hydrophobicity of the four lactic acid cultures and compared them with their attachment and biofilm behavior. All four strains were negatively charged and both EPS positive cultures had a greater negative charge compared with their EPS

negative genetic variants (Table 3), so no correlation was found with the different adhesion properties observed on RO membrane. Contact angle revealed the hydrophilic character of all the strains surfaces, however, different degrees of water affinity were observed between the LAB pairs (Table 3). JFR(+) is strongly hydrophilic (low adhesion to xylene) compared to its genetic variant, while ST3534 significantly less hydrophilic than its negative mutant ($P < 0.05$). MATH results were in agreement with contact angles showing greater adhesion to xylene for ST3534 and JFR(-) than their respective mutants, however JFR(-) showed a remarkable hydrophobic character by this technique compared with contact angle (Table 3). Also, cell aggregation or clumping could be observed visually in liquid M17 cultures of JFR(-) and ST3534 but not in JFR(+) nor ST5842 after biofilm formation assays on petri dishes (Figure 2), which was consistent with the hydrophobicity of the cells.

Cell hydrophobicity was associated with our biofilm findings and could explain the differences between the two EPS(+) cultures. The strong hydrophilicity characteristic of JFR(+) cultures clarifies why the EPS produced by JFR inhibits biofilm formation by interfering with attachment of the cells to the membrane. This strain has a stronger affinity for the liquid medium, and cells get washed off the membrane during the rinsing process, preventing the strengthening of the bonds with the membrane and between cells during biofilm formation.

Microscopic observations

The differences observed between genetic pairs were attributed to only EPS. No macroscopic attachment organelles were found on the cell wall of any of the four LAB studied by TEM (Figure 3). Capsule of EPS (cell-associated EPS) was present only on

both EPS positive cultures, and was absent in their respective mutants. In addition, no growth rate differences were found between genetic pairs (not shown).

The biofilm model used served to study the ability of EPS positive and EPS negative cells to attach to RO membranes and this was confirmed by SEM micrographs of three days old and one month old biofilms (Figures 4 and 5). After 3 days, the conditioned film formed by ST3534 consisted of a monolayer of cocci embedded within EPS, while the biofilm of its negative mutant was less abundant giving the larger uncovered membrane area. Biofilm formed by JFR(+) showed significantly larger uncovered membrane areas compared to its negative mutant in agreement with our previous results. After one month, the biofilm of ST 3534 was thicker and consisted of mountain and valleys structures that were not observed on its mutant. Such three-dimensional structures were not seen on any of the biofilms formed by JFR(+) or JFR(-) either, but both of these biofilms consisted of flat multilayers of cells leaving almost no uncovered membrane areas.

Influence of EPS producing cultures on biofilm formation by potential food pathogens

The EPS produced by JFR seem to interfere with *L. lactis* attachment and biofilm formation, and it also reduced the number of cells of *Bacillus* spp. 10/1 in the biofilm when both strains were co-cultured together compared with the consortia formed by *Bacillus* and JFR EPS negative mutant (Table 4). Absence of antimicrobial activity produced by JFR against *Bacillus* 10/1 (not presented here) was confirmed by agar spot assay (Mohankumar and Murugalatha, 2011).

Effects of surfactants on cell attachment and biofilm formation

Tables 5 and 6 show the outcome of the experiments of adhesion performed with surfactants. In general, it can be inferred that Span 80 significantly increased attachment and biofilm formation by the EPS(+) and EPS(-) LAB whilst Tween 20, decreased attachment and biofilm formation by both types of cultures. However, Span 80 decreased attachment of JFR(-) by 0.2 log cfu cm² and did not affect ST3534 ability to form biofilm even after attachment of preconditioned cells ($P < 0.05$). In the other hand, while Tween 20 was effective on reducing attachment on the four strains, and biofilm formation after attachment of preconditioned cells, a slight increase was observed during biofilm formation on both genetic pairs.

DISCUSSION

Evaluation of attachment and biofilm formation as affected by EPS production

Two opposite adhesive behaviors on RO membranes were found in EPS producing LAB. The EPS produced by ST3534 seem to help in adhesion and biofilm formation to RO membranes, but the EPS produced by JFR(+) seem to negatively interfere with both. JFR(+) produces free EPS that is highly hydrophilic and have high water binding capacity, what is a great feature in some non-fat dairy products, but the free EPS can cause undesirable slippery mouthfeel in some cheeses (Hassan, 2008). Molecular features including composition, molecular weight, charge and branching are likely to be responsible of the differences between EPS that supports or interferes with biofilm. The way the monosaccharides are linked contributes to the rigidity of the polymer and the viscosity and the beta-linkages have been associated to stiffer chains and hence a higher consistency of EPS solutions (Laws and Marshall, 2001). Also, bear acidic groups provide ability to interact electrostatically with cations and to enter into

hydrophobic interactions (Marshall et al., 1989). The characterization of EPS is very complex and often, the information available is incomplete. The current methodology used to determine monosaccharide composition of EPS involves extraction procedures that dramatically affect their properties, and therefore, this information has to be considered with caution. Future advances in the analytical techniques are necessary to determine the intact structure of EPS that will lead us to a better understanding of the structure/function relationship.

While the data of role of EPS produced by microorganisms on biofilms formation on RO membranes is scarce, some researchers have been able to associate production of EPS to strength of bacterial adhesion and biofilms on diverse surfaces (Quintero and Weiner, 1995, Langille et al., 2000, Michel et al., 2011).

Influence of EPS on biofilm formation by potential food pathogens

The interfering ability of the EPS produced by JFR(+) on its own cell adhesion and other microorganisms in consortia is a very interesting feature that, to our knowledge, is first time described for *Lactococcus*. Others have previously described antiadhesion interference bacterial polysaccharides some of which exhibited anti-biofilm activity against pathogenic strains (Valle et al., 2006, Kim et al., 2009), or even also disrupt established biofilm of some strains (Jiang et al., 2011). Furthermore, the treatment of abiotic surfaces with some polysaccharide reduced the initial adhesion and biofilm development of strains, such as *Escherichia coli* and *Pseudomonas fluorescens* (Abu Sayem et al., 2011). Some antiadherent bacterial EPS shows technological applications as anti-biofilm agent in food industry and medicine (Kavita et al., 2014). The dairy industry could benefit of using these antiadherent EPS producing starter cultures since the

reduction of biofilm adhesion and their suspension into bulk fluids renders the bacterial populations vulnerable to less toxic biocides (Garrett et al., 2008).

Role of hydrophobicity on cell attachment and biofilm formation by EPS producing bacteria

Carbohydrates are viewed as entirely polar molecules, however it has been demonstrated that hydrophobic and van der Waals interactions between carbohydrate and protein contribute to their mechanism of interaction (Quioco, 1988), which might facilitate lectins binding to sugar moieties in oligo and polysaccharides substrates (Sharon and Lis, 2002). Nonionic surfactants contain a hydrophobic alkyl group that can combine with hydrophobic sites, such as lipids and lipopolysaccharides on the bacterial cell surface, and a hydrophilic polyethylene oxide chain that has high affinity for water (Noda and Kanemasa, 1986). Bacterial surfaces differ on their degree of hydrophobicity among genera, species, and strains. Adsorption of surfactants with different HLB values is expected to modify the bacteria cell surface and its neighboring hydrophobic interactions with surfaces (Clarke, 2002), ultimately affecting adherence and biofilm formation. Modification of the hydrophobicity of the cell surface by Span 80 and Tween 20 affected cell surface hydrophobicity and consequently attachment and biofilm formation. The hydrophobicity and biofilms formed by the EPS(-) cells were in general more affected by surfactant modification than those in the EPS(+) strains possibly due to low affinity of the EPS for the surfactant molecules. Generally, the increase in cell surface hydrophobicity was associated with more intense biofilm formation confirming that hydrophobicity of EPS plays the major role in biofilm formation on RO membranes. The finding of this study also highlights the importance of bacterial cell-membrane

surface hydrophobic/hydrophilic interactions in biofilm formation. This information would be of great interest for membrane filtration manufacturers as it suggests possible control of biofouling of filtration membranes by manipulating the membrane surface chemistry. An antiadhesion approach has been the main focus of membrane designers. In this context, polymer blendings, inorganic additives, grafting and coating methods have been developed to reduce macroscale roughness, preventing or slowing adsorption and microbial attachment. In a recent study on a number of commercial low-fouling membranes, increased hydrophilicity, lower surface charge and greater smoothness were associated with better antifouling performance (Norberg et al., 2007). However, another study found that bacterial adhesion to a substratum is not so much influenced by its hydrophobicity, but by the retention of adhering bacteria under fluctuating shear forces (Bos et al., 2000). The preference of bacteria to hydrophobic or hydrophilic surfaces has been debated. Opposite conclusions have been made over the past decades, and part of the confusion could be attributed to the use of inappropriate experimental systems to study bacterial adhesion, the variety of surfaces studied and the wide range of cell surface hydrophobicity among strains. A reference guide to microbial cell surface hydrophobicity based on contact angles with four diagnostic liquids (water, formamide, methyleneiodide and bromonaphthalene) on 142 isolates of Gram-positive and Gram-negative bacteria concluded that bacterial surfaces can be as hydrophilic as glass or as hydrophobic as wax, and that this diversity in the bacterial world makes it impossible to generalize (van der Mei et al., 1998).

In addition, an antimicrobial approach can be used to attack, disperse or suppress the activity of attached microorganisms. Biocidal agents or effects to kill the organisms,

and the suppression of biofilm formation by disrupting bacterial communication or EPS production are some of the research areas that offer opportunities for future implementation in membrane systems from the perspectives of water and wastewater treatments applications (Mansouri et al., 2010). These areas have potential for dairy industry, but need further investigation, and since harsh environmental conditions like presence of sub-lethal concentrations of antimicrobials actually promote exopolysaccharide synthesis (Wingender et al., 1999) a future research direction should be focused on the isolation and effective use of biological enzymes towards the hydrolysis of bacterial EPS that supports cell-cell adhesion to disrupt the biofilm structure in dairy separation membranes with the aim of facilitating the removal of foulants by the CIP protocols.

CONCLUSIONS

In conclusion, EPS produced by LAB may enhance biofilm or interfere with attachment and biofilm formation depending on their molecular composition. ST3534 produces EPS that supported biofilm formation while EPS produced by JFR(+) interfered with attachment and biofilm formation. Growth medium did not affect tendency of the test strains to form biofilm. Similarly, bacterial cell surface charge does not seem to affect attachment or biofilm formation on RO membrane. On the other hand, in general, high cell surface hydrophobicity was associated with greater biofilm formation on RO membranes.

The EPS produced by JFR(+) in milk under pH control reduced colonization of RO membrane by a β -hemolytic *Bacillus* strain but JFR(+) did not show any antimicrobial activity against *Bacillus* and shows potential in technological applications

as anti-biofilm agent.

Chapter 2 Tables and Figures.

Table 1. Counts (\log_{10} cfu/cm²) of EPS(+) and EPS(-) cultures in biofilm formed on RO membranes¹.

Growth medium	Type of culture ²	<i>S. thermophilus</i>		<i>L. lactis ssp cremoris</i>	
		Count ¹	Std Dev ³	Count ¹	Std Dev ³
M17	EPS(+)	6.46 ^a	0.13	4.55 ^b	0.29
	EPS(-)	5.50 ^b	0.33	6.02 ^a	0.44
		<i>S. thermophilus</i>		<i>L. lactis ssp cremoris</i>	
		Count ¹	Std Dev ³	Count ¹	Std Dev ³
WPC	EPS(+)	5.96 ^a	0.25	4.33 ^a	0.32
	EPS(-)	5.22 ^b	0.30	5.67 ^b	0.60

¹ Counts (\log_{10} cfu/cm²) are means of three replicates.

^{a-b} Means in the same column without common superscripts are significantly different ($P \leq 0.05$).

² EPS(+): exopolysaccharides-producing culture; EPS(-): exopolysaccharides-negative mutant.

³ Std Dev= Standard deviation.

Table 2. Counts (\log_{10} cfu/cm²) of EPS(+) and EPS(-) cultures² attached to RO membranes in the absence of growth.

Type of culture ²	<i>S. thermophilus</i>		<i>L. lactis ssp cremoris</i>	
	Count ¹	Std Dev ³	Count ¹	Std Dev ³
EPS(+)	5.92 ^a	0.60	4.78 ^b	0.21
EPS(-)	6.12 ^a	0.50	6.16 ^a	0.22

¹ Data are means of three replicates.

a-b Means in the same row (Tables 1-3) or column (Table 4-5) without common superscripts are significantly different ($P \leq 0.05$).

² EPS(+): exopolysaccharides-producing culture; EPS(-): exopolysaccharides-negative mutant.

³ Std Dev= Standard deviation.

Table 3. Hydrophobicity as determined by MATH (xylene) and Zeta-potential (mV) under different ionic strengths of cell surface of EPS(+) and EPS(-) cultures.

Strain	Contact angle	% cells bound to xylene ¹		Zeta-potential at different PBS concentrations ¹		
		Biof. cond. ²	Attach. cond. ²	1 mM	5 mM	10 mM
		ST3534	34.59 ^a	48.61 ^a	55.89 ^a	-19.03 ^a
ST5842	14.30 ^c	10.41 ^b	18.98 ^b	-6.52 ^c	-4.83 ^c	-4.05 ^c
JFR(+)	12.84 ^c	3.47 ^b	0 ^b	-18.33 ^a	-12.6 ^a	-9.74 ^a
JFR(-)	24.12 ^b	76.55 ^a	84.63 ^a	-10.31 ^b	-7.96 ^b	-5.72 ^b

¹ Data are means of three replicates.

^{a-c} Means in the same column without common superscripts are significantly different ($P \leq 0.05$).

² MATH was measured under same conditions used in biofilm formation and attachment experiments.

Table 4. Counts (\log_{10} cfu/cm²) of JFR(+) and *Bacillus spp.* (grown on milk under pH control) in biofilm formed on RO membranes¹.

Biofilm consortia	Organism⁴	Count¹	Std Dev³
JFR(+)	JFR(+)	3.79 ^{cd}	0.18
JFR(-)	JFR(-)	5.40 ^a	0.24
<i>Bacillus</i>	<i>Bacillus</i>	4.41 ^b	0.05
<i>Bacillus</i> + JFR(+)	<i>Bacillus</i>	3.41 ^e	0.18
	JFR(+)	3.59 ^{de}	0.25
<i>Bacillus</i> + JFR(-)	<i>Bacillus</i>	3.93 ^c	0.06
	JFR(-)	5.32 ^a	0.27

¹ Counts (\log_{10} cfu/cm²) are means of three replicates.

^{a-e} Means in the same column without common superscripts are significantly different ($P \leq 0.05$).

² Std Dev= Standard deviation.

³ Organism within the consortia.

Table 5. Counts (\log_{10} cfu/cm²) of EPS(+) cultures after attachment (a), biofilm formation (b) or attachment followed by biofilm (a) on RO membranes in the presence of surfactants¹.

	a.		b.		c.	
	ATTACHMENT		BIOFILM		ATTACHMENT+BIOFILM	
	ST3534	JRF(+)	ST3534	JRF(+)	ST3534	JRF(+)
Control	5.99 ^d	4.80 ^f	6.57 ^c	4.58 ^f	7.40 ^a	6.69 ^{bc}
Tween 20	5.37 ^e	4.74 ^f	7.05 ^{ab}	5.25 ^e	6.47 ^c	6.67 ^{bc}
Span 80	6.62 ^{bc}	5.63 ^{de}	6.61 ^{bc}	6.76 ^{bc}	7.47 ^a	6.72 ^{bc}

¹ Data are means of three replicates.

^{A-F} Means without common superscripts are significantly different ($P \leq 0.05$).

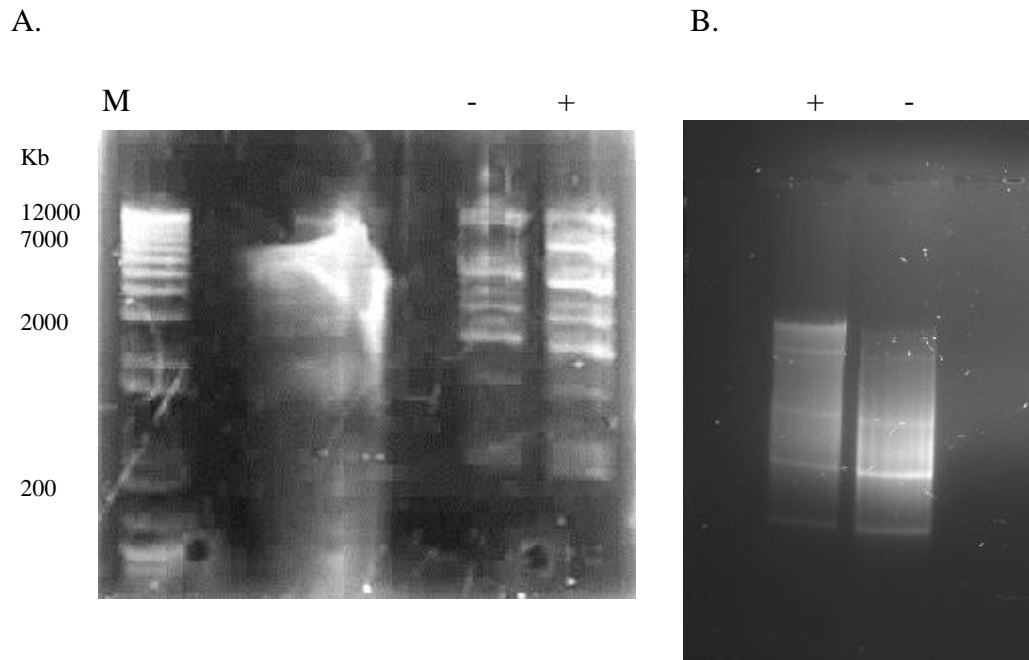
Table 6. Counts (\log_{10} cfu/cm²) of EPS(-) cultures after attachment (a), biofilm formation (b) or attachment followed by biofilm (c) on RO membranes in the presence of surfactants.

	a.		b.		c.	
	ATTACHMENT		BIOFILM		ATTACHMENT+BIOFILM	
	ST5842	JRF(-)	ST5842	JRF(-)	ST5842	JRF(-)
Control	6.34 ^{ef}	6.17 ^{ef}	5.23 ⁱ	6.04 ^{fh}	7.12 ^c	7.70 ^{ab}
Tween 20	5.22 ⁱ	5.65 ^{hi}	6.94 ^c	7.05 ^c	6.61 ^{hi}	7.13 ^c
Span 80	6.51 ^{de}	5.81 ^{gh}	6.93 ^{cd}	7.28 ^{bc}	8.03 ^a	8.13 ^a

¹ Data are means of three replicates.

^{A-I} Means without common superscripts are significantly different ($P \leq 0.05$).

Figure 1. Plasmid DNA comparison of JFR(+) and JFR(-).



A. Plasmid DNA digested with Eco R1:

- : EPS – mutant (missing 7 kb band plus small bands around 800-1000 bp)

+ : JFR wild type

M: 1 kb Plus DNA Marker (Invitrogen)

B. Undigested plasmid DNA.

- : EPS – mutant (missing large band at the top of the gel 800-1000 bp)

+ : JFR wild type

Figure 2. Liquid M17 cultures of ST3534 (A), ST5842 (B), JFR(+) (C) and JFR(-) (D) in petri dishes grown overnight showing cell aggregation in ST3534 and JFR(+), but not on ST5842 and JFR(-).

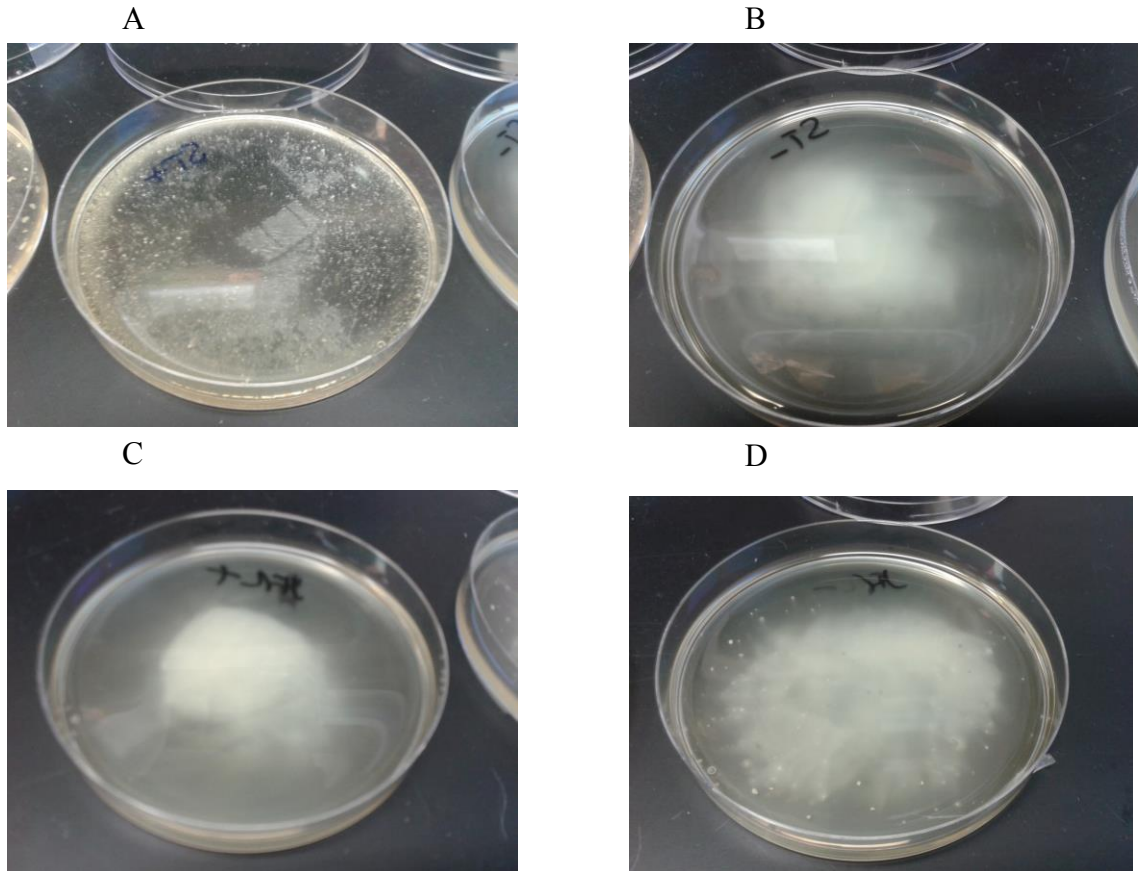
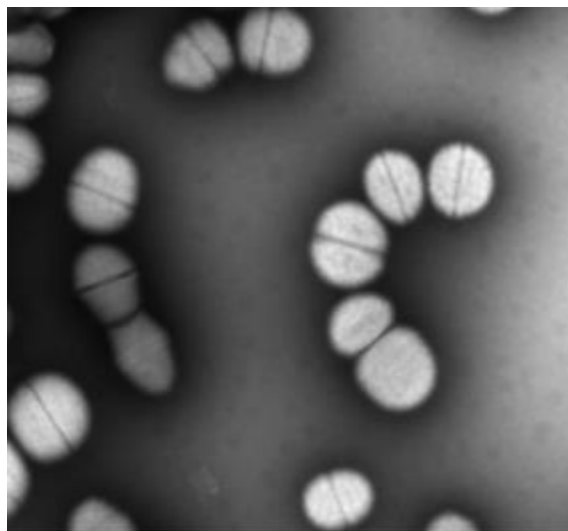
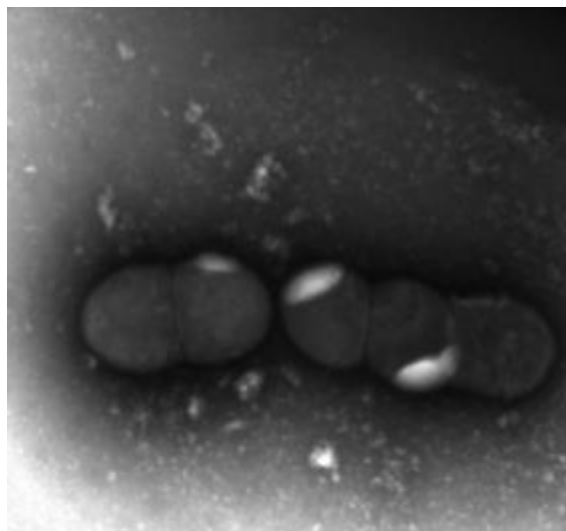


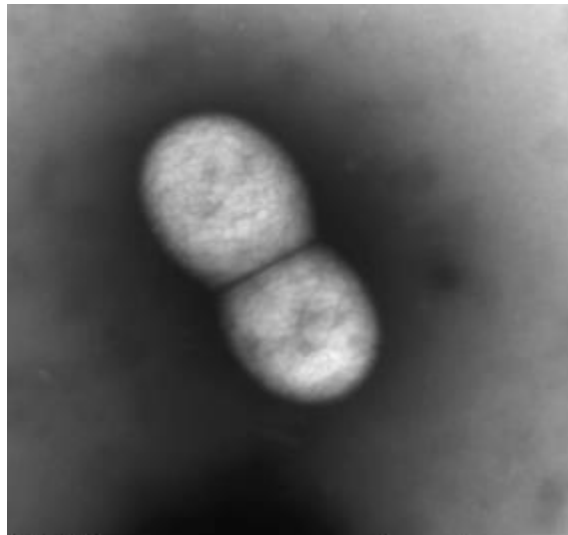
Figure 3. TEM micrographs of negatively stained ST3534 (top left), ST5842 (top right), JFR(+) (bottom left), JFR(-) (bottom right) showing lack of fimbria, pilli and flagella on both genetic variants.



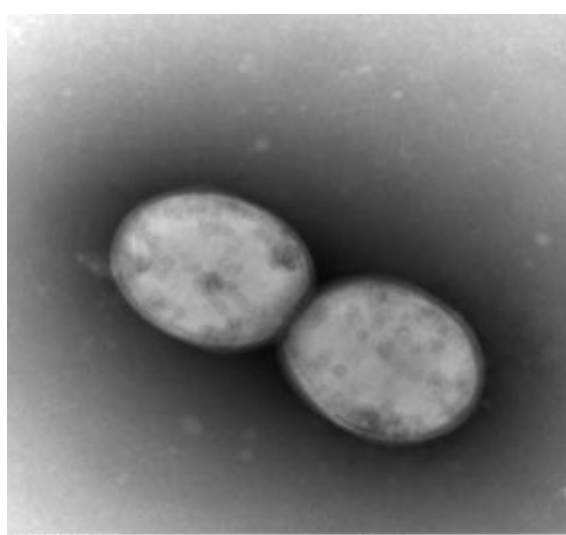
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ST- 42-1.tif
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JFR- unfixed unstained
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JFR- unfixed unstained
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Figure 4. SEM micrographs of 3 days old biofilm of ST3534 (A), ST5842 (B), JFR(+) (C), JFR(-) (D) on RO membranes.

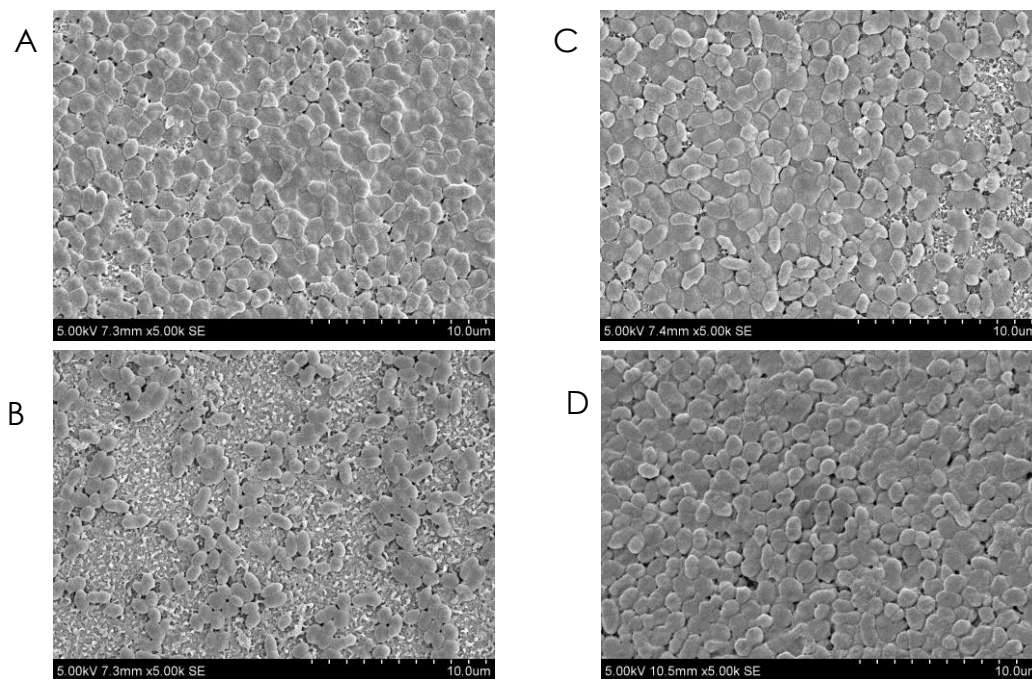
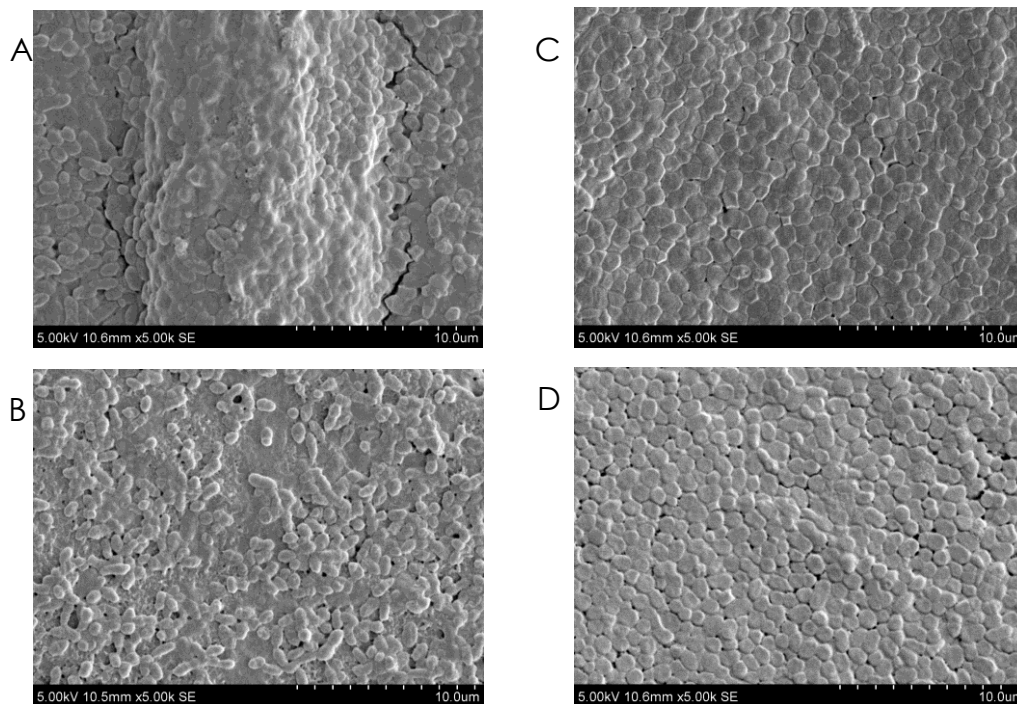


Figure 5. SEM micrographs of 1 month old biofilm of ST3534 (A), ST5842 (B), JFR(+) (C), JFR(-) (D) on RO membranes.



Chapter 3

Influence of slime produced by *Bacillus* spp. on membrane biofilm formation

ABSTRACT

Bacteria producing exopolymeric substances are thought to have an advantage in biofilm formation due to the role of slime in attachment of bacteria cells to a surface and in maintaining the three dimensional complex structure of the biofilms. To elucidate the role of slime production by non-starters on biofilm formation on RO membranes, the number of attached cells in biofilms formed by two different slime-producing *Bacillus* strains (Bc and K1) and their non-slime producing mutants (Bc-1 and K1-1) were compared. Biofilms were formed on three membrane pieces during 24 h using sterile TSB as growth medium. The number of attached cells were determined by viable cell enumeration. In addition, the number of cells that can attach to the membranes in the absence of growth was determined after incubating cells at 4°C during 24 h. Biofilms were formed on RO square pieces glued together in pairs exposing only retentate side. Contact angle of lawn of cells formed on TSB agar plates revealed major differences on the hydrophobicity of both organisms with an extreme water repulsion ability of Bc slime and a clearly hydrophilic character of the slime produced by K1. Both slimes resulted to be composed mainly by poly glutamic acid (PGA) although K1 contained a greater carbohydrate content than Bc, suggesting production of polysaccharides in addition to PGA. As reported in our previous work on biofilms formed by EPS producing LAB, hydrophobicity seemed to be clearly correlated with attachment and biofilm formation on RO membranes. Bacteria cell surface hydrophobicity was modified with surfactants and biofilm formation was again studied. Understanding the physic-chemical properties of the

bacterial EPS that supports biofilm formation on RO membranes will help to develop new strategies to reduce this problem, such as specific chemical formulations directed towards the most tenacious slimes. The observations from this investigation throw light on the adhesive characteristics of slime produced by *Bacillus* slime on dairy separation membranes and suggest that coating methods that intensifying membrane hydrophilicity and proteolytic treatments towards hydrophobic slimes are key features to focus on.

INTRODUCTION

In the processing of cheese whey, the modern dairy industry utilizes membrane separation technologies (microfiltration or MF, ultrafiltration or UF, nanofiltration or UF and reverse osmosis or RO) to separate and concentrate its valuable components, yielding a wide range of products (whey powder, whey protein concentrate, whey protein isolate, whey protein hydrolysate, lactose, etc). The performance of these filtration processes is compromised by fouling, or the deposition of dissolved solutes, particulates and colloids, that lead to a concentration polarization and cause a decline in flux and quality. One of the most common membrane configurations due to its compact arrangement is the spiral wound membrane module consisting of concentric layers of double membranes made of organic polymers (such as polyamide or polysulfone) wrapped around a tube. The combination of unavoidable fouling, long operation cycles and difficulty to clean these modules lead to the growth of biofilms in and on membrane surfaces. Biofilms are communities of bacteria associated with surfaces consisting of vegetative cells and spores (Semenyuk et al., 2014), which contribute to membrane biofouling, and increase risk of product contamination. Thermotolerant bacilli possess a major challenge for the dairy industry, due to their resistance to heat and cleaning agents. Some of the most persistent

strains that are isolated from fluid milk and dairy powders produce slime and pellicles at the air-liquid interphase, and their biofilms are very resistant to cleaning agents.

Production of exo-polymeric substances (EPS) is considered a pre-requisite for biofilm formation (Stoodley et al., 2002, Kolter and Greenberg, 2006), and it is commonly believed that slime-producing bacteria have an advantage over non-slime producing bacteria in terms of their adhesive characteristics and biofilm ability.

Our previous research (Chapter 2) showed that the hydrophobicity of the extracellular polysaccharides produced by lactic acid bacteria plays an important role in biofilm formation on dairy separation membranes, and reported a strain of *Lactococcus lactis* ssp. *cremoris* that produces high amounts of EPS (which is strongly hydrophilic) with anti-adherent properties that does not supports biofilm. In order to control biofilms in the dairy industry, information about specific composition of biopolymeric substances within the biofilm matrix is needed. But even more necessary is determining the slime composition and its biofilm function relationship, which would help in the design of chemical/biological formulations to disrupt the biofilm structure of these problematic microorganisms more effectively. Our hypothesis is that the surface hydrophobicity of slime producing *Bacillus* strains is associated with enhanced biofilm formation ability.

The objective of this work was to study the effect of slime production by *Bacillus* spp. on biofilm formation on separation membranes. Parameters related to bacterial adhesion (cell charge, capsule production, and hydrophobicity) were assessed to establish their contribution to differences in biofilm formation among strains.

MATERIALS AND METHODS

Source of bacterial cultures

Two slime-producing strains isolated from dairy powder kindly provided by Dr. Sanjeev Anand (South Dakota State University) were used to study biofilm formation (*Bacillus mojavensis* Bc and *Bacillus licheniformis* K1). One non-slime producing variant from each of the two strains (K1-1 and Bc-1 respectively) was used as a control. The mutants were produced by spontaneous mutation after successive passages in Tryptic soy broth (TSB), and selected for the lack of slime production in their colonies on agar. Mutant identity was confirmed by sequencing their ribosomal RNA genes at University of Cornell (NY). Production of slime and pellicles was observed only for Bc and K1 on agar and liquid medium.

Selection of slime-negative mutants

Three replicate clones of strain Bc and of strain K1 were inoculated into flasks with 10 mL of TSB liquid and were grown to stationary phase (37°C under static conditions). Fifty microliter were pipetted from the top layer of the liquid cultures and transferred to another tube with 10 mL of fresh medium and incubated under the same conditions. This was repeated during a period of 10 days and the cultures were checked for colony morphology daily. Eventually, colonies of the same morphology but lacking slime production were observed, isolated and their identity was confirmed by sequencing the 16S RNA genes at the Department of Food Science, Cornell University (Ithaca, NY). Culture samples from each line were stored frozen (-80°C in 5% glycerol). Growth curves were performed using TSB and no significant differences were observed between the slime-positive and negative mutants. TEM images of slime-positive and negative mutants were studied to detect structural differences in their surface that could potentially affect their adhesion properties, such as social motility or adventurous motility structures.

Attachment and biofilm development on RO membranes

Twenty mL of TSB containing 10^7 cfu/mL of the test strain were added to a petri dish containing 3 Reverse Osmosis (RO) membrane pieces (Toray membranes, Poway, CA) per treatment. Each membrane piece consisted of 2 membrane squares (2 x 2 cm) that were glued together to expose the retentate side. This method allowed us to study adherence to only the retentate side (Garcia et al, pending publication). Plates were incubated under static conditions at 37°C (24 h, 72h or 1 month, with daily changes of medium) for biofilm formation experiments, or at 4°C to study attachment in the absence of growth. Each treatment was repeated three times.

Evaluation of biofilm formation

Biofilms were evaluated by using both culturing techniques and microscopic observations. Membranes were rinsed for 30 s using three sterile beakers with 100 mL sterile distilled water to remove loosely attached cells and embedded cells were detached from membranes by stomaching at 230 rpm for 2 min. Counts of attached viable cells were determined by enumeration of microorganisms on TSA for 24 h at 37°C . Scanning Electron Microscopy (SEM) was used to observe three-day-old and one-month-old biofilms. Every 24 h membranes were taken using forceps and rinsed as described above to remove loosely attached cells and transferred to a sterile petri dish containing fresh medium. Each membrane piece with three-day-old and one-month-old biofilms was air dried at 22°C under laminar airflow for 24 h (Hassan et al., 2010). Membranes were then sputter coated with a 5 nm layer of gold using a CRC-150 sputtering system (Plasma Science, Inc. Lorton, VA). Specimens were observed using a scanning electron microscope model S-3400N (Hitachi Sci. Systems Ltd., Tokyo, Japan).

Study of the role of slime production on biofilm formation

To confirm the sole role of slime on biofilm differences between slime-positive and slime-negative pairs, factors affecting bacterial adhesion such as surface charge, hydrophobicity and presence of surface structures of the cell surface that are associated with attachment (pilli, fimbriae, flagella and capsule) were studied.

Bacterial surface charge at different ionic strengths were measured for cells (10^7 CFU/mL) suspended in 1, 5 or 10 mM solutions of PBS at pH 7.4 (Hassan and Frank, 2004). Zeta potentials were measured using Zetasizer Nano series instrument (Malvern Instruments Ltd, Worcestershire, UK).

Absolute cell surface hydrophobicity was determined by measuring contact angle on intact colonies grown on M17 agar plates by the sessile drop technique (Woodward, 1999). A lawn of cells was created by plating 1 mL of an overnight culture on the surface of the agar plate incubated for 12 h at the appropriate growth temperature. A cross section of 75 by 25 mm of the agar was cut with a scalpel and placed on a glass slide. A calibrated micropipette was used to deliver a drop of 10 μ l of sterile distilled water on the surface of the lawn of cells and an image was taken with a camera elevation angle of 0° with respect to the surface where the drop sits so that it looked exactly horizontally at the drop. The static contact angle was measured using ImageJ software (National Institutes of Health, Bethesda, MD) by manual point selection. If the contact angle was lower than 90° , the cells surface was considered hydrophilic, if the contact angle is wider than 90° , the cells surface was named hydrophobic. At least three images were taken on each cell layer preparation and the preparations and measurements were repeated three times.

The cell surface of each strain was examined microscopically by Transmission

Electron Microscopy (TEM) at the University of Georgia (Athens, GA). Twenty microliters of each culture grown on TSB overnight was pipetted and transferred to a sterile vial with 1 mL of fresh medium. A drop of the suspended cells was placed on Formvar coated 100 mesh copper grids (Electron Microscopy Sciences, Hatfield PA) and left to settle for 5 min. The excess liquid and any unbound cells were wicked off with a #1 Whatman filter wedge. A drop of 1% aqueous uranyl acetate was placed on each grid and grids were air dried before imaging on a FEI Tecnai20 TEM (FEI, Inc, Hillsboro OR) operating at 200KeV and images were taken digitally with an AMT camera (AMT, Woburn MA).

Modification of cell surface hydrophobicity by surfactants

Non-toxic nonionic surfactants were shown to modify bacterial cell surface thermodynamic properties (contact angle and surface free energy) and potentially influence the bacterium/surface interaction (Feng et al., 2013). Two nonionic surfactants, Tween 20 and Span 80, with different hydrophilic lipophilic balance (HLB) values were applied to cells to modify surface hydrophobicity as previously described (Chapter 2). Attachment and biofilm formation of the altered cells was then studied. Cells were suspended in a conical tube containing 20 mL of cold TSB broth that was previously stored at 4°C at a final concentration of 10^7 cells/mL. An excess of the surfactant (0.1 %) was added to the bacterial suspension and the tube was agitated by mechanical stirring in a vortex for 30 s and let stand for 1 min. Then the suspension was transferred to a sterile Petri dish containing 3 RO pretreated membrane pieces, each membrane consisting of two square membrane (2x2 cm) pieces glued together exposing the retentate and the plate was incubated for 24 h in static conditions at 4°C (for attachment in absence of growth)

or at 37°C (for biofilm formation). A control without surfactant was used in every experimental condition. The number of viable cells attached to the membranes was determined as described above.

Characterization of the slime of *Bacillus* strains

The slime of Bc and K1 was isolated from the supernatant of overnight cultures in TSB, and purified by ethanol precipitation, the pellet was washed three times with ethanol, dialyzed against water for 72 h at 4°C, and freeze-dried. Pellicles on the air-liquid interphase were collected and purified separately using the same method. The amount of total carbohydrates of the slime was determined by the phenol-sulfuric method (Dubois et al., 1951) and the amount of Total Kjeldahl Nitrogen was determined (AOAC, 2000). A glycosyl composition analysis was performed with Gas Chromatography-Mass Spectrometry (GC-MS) of TMS derivatives of methyl glycosides (TMS) at the Complex Carbohydrate Research Center (University of Georgia, GA). Samples were prepared as previously described by others (York et al., 1985, Merkle and Poppe, 1994). Briefly, a small amount of sample (0.2 mg) mixed with Inositol (20 µg) as internal standard, was hydrolyzed in 2M trifluoroacetic acid (TFA) at 120°C for 1 h. Methyl glycosides were then prepared from the dry hydrolyzed sample by methanolysis in 1M HCl in methanol at 80°C (16 h or overnight). The methyl glycosides were re-N-acetylated with pyridine and acetic anhydride in methanol (for detection of amino sugars) at room temperature for minimum 30 min. The sample was then per-O-trimethylsilylated by treatment with Tri-Sil (high purity pyridine) at 80°C (0.5 h). GC/MS analysis of the TMS methyl glycosides was performed using a Supelco EC-1 fused silica capillary column (30m × 0.25 mm ID). Glycosil composition of the sample and the molar percentages of each residue were

elucidated from the spectra. Values were expressed as mole percent of total carbohydrate. Total carbohydrate content was calculated using the Phenol–Sulfuric Acid method (Dubois et al., 1951).

Amino acid analysis of slimes and pellicles was carried out by HILIC direct method at the Redox Center (University of Nebraska, NE). The freeze-dried slime samples were suspended into 1.0 mL of 6N HCl and digested at 95°C overnight (Kambourova et al., 2001). Next day samples were cooled, methanol was added and the samples were evaporated using a stream of dry nitrogen. The final volume was adjusted to 1.0 mL with MeOH. An aliquot of 5 µL was injected into a BEH amide column (Waters, Parsippany, NJ) and a method for polar metabolites was used to quantify amino acids with the mobile phase starting from 85% ACN and 15% 20 mM NH₄OAc + 20 mM NH₄OH to 5% ACN and 95 % 20mM NH₄OAc + 20 mM NH₄OH. The transitions were monitored by MRM –MS. Concentrations were calculated using an external calibration curve and expressed in weight percentage.

The hydrophobicity of the amino acid content was studied using the hydrophobicity index, a measure of the relative hydrophobicity of the amino acids at pH 7. This index indicates how soluble an amino acid is in water at a certain pH. In a protein in solution, hydrophobic amino acids are likely to be located in the interior, whereas hydrophilic amino acids are likely to be exposed to the aqueous environment. Values used were normalized as per the method recommended by Monera et al., (Monera et al., 1995).

In addition the hydrophobicity of the slime pellicles was examined visually after gently lifting them and transferring into a beaker containing only water.

Statistical analysis

ANOVA test was performed to determine significant differences between treatment group means of three replicates at 5% of significance using SAS 9.3 (SAS Institute Inc., Cary, NC).

RESULTS

Attachment and biofilm formation by slime-producing *Bacillus* strains

The ability of the slime producing and non-slime producing strains to adhere (attach) and form biofilm on RO membranes was studied. Regardless of the slime production, the four strains studied attached to RO membranes and formed biofilms. The number of viable cells in biofilm formed by Bc was more than 1 log cfu/cm² greater than its less hydrophobic slime-negative mutant ($P < 0.05$), although both strains have similar counts after initial attachment in absence of growth (Table 1). Since Bc and Bc-1 were genetically identical, except for the slime character, this suggested that the slime produced by Bc helps in cell-cell adhesion during biofilm formation. However, counts lower by about 0.7 log cfu/cm² were found in biofilm formed by K1 compared to K1-1 ($P > 0.05$) (Table 2). Our observations on cell-surface attachment indicated that K1, had an advantage over its slime-negative mutant when it comes to the first step of biofilm formation, which is adhering to the membrane, but did not enhance cell-cell adhesion, or biofilm formation (Table 2).

Hydrophobicity and surface charge

The contact angle of slime-producing *Bacillus* strain Bc was significantly greater than that of slime-producing strain K1 (136.28° and 55.39° respectively). Considering a contact angle of 90° as a threshold, Bc is considered hydrophobic while K1 is hydrophilic

(Förch et al., 2009). Their mutants showed a slightly lower contact angle than their parental strains (91.29° for Bc-1 and 30.10° for K1-1). The greater contact angle on the parental strains could be attributed to some of the molecules that are present in the composition of their slimes. The strain Bc, was highly slimy and formed a skin layer that acted as a barrier against water. The EPS(-) mutant of this strain was not slimy but still exhibit a hydrophobic phenotype.

The zeta-potential of Bc, K1 and their slime-negative mutants revealed that the four strains had similar negative surface charge values, and only K1-1 was significantly more negative than its parental strain (Tables 1 and 2). These results were not well correlated with their biofilm or attachment counts, and therefore did not seem to play a major role in attachment and biofilm formation of the test strains.

The slime of the hydrophobic strain Bc seems to contribute to biofilm formation as manifested by greater viable counts, while the slime produced by the hydrophilic strain K1 only improves the initial attachment to the membrane surface but does not enhances biofilm formation (Tables 1 and 2). Since the hydrophobicity of the slime-producing cultures was strongly associated with boosted biofilm on RO membranes, we therefore hypothesized that modifying the surface hydrophobicity of the slime-producing and non-slime-producing bacteria would have an impact on their attachment and biofilm abilities. Surfactants with different hydrophilic lipophilic balance (HLB) values were applied to cells to modify surface hydrophobicity, and results of the attachment and biofilm formation of the altered cells is shown in Table 3. Tween 20 (high HLB) resulted in lower attachment of Bc, while Span 80 (low HLB) improved biofilm formation by K1 ($P < 0.05$).

Microscopic observations

Microscopic examinations were carried out to observe cell surface structures that are known to be involved in adhesion. Individual cells of the slime-producing and non-slime-producing mutants were negatively stained and observed by TEM (Figure 2).

While none of these structures were observed on any of the slime positive or negative strains, the slime layer seems as a dark fibrillary network which is lack in the mutant Bc-1 and less intense in K1-1. These observations supported the validation of our mutants as controls to study role of slime on biofilm formation by *Bacillus* strains.

SEM observations revealed a denser biofilm formation by Bc in comparison with Bc-1, and a weaker biofilm formed by K1 compared to K1-1 (Figure 1) showing large areas of cell mass detachment and formation of channels in K1, which was in agreement with the viable cell counts.

***Bacillus* slime composition**

The purified slime of Bc and K1 extracted by ethanol precipitation resulted to be composed mostly by protein, as the hydrophobic and hydrophilic strains had 11.68 and 11.20% TKN respectively, and a total carbohydrate content of 3.6 and 6.5% respectively (Table 4). The carbohydrate fraction in both samples, analyzed by GC/MS analysis, contained Glutamic acid TMS derivative along with the standard monosaccharides. The glycosyl composition of both samples Bc and K1 is fairly similar to each other and the molar percentages of each residue are presented in Table 5. Mannose (Man) was the major monosaccharide in both samples, some Galactose (Gal) and Glucose (Glc) were detected, and Arabinose (Ara) and Xylose (Xyl) were detected in minor amounts.

Amino acid analysis of the EPS revealed very similar composition between

strains and also between supernatant and pellicle fractions, with minor differences in some amino acids (Table 6). Glutamate (Glu) was the most abundant amino acid found in the slimes of Bc and K1 (55.72% 59.51% respectively) and also in their pellicle fractions (44.85% and 50.21% respectively). No significant difference was detected for Glu between Bc and K1 slimes or pellicles ($P>0.05$). The second most abundant amino acid found in Bc and K1 slimes (15.52% and 14.01%) and on their pellicles (14.41% and 16.43%), was Aspartate (Asp). Asp content was slightly greater in Bc slime but slightly greater in K1 pellicle ($P<0.05$). The amino acid Lysine (Lys) was the third followed by Glutamine (Gln) with similar levels on Bc and K1 slimes, however, on their pellicles Arginine (Arg) was significantly higher than Lys. These amino acids that are in most abundance in both slimes are classified as hydrophilic. The level of Arg and Serine (Ser) in the slime of Bc was slightly higher than K1. Ornithine (Orn) was significantly higher in K1 slime but it was low on K1 pellicle. Finally, the pellicle composition of Bc and K1 was also similar in composition but the levels of Arg, Ser and Histidine (His) were higher in Bc ($P< 0.05$). Arg and His are classified as moderately hydrophobic at pH 7 (Kovacs et al., 2006). In general, pellicles of both strains were notably richer in hydrophobic amino acids such as Phenylalanine (Phe) or Arg, and lower in Ser.

It is worth mentioning that, even though we found only minor compositional differences between both pellicles, there was a substantial difference in the floating abilities of these pellicles, as after gently lifting them and transferring into a beaker containing only water, Bc's pellicle migrated to the surface, while K1's remained in the bottom of the beaker (Figure 3). This could be due to conformational differences between both polymer structures, with a hydrophilic PGA core, and hydrophobic amino acids

bonded to the exterior of the chains. Also, the greater content of carbohydrates in K1 might contribute to its greater hydrophilicity, as monosaccharides have water-binding capacity.

DISCUSSION

The extracellular matrix has been described as an essential part of biofilm formation by many different organisms (Branda et al., 2006). The variety of exopolymeric substances produced by microorganisms is significant. The structure and composition of these substances determine their biological function, and in the case of some cultures used in fermented foods, their textural properties. The majority of research that has been published on *Bacillus* slime is limited to the models *Bacillus subtilis*, and *Bacillus licheniformis*. It is difficult to exactly compare data presented in our study with previous reports, taking into account that the biofilm ability of a microorganism varies with the surface material, and that the information available on biofilm formation by *Bacillus* isolates from dairy origin on dairy filtration membranes is scarce. Therefore, there is a need for understanding what characteristics make certain types of biopolymers more adhesive than others, to a particular surface material, which in the biofilm context may lead in future to the development of novel control strategies.

In this work, two highly slimy *Bacillus* isolates, obtained from dairy powders and showing different colony morphologies on agar, were studied. The colonies of Bc and K1 were both mucoid but Bc had a velvety layer on top. Both spore-forming organisms were observed to have rather opposite biofilm abilities on RO membranes. To explore the connection between slime-production and biofilm formation and/or attachment in the absence of growth, slime-negative mutants were used. Bc's biofilm forming ability was

greater as compared to its mutant Bc-1, suggesting that Bc slime supports biofilm formation. However, K1's slime did not bear on any supportive effect on biofilm formation judging on the greater counts of its slime-negative mutant K1-1. The observations from this investigation are in agreement with a previous study that highlights the importance of analyzing multiple isolates when attempting to characterize the adhesion of a bacterial species (Joshi et al., 2012).

In our laboratory, cleaning studies with an antimicrobial enzyme showed that biofilm formed by K1 was very weak and easily removable compared with Bc (Chapter 4, Table 9), highlighting the importance of a strong adhesion to resist the action of cleaning agents. From an industrial point of view, it seems more directly applicable to focus on what characteristics of the slimes make them more adhesive and/or biofilms more difficult to eliminate. Since our study revealed the important role of the slime produced by Bc in biofilm formation, we focused on screening physicochemical characteristics that matters to adhesion. Capsulated strains generally possess cell surfaces that are approximately twice as electronegative as those of naturally-occurring noncapsulated strains (Wilson et al., 2001), what seem to apply to most polysaccharide capsules. Both slime-producing strains were negatively charged, however, the differences in zeta-potential observed were not consistent with attachment or biofilm formation due to surface adhesion.

In our previous work, we determined that the hydrophobicity of the EPS formed by *Streptococcus thermophilus* and *Lactococcus lactis* subsp *cremoris* has an important role in the biofilm ability of these lactic acid bacteria to RO membranes. Once again, hydrophobicity, but not charge, was shown to be important to form biofilm on RO

membranes independently of the growth medium. Contact angle measurement revealed an extremely hydrophobic surface of the slime-producing Bc strain. The lawn of cells produced by this strain had the tendency to exclude the contact with water and resulted in an almost perfect water sphere. Other authors have observed similar resistance to wetting and gas penetration in *Bacillus subtilis* (Epstein et al., 2011). We previously observed that lactic cultures formed lawn of cells on agar that seem to be strongly hydrophilic, as the water droplet occupied a big area wetting the surface, which was also associated with reduced attachment and biofilm formation on RO membranes (Chapter 2). Similarly, spores hydrophobicity of different species of spore forming bacteria has been associated with greater adhesion to hydrophobic surfaces (Husmark and Ronner, 1990, Ronner et al., 1990).

We modified cell surface hydrophobicity using surfactants with opposite HLB, and observed variations in their adhesive behaviors depending on the initial level of surface hydrophobicity of the strains, and the presence or absence of slime-production. Adsorption of surfactant molecules and their effect on cell surface hydrophobicity was demonstrated previously (Zhong et al., 2008). Interestingly, an increased biofilm formation was observed for K1 cells modified with Span 80, but the highly hydrophobic Bc cells exposed to Span 80 exhibited reduced attachment and significantly lower biofilm. The later could be explained by a reduced number of hydrophobic areas exposed due to the interaction with the hydrophobic moiety of the surfactant molecules, and therefore with the polar moiety directionally oriented towards the environment. Similarly, Tween 20 reduced attachment of Bc significantly, which might be due to their different ability to form multiple layers and micelles under certain circumstances (Zhong et al.,

2008) such as presence or absence of capsule and slime. Our results display the importance of choosing the right agent when cleaning biofilms, as some surfactants may improve bacterial attachment and biofilm formation by increasing their adherence. These findings are of significance to membrane designers, and chemical companies as they suggest that intensifying membrane hydrophilicity and development of cleaning formulations specifically designed towards hydrophobic slimes are key features to focus on to reduce the formation of biofilm on dairy separation membranes by spoilage and potentially disease causing bacteria. Low biofilm forming membranes require low adhesion, and this can be tailored by alteration of the material composition and surface roughness. Surface roughness can amplify the contact angle of water drops (Bico et al., 1999), and microbial cells and other debris can be retained in the cavities. Smoother surfaces with lower hydrophobicity are associated with lower cell adhesion (Myint et al., 2010). Therefore we suggest that efforts must be directed towards exploring new membrane materials or improving coating methods that intensify current membrane hydrophilicity.

The slime composition analysis in our study revealed the glutamic acid as the major component of both slimes, along with minor amounts of other amino acids and monosaccharides. Production of a homopolyamide of Gamma -poly- DL -glutamic acid (γ -PGA) have been reported across several species of *Bacillus* (Ivanovics, 1937, Gardner and Troy, 1979, Morikawa et al., 2006, Cachat et al., 2008). γ -PGA is a commercially important biopolymer with many applications in biopharmaceutical, food, cosmetic and waste-water treatment industries (Manocha and Margaritis, 2010). Some strains have been reported to produce a mixture of PGA and levan (Feng et al., 2015). We believe that

mixed production of PGA and polysaccharide could also likely be the case for K1, given the greater polysaccharide content of its slime. These findings suggest that the PGA production by *Bacillus* strains is likely to be associated with enhanced biofilm formation to RO membranes, however, production of certain exopolysaccharides may counteract the adhesive power of PGA.

Pellicle formation in *Bacillus subtilis* has been associated with cell aggregation in clusters, and microscopic observations showed that the extracellular matrix seems necessary for cell chain clustering during pellicle formation (Kobayashi, 2007). In one study, *tasA*, encoding a major protein component of the biofilm matrix (Branda et al., 2006), and *pgsBCA* cluster (poly- γ -glutamate synthase) were intentionally knocked out to enhance levan production in the strain *B. amyloliquefaciens* NK-1 that produces levan and polyglutamic acid. Mutants deficient only in TasA resulted in deficient biofilm mutants with incomplete pellicles, but surprisingly *pgsBCA* mutants were unable to form pellicle, a finding that demonstrated the important role of PGA in both pellicle and biofilm formation (Feng et al., 2015). The backbone of PGA is hydrophilic, and can be modified by other amino acids to become amphiphilic (Shima et al., 2013). The great similarity of the composition of *Bacillus* pellicles and slimes suggests that pellicles are not formed by cells floating and growing on the surface as previously suggested (Kobayashi, 2007), but are constituted by the same biopolymers that are modified by amino acids transforming these polymers into amphiphilic molecules that coalesce together and separate from the liquid phase. This postulation would represent a genius biological strategy of certain strains that self-produce a semipermeable physical barrier against the external environmental aggressions that could also serve as a solid surface, to

which these aerobic organisms could attach to gaining access to Oxygen when they grow in liquid medium. Biofilm cleaning experts often describe the formation of membranes over the surface of biofilms incrusting on industrial pipes and other surfaces to be very difficult to disintegrate by chemical treatments. Also, the formation of resistant pellicles would actually contribute significantly to fouling of filtration membranes.

PGA has previously been described as an exopolymer that increases biofilm formation, by means of enhancing cell–surface interactions (Stanley and Lazazzera, 2005). It has also been linked to the mucoid appearance of colonies of *B. subtilis* (Stanley and Lazazzera, 2005, Morikawa et al., 2006). This unusual anionic polyamide consists of glutamate monomers linked via γ -amide linkages and not α -peptide bonds (Ashiuchi and Misono, 2002, Kamei et al., 2010). This remark is of importance when designing cleaning formulations including proteolytic enzymes, as simple amphiphilic α -peptides do not survive proteolysis, but γ -peptides (which have completely different geometries and chemical and biological properties) are known to be extraordinarily stable to the enzymatic cleavage (Seebach et al., 2004). *Bc* showed marked proteolytic activity when grown in milk (data not presented here), but its pellicle remains intact to the action of its own proteinases. Cleaning formulations often include proteolytic enzymes, which could be inefficient in the degradation of PGA. This information can be used to design an effective CIP treatment, which might include the inclusion of specific proteolytic enzymes that are able to digest this type of amino acid polymers, and disrupt the biofilm matrix to assist in biofilm removal. γ -DL-glutamyl hydrolase (EC 3.4.19.9) has been described as an enzyme that is able to degrade PGA, cleaving the γ -glutamyl bond only between D- and L-glutamic acids of γ -PGA (Suzuki and Tahara, 2003). γ -

Glutamyltranspeptidase (GGT, EC 2.3.2.2) participates in γ -PGA degradation in *B. subtilis* that supplies constituent glutamates when nitrogen is limited during stationary phase (Abe et al., 1997). GGT is currently available commercially, which is derived from equine or porcine kidney, but a microbial version could be produced at a lower cost. These enzymes alone or in combination would have potential as new biodegradable-cleaning agents for the food industry. To our knowledge these enzymes have not been used in cleaning formulations yet, and future research is necessary to evaluate their potential as cleaning agents for dairy filtration membranes.

This work showed that the hydrophobicity of Bc slimes and pellicles enhances the biofilm formation ability of *Bacillus* spp. and its resistance to cleaning processes and that the mixed production of other carbohydrates polymers in K1 would reduce its hydrophobicity and counteract its adhesiveness. Further studies are necessary to elucidate conformational differences attributed to Bc and K1 slimes that would explain the increased hydrophobicity and adhesive properties of the biofilm supporting slime, and to test specific enzyme candidates in order to find the right tool to disrupt these tenacious biofilms more easily.

CONCLUSIONS

In this study, we compared the attachment and biofilm ability on RO membranes of two *Bacillus* strains that produce slime and mutants lacking slime production. The role of slime production in biofilm formation was studied and related to parameters that affect adhesion such as surface charge and hydrophobicity. This study showed evidence that support our previous observations on lactic acid bacteria and confirms that the hydrophobicity of the EPS, regardless of whether it is composed of extracellular

polysaccharides or polyamino acids, plays an important role in biofilm formation on dairy separation membranes. The slime produced by spore forming bacteria enhances biofilm formation on RO membranes by increased hydrophobic interactions between cells and the surface and between cells. The hydrophobicity of the slimes and pellicles might be associated with their amino acid content and their conformation.

The pellicles formed on the air liquid interphase seem to be formed by the same composition as the slime with slight differences, which could be due to a rearrangement of some hydrophobic amino acids into the hydrophilic backbone of PGA. We postulate that the newly amphiphilic PGA molecules would coalesce and separate from the liquid phase functioning as a protective barrier.

We believe that this information is crucial to develop targeted approaches to prevent and disrupt established biofilms in the dairy filtration systems, to extend the life of membranes and to improve the safety and quality of the processed whey.

Chapter 3. Tables and Figures.

Table 1. Attachment, biofilm formation, contact angle and surface charge of Bc.

Strain	Contact angle (°)	Zeta-potential at different PBS concentrations (mV)			Biofilm (Log cfu/cm ²)	Attachment (Log cfu/cm ²)
		1 mM	5 mM	10 mM	TSB	TSB
		BC	136.28 ^a	-35.98 ^{A,a}	-24.50 ^{B,a}	-18.75 ^{C,a}
BC-1	91.29 ^b	-35.97 ^{A,a}	-23.7 ^{B,a}	-18.8 ^{C,a}	4.24 ^{A,b}	3.00 ^{B,a}

A-C: superscripts with the same letter in a row are not significantly different ($P>0.05$), a-b: superscripts with the same letter in a column are not significantly different ($P>0.05$).

Table 2. Attachment, biofilm formation, contact angle and surface charge of K1.

Strain	Contact angle (°)	Zeta-potential at different PBS concentrations (mV)			Biofilm (Log cfu/cm ²)	Attachment (Log cfu/cm ²)
		1 mM	5 mM	10 mM		
		K1	55.39 ^a	-31.65 ^{A,b}		
K1-1	30.10 ^b	-36.783 ^{A,a}	-29.73 ^{B,a}	-25.68 ^{B,a}	6.58 ^{A,a}	1.48 ^{B,b}

A-C: superscripts with the same letter in a row are not significantly different ($P>0.05$). a-b: superscripts with the same letter in a column are not significantly different ($P>0.05$).

Table 3. Study of attachment and biofilm formation by surfactant-modified cells.

Strain	Biofilm (Log cfu/cm ²)			Attachment (Log cfu/cm ²)		
	TSB	T20	S80	TSB	T20	S80
BC	6.16 ^{A,a}	6.07 ^{A,a}	4.53 ^{B,c}	3.18 ^{C,a}	2.06 ^{D,bc}	2.52 ^{D,a}
BC-1	4.24 ^{A,b}	4.08 ^{A,c}	3.66 ^{B,d}	3.00 ^{C,ab}	1.92 ^{D,c}	2.10 ^{D,a}
K1	5.78 ^{B,a}	5.29 ^{B,b}	6.48 ^{A,a}	2.52 ^{C,b}	2.50 ^{C,ab}	2.36 ^{C,a}
k1-1	6.58 ^{A,a}	6.21 ^{AB,a}	5.72 ^{B,b}	1.48 ^{D,c}	2.66 ^{C,a}	2.57 ^{C,a}

A-F: superscripts with the same letter in a row are not significantly different ($P>0.05$), a-d: superscripts with the same letter in a column are not significantly different ($P>0.05$).

Table 4. Total carbohydrate content and total Kjeldahl Nitrogen (TKN) of the slime of the *Bacillus* strains Bc and K1.

Sample	CHO (wt/wt%)	TKN (% wt/wt)
Bc	3.6	11.675
K1	6.5	11.195

Table 5. Monosaccharide analysis of slime of the *Bacillus* strains Bc and K1.

Glycosyl residue	Bc	K1
Arabinose	3.2	2.5
Xylose	1.1	0.6
Mannose	67.4	63.7
Galactose	12.6	22.9
Glucose	15.8	10.2

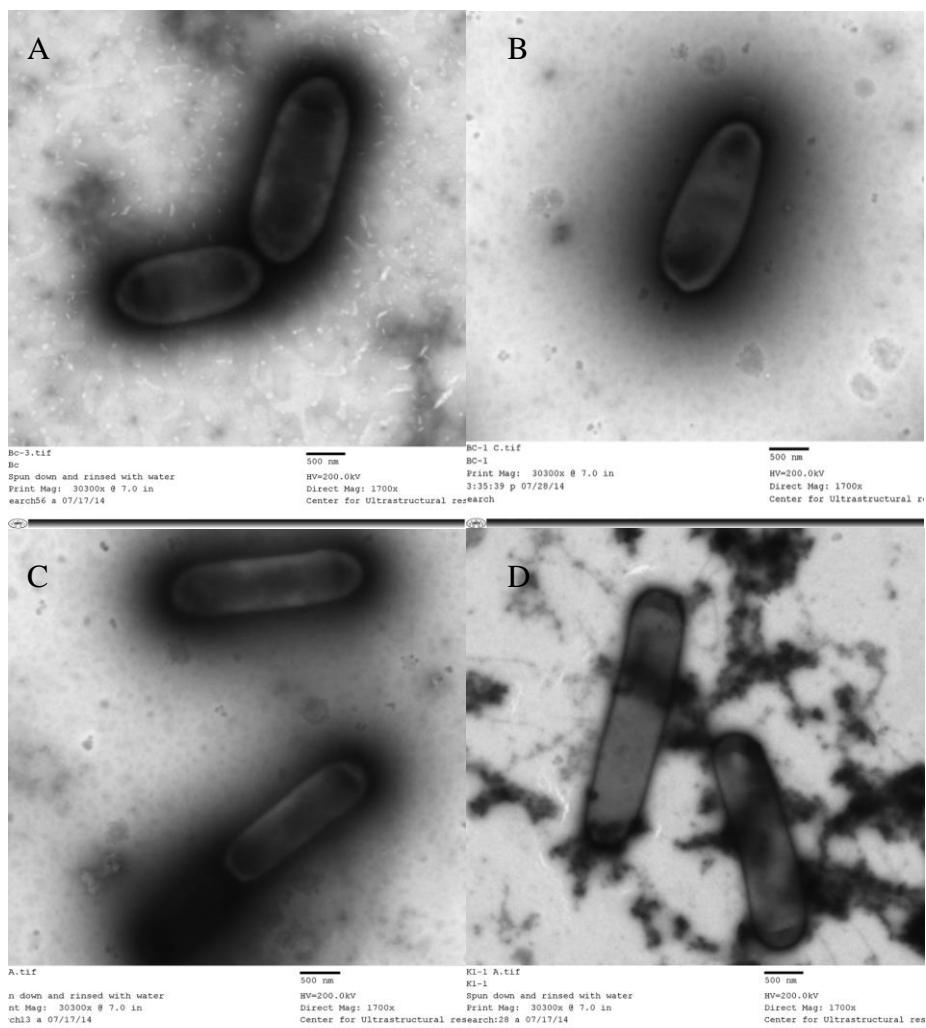
Values are expressed as mole percent of total carbohydrate.

Table 6. Amino acid analysis of slime of *Bacillus* strains Bc and K1.

Strain	BC		K1	
	Slime	Pellicle	Slime	Pellicle
Glutamate	55.719	44.848	59.505	50.206
Aspartate	15.526	14.410	14.006	16.427
Lysine	7.148	9.073	5.683	6.901
Glutamine	6.733	7.861	5.543	6.945
Arginine	6.267	12.354	4.038	9.535
Serine	3.723	1.520	2.366	1.021
Histidine	0.979	2.054	0.943	1.169
Phenylalanine	0.928	3.510	1.294	3.621
Cystine	0.909	0.983	0.524	0.640
Leucine	0.692	0.925	0.886	1.170
Isoleucine	0.631	0.896	0.726	0.871
Ornithine	0.338	0.225	4.118	0.906

Values are expressed in weight percentage (wt/wt).

Figure 1. TEM images of slime positive and negative *Bacillus* strains.



A. Bc. B: Bc-1. C: K1. D: K1-1.

Figure 2. SEM images of biofilms formed by slime-positive and negative *Bacillus* at different developmental levels. Three days-old biofilms of Bc (A), Bc-1 (B), K1 (C), and K1-1 (D), and one-month-old biofilms of Bc (E), Bc-1 (F), K1 (G), and K1-1 (H).

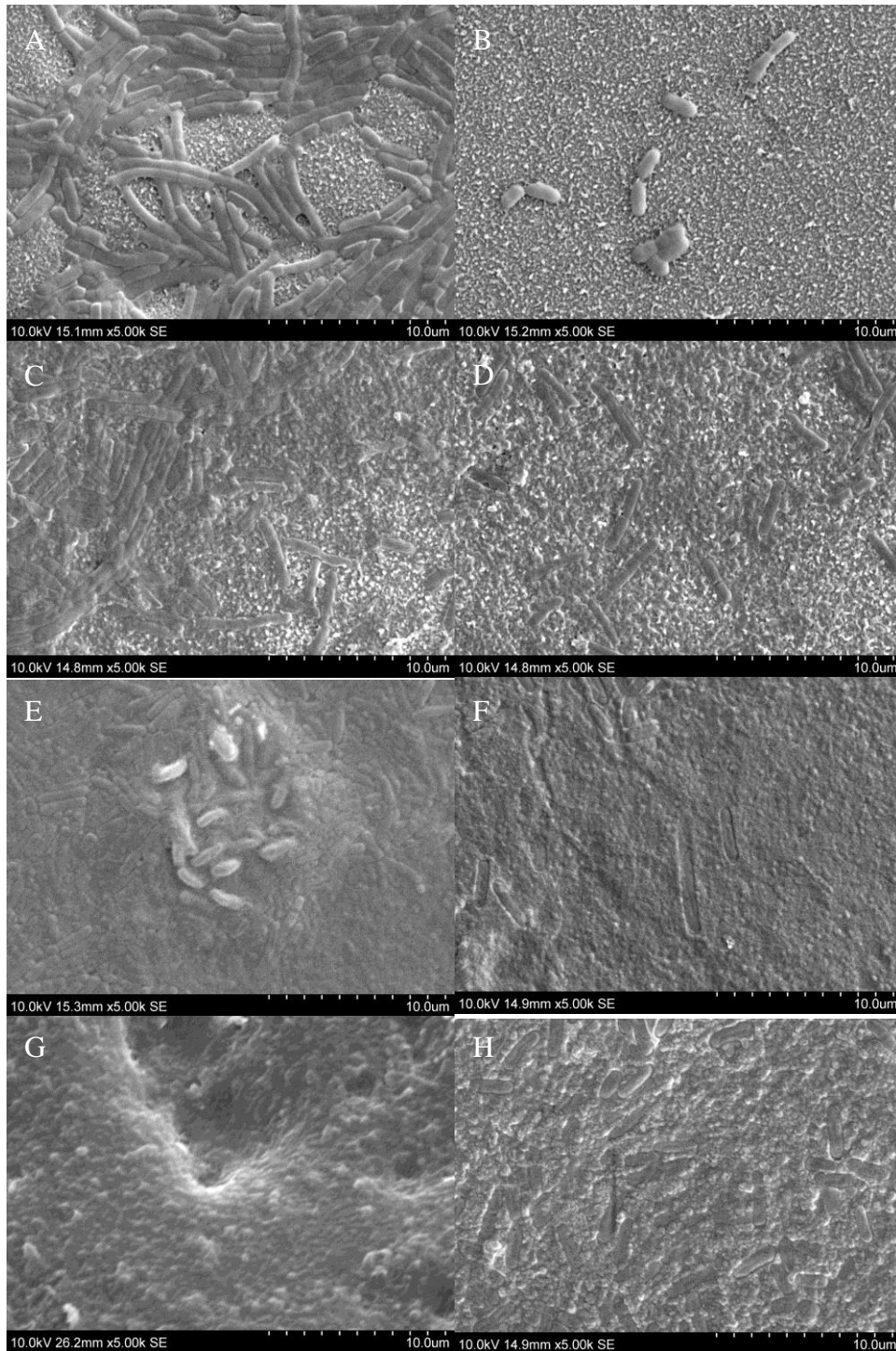
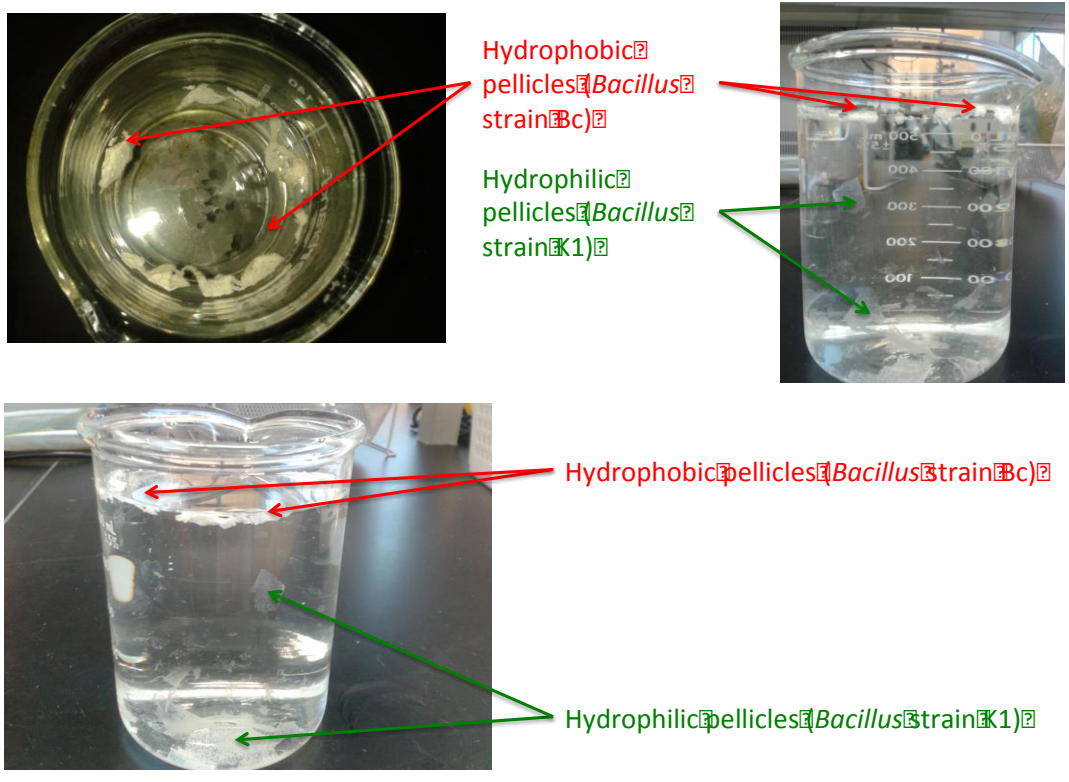


Figure 3. Images of flocculation ability of pellicles lifted from slime-produced liquid cultures and suspended in water.



Red arrows point to the hydrophobic pellicle sample of a Bc liquid culture grown in TSB for 24 h at 37°C, which stays at the top of the water solution. Green arrows point to the hydrophilic pellicle sample of a K1 liquid culture grown in TSB for 24 h at 37°C, which sink to the bottom when suspended in a water solution.

Chapter 4

A Novel application of a fungal catalase preparation to control spore-forming bacteria in the dairy industry

ABSTRACT

Spores can resist pasteurization, germinate, and grow in the dairy products during storage, causing spoilage. The aim of this work was to investigate the antimicrobial properties of several catalase preparations (Cat) against spore-forming bacteria isolated from dairy sources and their possible applications in milk and cleaning of separation membranes. The antimicrobial activity of a food grade (FG) commercially available Cat produced by *Aspergillus niger*, a non-food grade Cat (NFG), and a Cat from bovine liver (BL) against *Bacillus sporothermodurans* (Bs), *Geobacillus stearothermophilus* (Gs), *Bacillus mojavensis* (Bc), *Bacillus licheniformis* (K1), and *Bacillus* spp. (10/1) was assessed by the agar diffusion assay and broth microdilution. The effect of FG Cat on biofilms formed by two single strains of slime-producing *Bacillus* (Bc and K1) and a cocktail of four strains (Bc, Bs, Gs and 10/1) was studied alone (150 mg/mL) and after a pretreatment with 0.1% Tween 20-PBS buffer. Lastly, a cocktail of 10^3 spores/mL of four strains of *Bacillus* (Bc, Gs, Bs, and 10/1) inoculated in UHT milk was challenged with 0, 6.25 and 12.5 mg/mL of FG Cat for 30 min and the number of survivors was determined. The FG and NFG Cat inhibited the growth of vegetative cells of all tested species of *Bacillus* at 12.5 mg/mL with inhibition zones of up to 25 mm, while BL did not show any inhibition. The FG Cat reduced the number of viable cells in one-day-old Bc and K1 biofilms by 2 and 4.87 log cfu/cm² respectively ($P < 0.05$). While the application to a three-day-old multispecies biofilm reduced the viable counts by only 0.73 log cfu/cm², a

pretreatment with Tween 20 followed by FG, resulted in a 1.18 log cfu/cm² reduction ($P < 0.05$). The FG Cat at 12.5 mg/mL reduced the number of germinating spores in milk by 36.7%. The FG Cat lost antimicrobial activity after heating at 100°C for 10 min. This work describes for the first time the antimicrobial activity of Cat preparations against bacterial spores, which would create new opportunities for the dairy industry to control germination and outgrowth of sporeforming bacteria (patent pending).

INTRODUCTION

One of the important microbiological problems faced by the dairy industry is the contamination by sporeforming bacteria and their spores. The presence of high counts of spores of thermophilic bacilli is a common issue encountered by dairy powder manufacturers in the USA (Murphy et al., 1999). The number of spore forming bacteria in raw milk is normally low if good hygiene practices are implemented in the dairy farms (McGuiggan et al., 2002), but the number gets raised within the manufacturing plant likely due to a multiplication of cells during pasteurization and concentration treatments

Sporeformers cause problems in the processing of dairy and food products such as UHT drinks, infant formulas, sport beverages, and cheese. Spores are naturally resistant to heat, and therefore are not destroyed by pasteurization and can germinate and grow at a wide range of water activity, pH, and oxygen levels. Thermophilic aerobic bacilli are the most frequent contaminants in the dairy industry and their presence indicates failure to control their growth rather than high counts in the raw milk entering the plant. These thermophilic bacilli have a maximum growth temperature of 45-70°C which is common in several sections of dairy manufacturing plants (Burgess et al., 2010), with the optimum growth temperature of 28-35°C and an optimum pH range of 4.9-9.3

(Apostolos, 2014). Most sporeforming strains are able to degrade casein and starch and produce enzymes and metabolic products that lead to flavor and texture defects (Ledenbach and Marshall, 2009).

Biofilms are bacterial communities consisting of vegetative cells and bacterial spores (Semenyuk et al., 2014). Spore forming bacteria can adhere and form biofilms in heat exchangers, evaporators, etc., and proliferate when they are exposed to high temperatures. Biofilm formation by heat stable microorganisms on milking machines and dairy processing equipment such as heat exchangers and separation membranes has been reported (Langeveld et al., 1995, Tang et al., 2009). Precisely, the foulant of the preheat section of the evaporator has been associated with an increase in vegetative cell growth and sporulation, and therefore constitute a source of contamination (Scott et al., 2007). The formation of biofilm on food contact surfaces can lead to product contamination and inefficient processes.

Strategies to control spore-forming bacilli in dairy plants and dairy products include sanitation, in-process sampling to monitor contamination within the plant, elimination of biofilms, optimization of CIP, and limiting the production run time. The US dairy processors are looking for solutions to produce dairy products containing low spore counts.

Microorganisms in biofilms are more resistant to disinfection agents than in their planktonic state and often, very toxic chemicals are necessary to achieve a “total reduction or no growth”. Many fungi’s secondary bioactive metabolites are well known for their antimicrobial activities, and are considered food grade. This study investigated the antimicrobial activities of fungal catalase (Cat) preparations and their possible

applications in the dairy industry to control sporeforming bacteria. Catalase or hydrogen-peroxide oxidoreductase is a hemoprotein that catalyzes the reaction: $2 \text{H}_2\text{O}_2 = \text{O}_2 + 2 \text{H}_2\text{O}$. Catalase is used in industrial processes to remove residual hydrogen peroxide used as a bleaching and antimicrobial agent in equipment and aseptic packaging of foods and drinks. A few reports showed antimicrobial properties of bovine catalase, but to our knowledge, this is the first time that a fungal catalase is tested against sporeforming bacteria.

MATERIALS AND METHODS

Bacterial strains

Sporeforming bacteria (beta-hemolytic *Bacillus* spp., *Bacillus sporothermodurans*, *Geobacillus stearothermophilus*, *Bacillus mojavensis* and *Bacillus licheniformis*) isolated from used membranes and dairy powders were kindly provided by Dr. Sanjeev Anand, South Dakota State University. *Streptococcus thermophilus* was provided by Chr Hansen (Table 1). *Bacillus mojavensis* and *Bacillus licheniformis* were selected for the biofilm experiments because of their ability to produce slime and one non-slime-producing mutant from each strain was isolated in our laboratory by spontaneous mutation. Strains identification by 16S RNA gene sequencing was done at the Department of Food Science, Cornell University (Ithaca, NY). Cultures were stored in cryovials at -80°C and activated by growing at their optimum temperatures in the appropriate medium broth for 24 h, and then on agar plates to obtain pure culture.

Enzymes

In this study we tested different catalase sources to evaluate their antimicrobial properties. A food grade (FG) catalase 7500 B/g from fungal origin (Bio-cat, Inc. Troy,

VA), a non-food grade (NFG) catalase 500 B/g (Aqua Phoenix, Inc., Hanover, PA), a catalase from bovine liver (BL) 2,000-5,000 U/mg of protein (Sigma-Aldrich, Inc. WI) and peroxidase (P) from horseradish 120 - 180 U/mg (Sigma-Aldrich, Inc. WI) were tested. Peroxidase was included to evaluate whether the antimicrobial activity of catalase is due to its peroxidatic activity. One unit of catalase decomposes 1.0 micromole of hydrogen peroxide per min at pH 7.0 and 25°C, when the hydrogen peroxide concentration is between 10.3 and 9.2 millimolar (Sigma-Aldrich, 2011). One Baker Unit is defined as the amount of catalase that decomposes 264 mg of hydrogen peroxide under the conditions of the assay. Nonfood grade was dissolved in 10 mM PBS, FG was dissolved in 100 mM PBS, BL was dissolved in 10 mM PBS and P was dissolved in 50 mM K-phosphate containing 0.1% Bovine Serum Albumine at pH 6.0.

Spore preparation

The spores used in this study were prepared by growing each strain separately at 37°C (except for Gs, which was incubated at 55°C) in 10 mL of tryptic soy broth (TSB) for 24 h, and using this culture to inoculate the sporulation medium (Seale et al., 2008) at 1%. The sporulation medium was incubated for a week at the same growth temperature. Ten mL of the sporulation medium were transferred to glass sterile culture tubes and heated in a water bath (Isotemp model 205; Fisher Scientific, Waltham, Massachusetts) at 85°C for 15 min to kill vegetative cells. The tubes were cooled in iced water and spores were harvested by centrifugation (10,000 xg, 15 min at 4°C), and then washed three times in sterile distilled water. After 24 h of storage at 4°C, spores were counted by enumeration on Tryptic Sor Agar (TSA).

Inhibition of spores in milk

Spores of a cocktail of four strains of *Bacillus* (Bc, Gs, Bs, and 10/1) were challenged for 30 min with low concentration of FG catalase. Commercial UHT milk was inoculated with an equal number of spores of each of the four species to achieve a final concentration of 10^3 , 10^2 , and 10^1 spores mL^{-1} . The catalase at two concentrations (6.25 or 12.5 mg/mL) or PBS 100 mM (negative control) was added and tubes were incubated at 37°C with agitation (150 rpm) for 30 min and the number of viable cells was determined by serial dilution and plating on TSA plates. The challenged microorganisms were confirmed by Gram stain and colony morphology to be consistent with *Bacillus* strains. A sterility controls was used to confirm no growth. This experiment was repeated three times. Percent reduction and \log_{10} reduction were calculated using the following equations:

$$[\text{Initial count (CFU/mL)} - \text{Test result (CFU/mL)} / \text{Initial count (CFU/mL)}] \times 100$$

= Percent Reduction.

$$\text{Log}^{10} \text{ Initial Count (CFU/mL)} - \text{Log}_{10} \text{ Test Results (CFU/mL)} = \text{Log}_{10}$$

Reduction.

Agar diffusion test on vegetative cells

To determine the potency of catalase to inhibit the growth of the sporeforming bacteria, the agar diffusion bioassay was used due to its high sensitivity, simplicity and cost effectiveness (Pongtharangkul and Demirci, 2004). A number of wells were made in TSA agar plates using the back of sterile Pasteur pipets and 50 microliters of the enzyme solutions were deposited. Plates were incubated for 1-2 h to allow diffusion of the enzyme into the medium. Then, a lawn of *Bacillus* cells was prepared by spreading each of the test strains separately with a sterile swab in three different directions using 0.1 mL

of the inoculum grown overnight in TSB and containing 10^7 cfu/mL. In addition, two non-sporeforming lactic acid bacteria were tested using M17 broth as a growth medium. Plates were incubated at 37°C overnight and the zone of inhibition was read using a caliper. This experiment was repeated three times.

Determination of MIC by microdilution testing

The minimum inhibitory concentration (MIC) assay was used to determine the concentration of catalase that inhibits the visible growth of the microorganisms (vegetative cells and/or spores) using 96-well microdilution trays (USA scientific). For each of the test strains, 100 µl of the enzyme (4.68-150 mg/mL binary dilutions) were added into the respective well followed by application of 100 µl of the test strain at a predetermined density of 10^5 cfu/mL of vegetative cells in PBS (Wiegand et al., 2008). Spores in water were tested at a concentration of 10^3 cfu/mL, which is similar to the typical level of spores found in dairy powders in the USA (Watterson et al., 2014). Finally 100 µl of TSB was added and plates were immediately transferred to the incubator and incubated for 24 h at 37°C under aerobic conditions. The lower concentration that inhibited the visible growth was recorded as the MIC. This experiment was repeated three times.

Effect of catalase on biofilm formed by slime-producing *Bacillus*

Three double pieces of a Reverse Osmosis (RO) membrane (Toray membranes, Poway, CA), each of them consisting of 2 membrane squares (2 x 2 cm) glued together to expose the retentate side, were pretreated by soaking in sterile distilled water for 5 days with daily water changes and then treated with hydrogen peroxide (0.5 % solution of H₂O₂) followed by rinsing in sterile distilled water three times, and placing in a sterile

petri dish.

Two slime-producing *Bacillus* strains, one highly hydrophobic (Bc) and one hydrophilic (K1), and mutants of those strains lacking the slime production (Bc-1 and K1-1 deriving from Bc and K1 respectively by spontaneous mutation in our lab) were tested for their ability to form biofilm. In addition, a cocktail of four strains (Bc, Bs, 10/1 and Gs) was used to form multiple-species biofilm. Strains designation is found in Table 1. Removal of single- and multiple-species biofilm by FG catalase was studied. Twenty mL of TSB were inoculated with 0.1 mL of the culture in late exponential phase containing 10^7 cfu/mL of a single test strain (Bc, Bc-1, K1 or K1-1), or a cocktail of four strains (Bc, Bs, 10/1 and Gs) and added to the petri dish containing the membrane pieces as described above. Petri dishes containing the membrane pieces were incubated for 72 h at 37°C for biofilm formation under static conditions. Every 24 h membranes were rinsed and transferred to a sterile petri dish containing 20 mL of sterile TSB. Membranes were then rinsed three times in distilled water to remove loosely attached cells and treated with FG catalase in PBS buffer at 150 mg/mL (pH 7) for 30 min at 37°C and agitation at 150 rpm. A treatment of PBS without enzyme was used as control.

Since surfactants increase the wettability of biofilms enhancing the cleaning efficiency (Parker et al., 2004, Lequette et al., 2010), a pre-treatment with surfactant Tween 20 (0.1%) was also applied for 30 min at 37°C prior to the catalase treatment. A control treatment with only surfactant was included.

For quantification of viable bacteria attached to RO membranes, the membrane pieces were rinsed three times in distilled water to remove unattached cells (Hinsa-Leasure et al., 2013) and embedded cells were detached by stomaching at 230 rpm for 2

min. Counts of attached viable cells were determined by enumeration of microorganisms on TSA agar incubated for 24 h at 37°C. Each test was repeated 3 times.

Characterization of the antimicrobial activity

Effect of heat on the antimicrobial properties of catalase

Cat preparations were heated at different temperatures and the effect of heat on the antimicrobial properties of Cat was studied. One mL of catalase at 150 mg/mL was transferred to a 1.5 mL microtube and heated in a dry bath incubator (Fisher Scientific, Waltham, Massachusetts) at 100°C for 10 min or pasteurized at 63°C for 30 min following by cooling in iced water for 5 min. Inhibition of growth of strains 10/1, Bs, Bc and Gs was tested with the enzyme before and after the heat treatment. Briefly, 100 µl of the enzyme was mixed with 100 µl of an overnight culture of each of the *Bacillus spp.* strains and 100 µl of sterile TSB. A growth control without enzyme was also included. Tubes were incubated for 24h at 37°C. Since the boiled samples were cloudy due to protein aggregation during denaturation by heat, turbidity could not be used to indicate growth, so 10 µl of each tube was spotted onto TSA plates and visible growth was recorded after 24 h of incubation at 37°C. The MIC of the pasteurized enzyme (at 1.5-25 mg/mL binary concentrations) was tested by microdilution assay following the method described above. Each culture was added at 10^5 cfu/mL and a control without enzyme was included. Each test was repeated three times. In addition, the catalase activity of the FG was tested by mixing one drop of FG at 150 mg/mL before the heat treatment (N), or after pasteurization (P) or boiling (H) and one drop of 3% hydrogen peroxide over a microscope glass slide. The immediate apparition of bubbles was indicative of positive reaction for catalase activity.

Sporostatic test

The sporostatic test was used to determine at what stage does Cat inhibits the development of a spore to a vegetative cell, or whether it is the actual prevention of germination or outgrowth (Hugo, 1971).

A spore preparation of *B. mojavensis* (Bc) was prepared as described above, and 100 μ l of spore solution at 10,000 cfu/mL was added to a sterile microtube containing 800 μ l of sterile TSB and treated with 100 μ l of FG Cat (150 mg/mL), and incubated for 24 h at 37°C. No growth was observed. Then catalase was removed from the spore suspension by microfiltration and diafiltration using a 0.45 μ m filter, and spores were washed three times with TSB. Later spores were resuspended in TSB and incubated in this growth medium for 24 h at 37°C to study reactivation of germination and growth. Growth was confirmed by the apparition of turbidity. A loop fool of the liquid culture was streaked on TSA for evaluation of colony morphology and culture purity. The test was repeated three times.

Identification of the major proteins of Cat preparations

The FG and NFG Cat preparations were separated by SDS Poly Acrylamide Gel Electrophoresis. Samples prepared at 6.25, 12.5, and 25% (wt/vol) were diluted in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue) at 5%. One extra sample of each enzyme (prepared at 25% and diluted as previously described) was reduced with 2% β -bercaptoethanol and heated at 95°C for 10 min. Fifteen μ l of each sample and of the molecular weight marker Precision plus dual color standard (Bio-rad, Hercules, CA) were loaded in a Mini-PROTEAN® TGX™ precast gel (Bio-rad, Hercules, CA) and the electrophoresis was run for 30 min at

200 V.

Protein bands were observed after staining gel by submerging in 0.1% Amido Black stain solution for 1 h, followed by one step of de-staining with 10% Acetic Acid overnight, and a second step of de-staining with 10% Acetic Acid for two days. The molecular weight of the bands was compared with the standard and gels were sent to the Redox Biology Center, University of Nebraska-Lincoln (Lincoln, NE) for protein sequencing. Bands were cut from the gels with a clean scalpel into small pieces. Proteins were extracted 3 times with 50 mM NH_4HCO_3 (ABC) in 50% acetonitrile (ACN). The gels were then dried with 100 μL of ACN and the solvent evaporated at 50°C. The gels were then rehydrated in 50 mM ABC and trypsin was added at 5% of the amount of protein. The digestion proceeded overnight at 37°C. The supernatant was then removed and the peptides were extracted from the gel using acidified 60% ACN 3 times. The extracts and supernatant are combined, speed vacuum dried and resuspended in 0.1 % formic acid before running on the Thermo LTQ. This instrument performs shotgun proteomics and selects precursor ions for fragmentation. The MS/MS spectra are later used for confirmation and assignment of the peptides.

RESULTS

Inhibition of spores in milk

The study was conducted to evaluate the inhibitory activity of Cat, especially a commercially available food grade preparation (FG), on spores in milk. The outcome of the Cat preparations reducing the number of germinating spores in milk is displayed in Table 2.

A cocktail of spores from four isolates (Bc, Bs, Gs, 10/1) was inoculated into

UHT at different concentrations and challenged with FG at two concentrations during 30 min at 37°C. Table 2 shows the percentage of reduction as calculated by the number of survivors after the exposure to FG. The percentage reduction of germinating spores was correlated with the concentration of spores and enzyme in the solution. The food grade catalase at 12.5 mg/mL reduced the number of germinating spores by 36.7% in milk inoculated with 1000 spores/mL. The highest percentage reduction (68.4%) in germinating spores was achieved in milk inoculated with 10 spores/mL.

Determination of the MIC of Cat

Once we confirmed the inhibitory effect of Cat in spores we proceed to evaluate the susceptibility of vegetative cells of different species of spore forming bacteria to different enzyme preparations in agar (Table 3, Figure 1), and also in broth to determine which is the minimum inhibitory concentration (MIC) of Cat against spores (Table 4) and vegetative cells (Table 5). The NFG Cat produced inhibition zones on TSA of up to 25 mm and FG produced zones of up to 21 mm (Figure 1, Table 3). Of all strains tested, vegetative cells of *Bacillus* spp. 10/1 were the most susceptible to NFG and FG Cat while the less susceptible one was Gs. Increasing the concentration of both FG and NFG resulted in a greater inhibition of growth in TSA. In general, NFG was more effective in inhibiting the growth of sporeforming bacteria at lower concentrations than FG. The NFG enzyme at 10 mg/mL produced a halo of inhibition in TSB agar plates by the agar diffusion method while the FG enzyme did not. No antimicrobial activity was observed using BL or P for any of the sporeforming vegetative cells or spores (Table 3). The effectiveness of FG on spores and vegetative cells by the broth microdilution method was species-dependent (Tables 4-5). The MIC of FG for three of the four spores tested (Bs,

Bc, and 10/1) was 12.5 mg/mL. The MIC for Gs was one fold higher. *G.*

stearothermophilus spores, which is reported to have high resistance to heat and oxidizing chemicals than other *Bacillus* species, required a relatively high enzyme concentration to completely inhibit germination. The susceptibility of vegetative cells of strains Bs and 10/1 was greater than their respective spores. Nevertheless, very similar MIC was observed for Bc and Gs vegetative cells and their spores.

Effect of catalase on biofilm formed by slime-producing *Bacillus*

The results of applying Cat to biofilms on reduction of viable counts are shown in Tables 8-10. The NFG Cat reduced ($P < 0.05$) the number of viable cells in biofilm formed by slime-producing *Bacillus* strain Bc in more than 1 log cfu/cm² (Table 8). The FG Cat reduced ($P < 0.05$) the number of viable cells in the one-day-old biofilm of the slime-producing strains Bc and K1 by 2 and 4.87 log cfu/cm² respectively (Table 9). Interestingly, the highly hydrophobic slime of Bc seem to have a better protective action than the slime of K1 (Chapter 3). However, the application of FG Cat to a three-day-old multispecies biofilm reduced the viable counts by only 0.73 log cfu/cm² (Table 10). A pretreatment with Tween 20 followed by FG, resulted in a 1.18 log cfu/cm² reduction (Table 10).

Characterization of the antimicrobial activity

Tables 6-7 and Figure 2 show the effect of heat on the stability of the antimicrobial activity of Cat. Heating the FG Cat solution at 100°C for 10 min abolished both, the Cat activity (hydrogen peroxide hydrolysis), and its antimicrobial activity against all *Bacillus* species (Table 6, Figure 2), although pasteurization (63°C for 30 min) did not affect any of the two activities (Table 7, Figure 2). This confirmed that the

inhibition compound was a protein in nature.

The sporostatic test confirmed that the antimicrobial activity of the Cat preparations was sporostatic, non sporicidal, as the spores were able to germinate and grow normally in TSB after the Cat was removed by microfiltration.

Finally the identification of size of the proteins in Cat preparations is revealed in Figure 3 and their sequence results are showed in Figure 4. Both NFG and FG preparations were separated by SDS-PAGE, which showed two major protein bands. One upper protein band migrated in the agarose gel with a relative molecular mass of 80 kDa, which is similar in size to Cat from *A. niger*, and the lower band corresponded to a protein of 225 KDa, which is comparable to the Cat produced by *Streptomyces coelicolor* (Kim et al., 1994) (Figure 3). Both bands were purified and identified by shotgun sequencing. Mascot searches were done with the mass spectrometry data to identify proteins from the Fungi database (Figure 4). The higher molecular weight protein was identified as Catalase R and the lower molecular weight was identified as Glucose Oxidase. Glucose Oxidase (GOx) is an enzyme that catalyzes the reaction between oxygen and glucose forming hydrogen peroxide and gluconolactone. Gluconolactone can react spontaneously with water forming gluconic acid, which lower the pH of the solution. We actually observed a pH decrease in the milk inoculated with Cat, which we had to control by increasing our buffering capacity and reducing the exposure time to rule out a pH inhibition. The production of hydrogen peroxide could explain at least a portion of the antimicrobial activity of the Cat preparation.

DISCUSSION

Catalase belongs to the superfamily of hemoproteins. The exact mechanism of the

antimicrobial activities of the fungal Cat preparation is unknown. It has been suggested that the bactericidal activity of apohemoproteins (called hemocidins from: HEMOGlobin microbiCIDal peptides) is related to their polycationic nature and exceptional α -helical conformation in hydrophobic environments. They would act on the negatively charged bacterial membranes by a 'carpet-like' mechanism forming aggregates that lead to membrane destabilization and disruption (Mak et al., 2000). It was also proposed that the antimicrobial activities of hemoproteins is primarily associated with the abundance of amphipathic α -helical domains of a characteristic bipolar distribution of charged and hydrophobic amino-acid residues (Dubin et al., 2005). This suggested that the mode of action is similar to that of the classical peptide antibiotics (Dubin et al., 2005).

Hemocidins have strong antimicrobial activities in the micromolar range (Mak et al., 2000). Both production and application of hemocidins as drugs or preservatives were claimed in a patent (PL 187999) (Dubin et al., 2005). There are three classes of heme proteins involved in detoxification of H_2O_2 , catalase, catalase-peroxidase, and peroxidase. There are few reports of the antimicrobial properties of Cat enzymes, and none of those from the fungal origin.

On exposure to acids, Cat completely dissociates into subunits at pH values lower than 3, which is accompanied by a reduction in the Cat activity and an increase in peroxidatic activity (Samejima and Yang, 1963, Klebanoff, 1969, Paul et al., 1973, Jackett et al., 1980). The pH of the medium containing the enzyme preparation decreased to about 3.0 at the enzyme concentration of 150 mg/mL, 4 at 75mg/mL, 4.7 at 37.5mg/mL, and 6.5 at 18.75mg/mL, while it remained unchanged at lower enzyme concentrations. The duration of Cat exposure in our experiments (30 min) did not allow

the pH to drop by more than 0.1 unit. We observed that germination of lower concentrations of spores in water (10^1 , and 10^2 cfu/mL) was completely inhibited with 6.25 mg/mL of FG Cat at pH 6.5 in the broth microdilution.

Multiple-species biofilms showed a greater resistance to FG Cat than single-species biofilm. The higher resistance of multiple-species biofilm to cleaning was previously reported (Singh, 2012). Our results suggest that the success of Cat preparations in removing biofilm is dependent on the thickness of biofilm (number of attached cells) and the strength of the biofilm (which is directly affected by hydrophobicity of bacterial and membrane surfaces, Chapter 3). Cat preparations, however, shows potential in the cleaning and sanitation of RO membranes in combination with other agents such as surfactants. Finally its inhibitory power against spoilage bacteria can be exploited in cleaning of food contact surfaces.

The inhibition of the antimicrobial activity by temperature indicated the protein nature of the antimicrobial agent in fungal Cat preparations. Our work offers evidence of a strong contamination of GOx in the preparation, which is commonly extracted in combination with Cat. In *A. niger*, Cat enzymes are typically located in the cytoplasm and peroxisomes, although some mutants produce extracellular Cat into the medium, which is more suitable for industrial production and purification (Rogalski et al., 1998). The separation of the two enzymes from the extracted matter is costly, and often avoided, to reduce the cost of the commercial preparation. GOx system itself has antimicrobial activity due to the hydrogen peroxide formed, and the lowering of pH produced by gluconic acid production. In example, honey bees produce GOx and deposit it in the honey, acting as a natural preservative (Eteraf-Oskouei and Najafi, 2013). The enzymatic

production of H_2O_2 by GOx would be limited by the simultaneous Cat hydrolysis, and therefore we had a rational motive to question its contribution to the total antimicrobial properties of the enzymatic preparation. However, it is possible that in the presence of glucose in the system, GOx can generate H_2O_2 at a significant greater rate than Cat is transforming it. H_2O_2 is a very toxic substance and due to its strong oxidizing power, rapidly inactivates the catalase (Sizer, 1944). That is why a continuous synthesis of new catalase is necessary in living cells to obtain long-term peroxide protection (Cho and Bailey, 1976). Although the role of catalase R (CatR) on the antimicrobial properties of the fungal Cat preparations is still unclear, it is well known that some hemoproteins are bactericidal (ACA hemocidins) with a similar mode of action to that of the classical peptide antibiotics. Blastp analysis revealed that CatR from *A. niger* has 54% identity to CotJC from *B. subtilis*, which affects spore coat composition. Future studies need to be conducted to test whether CatR inhibits *Bacillus* and or other spore forming bacteria by mimicking a spore coat protein letting the cell think that it is in sporulation phase, and therefore inhibiting growth. Other hypothesis would be that the effect of Cat is due to the peroxidase activity of the enzyme. It could have the ability to (under low steady state concentrations of H_2O_2) use H_2O_2 in peroxidatic reactions responsible of the antimicrobial activity (by conversion of an oxidizable cofactor from a weak to strong antimicrobial agent) (Klebanoff, 1969). We observed no antimicrobial effect using peroxidase from horseradish.

It is worth mentioning that the commercial enzyme preparations were prepared from powder presentation and dissolved in a phosphate buffer solution and no additives were added. This means that a stabilization work could potentially improve the

antimicrobial activity of the enzymes. Stabilization of enzymes to prevent inactivation due to unfolding during industrial applications is crucial in order to be economically viable (Tavares et al., 2013), and this can be achieved by means of additives such as ionic species, hydrophobic co-solvents and polymers (Costa et al., 2002, Nita et al., 2007).

CONCLUSIONS

This work describes for the first time the marked broad-spectrum antimicrobial activity of Fungal Cat preparations against spore-forming bacteria that causes spoilage of milk and dairy products. The antimicrobial activity of food grade Cat inhibited the growth of spores in milk, and reduced the viable counts of biofilms formed by slime-producing sporeforming bacteria on RO membranes.

A severe heat treatment (boiling) abolished the antimicrobial activity and the catalase activity, but a moderate heat treatment (pasteurization) did not affect any of those activities of the Cat preparations. The spore inhibitory activity of the Cat preparations was sporostatic, as confirmed by the observation of germination and growth after removal of the Cat from spore suspensions.

Sequencing of the Cat preparations revealed presence of CatR and GOx. GOx would contribute to the antimicrobial activity by production of the bactericidal compound hydrogen peroxide. This naturally occurring enzyme preparation could serve as an attractive natural food grade additive to control germination and outgrowth of heat stable microorganisms and enhance product safety and quality in the food and non-food industry.

Chapter 4. Tables and Figures.

Table 1. List of bacterial strains used in this study

Strain	Identification	Source
Bs	<i>Bacillus sporothermodurans</i>	Dairy powder
Gs	<i>Geobacillus stearothermophilus</i>	Dairy powder
10/1	<i>Bacillus spp.</i>	Used dairy RO membrane
Bc	<i>Bacillus mojavensis</i>	Dairy powder
Bc-1	<i>Bacillus mojavensis</i>	Mutant derived from Bc
K1	<i>Bacillus licheniformis</i>	Dairy powder
K1-1	<i>Bacillus licheniformis</i>	Mutant derived from K1
ST	<i>Streptococcus thermophilus</i>	Chr Hansen®

Table 2. Effect of FG catalase on a cocktail of spores in milk

Catalase concentration (mg/mL)	Percentage of reduction (%) of a cocktail of spores inoculated at different concentrations in UHT milk (pH) ¹					
	log 3		log 2		log 1	
	Avg	SD	Avg	SD	Avg	SD
6.25	22.86 (6.63)	14.16	28.59 (6.63)	7.77	29.12 (6.64)	16.08
12.5	36.69 (6.51)	13.93	49.86 (6.51)	15.51	68.45 (6.51)	28.20

Percentage or reduction of spores (cfu/mL) in milk are means of three replicates.

Avg: Average. SD: Standard deviation.

¹ In parentheses, pH of the milk at the end of the 30 min treatment.

Table 3. Zone of inhibition (mm) of catalase against vegetative cells of sporeforming bacteria by agar diffusion.

Organism	Enzyme																			
	FG					NFG				BL						P				
	150	75	37	18	9	100	50	25	12.5	10	5	2.5	1.25	0.6	0.3	100	50	25	12.5	6
Bs	18	16	14	12	10	20	19	18.5	18	0	0	0	0	0	0	0	0	0	0	0
Bc	13.8	12.4	11	10	10	21	20	19	19	0	0	0	0	0	0	0	0	0	0	0
10/1	ND	21	19	16	10	25	22	21	20	0	0	0	0	0	0	0	0	0	0	0
Gs	ND	15	11.5	5	0	11.5	11	10	9	0	0	0	0	0	0	0	0	0	0	0

ND: Not determined.

FG: Food-grade catalase. NFG: Non-food-grade catalase. BL: catalase from bovine liver. P: Peroxidase.

Table 4. Estimation of the MIC of FG catalase on spores of *Bacillus* by broth microdilution.

Strain	Catalase concentration (mg/mL)							Growth control	Sterility control
	50	25	12.5	6.25	3.12	1.56	0		0
Bs	-	-	-	+	+	+	+		-
Bc	-	-	-	+	+	+	+		-
10/1	-	-	-	+	+	+	+		-
Gs	-	-	+	+	+	+	+		-

Record of growth after 24 h of incubation. +: Visible growth. -: No visible growth. The test was repeated three times.

Table 5. Estimation of the MIC of FG catalase on vegetative cells of *Bacillus* by broth microdilution.

Strain	Catalase concentration (mg/mL)					Growth control	Sterility control
	25	12.5	6.25	3.25	1.56	0	0
Bs	-	-	-	-	-	+	-
Bc	-	+	+	+	+	+	-
10/1	-	-	-	-	-	+	-
Gs	-	+	+	+	+	+	-

Record of growth after 24 h of incubation. +: Visible growth. -: No visible growth. The test was repeated three times.

Table 6. Effect of heat treatment on antimicrobial properties of catalase

Strain	Heat treatment			
	C	N	H	P
Bs	+	-	+	-
Bc	+	-	+	-
10/1	+	-	+	-
Gs	+	-	+	-

Record of growth after 24 h of incubation. +: Visible growth. -: No visible growth. C: Control without enzyme. N: Non-heated catalase (at final concentration of 50 mg/mL). H: 100°C 10 min (50 mg/mL). P: 63°C 30 min (50 mg/mL). The test was repeated three times.

Table 7. Effect of moderate heat treatment (63°C for 30 min) on MIC of catalase by broth microdilution

Strain	Non heated catalase (mg/mL)					Pasteurized catalase (mg/mL)					Growth control	Sterility control
	25	12.5	6.25	3.25	1.56	25	12.5	6.25	3.25	1.56	0	0
Bs	-	-	-	-	-	-	-	-	-	-	+	-
Bc	-	+	+	+	+	-	-	+	+	+	+	-
10/1	-	-	-	-	-	-	-	-	-	-	+	-
Gs	-	+	+	+	+	-	-	+	+	+	+	-

Record of growth after 24 h of incubation. +: Visible growth. -: No visible growth. The test was repeated three times.

Table 8. Effect of NFG catalase on single species biofilm removal

Strain	Treatment	Counts	SD
Bc	Control	6.10 ^a	0.13
Bc	Cat15	4.88 ^b	0.51

Control: PBS 10 mM without enzyme. Cat15: NFG catalase at concentration of 15

mg/mL. The counts (log cfu/mL) are means of three replicates was repeated three times.

SD: Standard deviation.

^{a-b} Means with the same letter superscript are not significantly different.

Table 9. Effect of FG catalase on single species biofilm removal

Strain	Treatment	Counts	SD
Bc	Control	3.84 ^c	0.19
Bc	Cat150	1.80 ^e	0.63
K1	Control	4.87 ^d	0.30
K1	Cat150	0.00 ^f	0.00
Bc-1	Control	2.31 ^b	0.23
Bc-1	Cat150	0.00 ^f	0.00
K1-1	Control	5.52 ^a	0.29
K1-1	Cat150	4.23 ^c	0.18

Counts are means (Log cfu/cm²) of three replicates. SD: Standard deviation. Control: PBS 100 mM without enzyme. Cat150: FG catalase dissolved in PBS 100 mM at concentration of 150 mg/mL.

^{a-f} Means with the same letter superscript are not significantly different.

Table 10. Effect of FG catalase on multiple species biofilm removal

3 days old biofilm		
Treatment	Counts	SD
Control PBS	4.64 ^a	0.19
Catalase 150 mg/mL	3.91 ^b	0.29
Control T20 0.1% only	4.10 ^b	0.14
Sequential 0.1%T20 + Catalase 150mg/mL	3.47 ^c	0.18

Counts are means (Log cfu/cm²) of three replicates. SD: Standard deviation. Control: PBS 100 mM without enzyme. Cat150: FG catalase dissolved in PBS 100 mM at concentration of 150 mg/mL. T20: Tween 20 dissolved in PBS 100 mM at 0.1% (wt/vol).

^{a-c} Means with the same letter superscript are not significantly different.

Figure 1. Determination of susceptibility of sporeforming and non-sporeforming Gram-positive organisms to FG catalase (150 mg/mL) by agar diffusion.

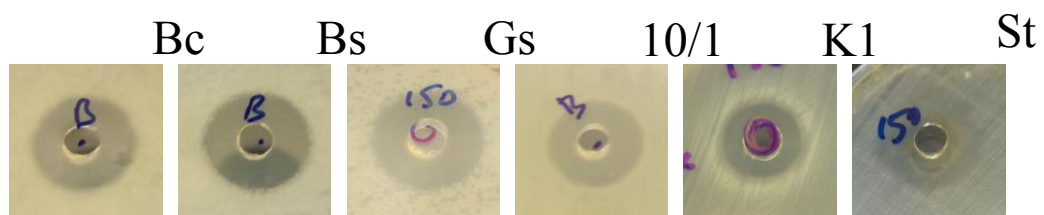
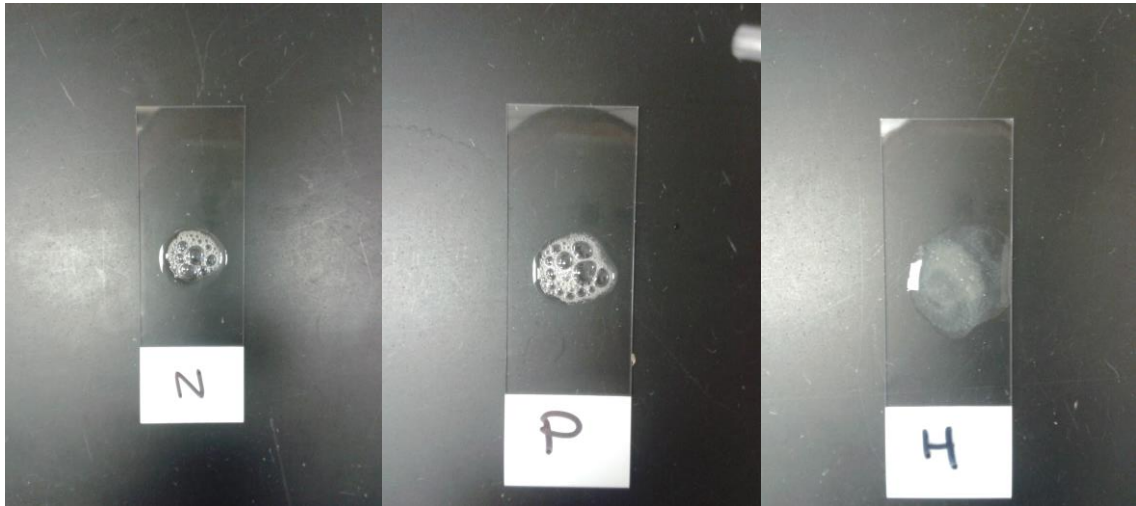
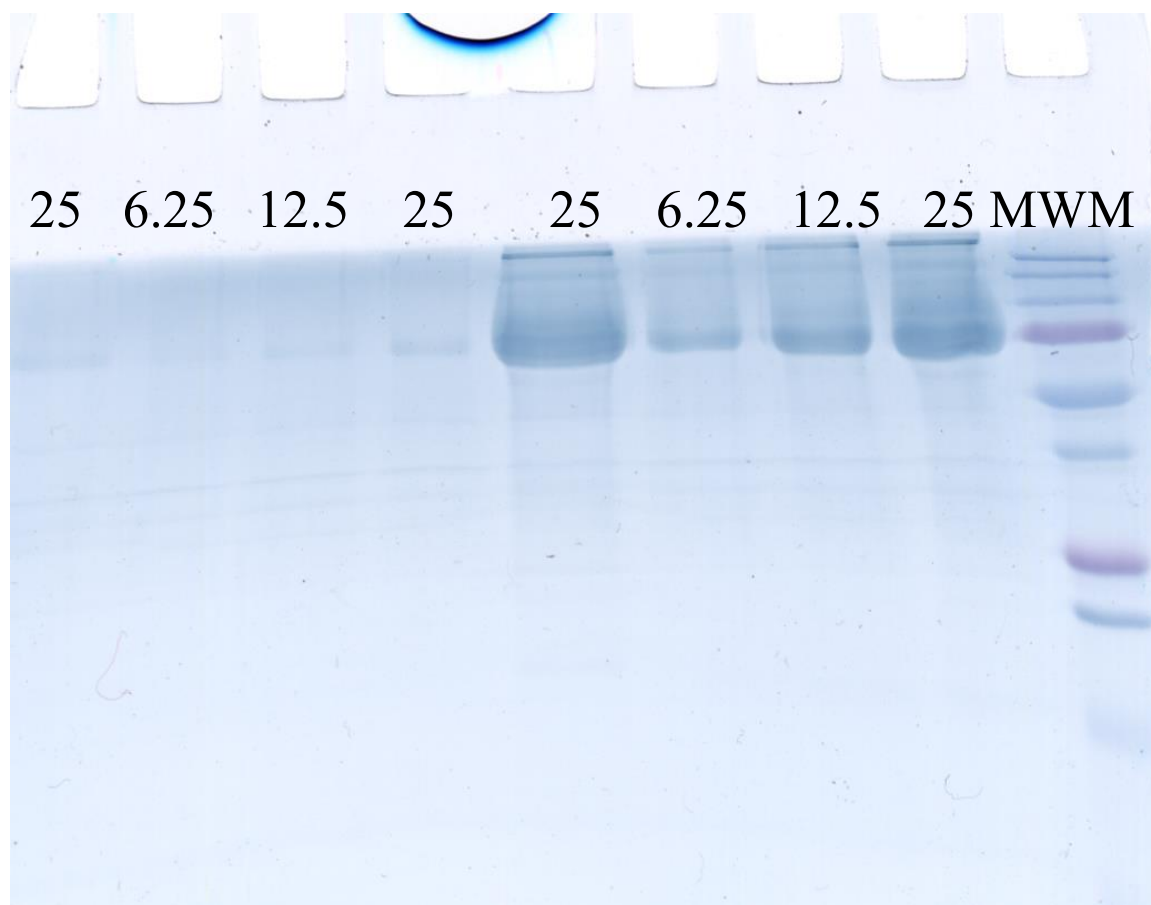


Figure 2. Effect of heat treatment on the catalase activity of FG (150 mg/mL) when tested with 3% hydrogen peroxide.



N: Non-heated FG. P: FG pasteurized at 63°C for 30 minutes. H: FG heated at 100°C for 10 minutes.

Figure 3. SDS-PAGE of catalase preparations.



From left to right, the first four wells were loaded with NFG using a non-reduced sample at 25 mg/mL and reduced samples at 6.25 mg/mL, 12.5 mg/mL and 25 mg/mL respectively. The last four wells were loaded with FG using a non-reduced sample at 25 mg/mL, and reduced samples at 6.25 mg/mL, 12.5 mg/mL and 25 mg/mL respectively. The last well to the right was loaded with the molecular weight marker (MWM).

Figure 4. Analysis of the sequence of the proteins of FG preparation purified from gel.

A. Upper protein band

Match to: **CATR_ASPNG** Score: **1258**
Catalase R (EC 1.11.1.6) - Aspergillus niger
 Found in search of G:\Core\LTQ\2015_06_01 South Dakota\C1.mgf

Nominal mass (M_r): **80411**; Calculated pI value: **5.44**
 NCBI BLAST search of [CATR_ASPNG](#) against nr
 Unformatted [sequence string](#) for pasting into other applications

Taxonomy: [Aspergillus niger](#)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
 Sequence Coverage: **22%**

Matched peptides shown in **Bold Red**

```

1 MRHFVLLPAV AGIAGAQCPCY LSGEMSFTQE QDNAGDTIEV TEQPIDNTLY
51 VNDTGSYMTT DFGTPISDQT SLKAGPRGPT LLEDFIFRQK LQRFDHERVP
101 ERVVHARGAG AYGTFKSYAD WSNVTAADFL SANDKETPMF CRFSTVVGFR
151 GSVDTARDVH GHACRFYTDE GNYDIVGINF APFFIQDAIQ FPDLVHAIKP
201 MPNNEIPQAA TAHTSAWDFE SQQSTALHSA LWLMSGNGIP RSFRHMNGYG
251 VHSFRFVAAN GTSKVVRTPW KSQQGVASLV WDEAQAAAGK NSDYHRQDLY
301 NAMPNGHYPK YELQAQIMDE ADMLRFGFDL LDPTKLVPEE VVPYTPLGMM
351 ELNANPTNYF AVEVEQAGFQP GHVVPIDFT DDPLLQGRLF SYLDTQLTRH
401 GGPNFEQIPV NRPRKPVHNN NRDGFGQQQI PTNNWAYTPN SMSNGYPMQA
451 NQTQGHGFFT APYRYASGHL VRQTSPTFND HWSQPAMFVN SLIPAEQQMV
501 VNAIVFENSK VNSPHVRKNV VNQLNMVNNN LAVRVARGLG LDEPSNPNTY
551 YTSNKTSNVG TFGKPLLSIE GLQVGFASN SHPESIKQGG AMAAQFSAAG
601 VDLNIVTEAY ADGVNTTYAL SDAIDFDALI IADGVQSLFA SPALANQMNS
651 TATSTLYPPA RPFQILVDSF RYGKPVAAVG SGSVALKNAG IDSSRSGVYT
701 GSSETTEKIA KEVLEGLYTF RFVDRFALDE

```

B. Lower protein band

Match to: **GOX_ASPNG** Score: **1276**
Glucose oxidase precursor (EC 1.1.3.4) (Glucose oxyhydrase) (GOD)
 Found in search of G:\Core\LTQ\2015_06_01 South Dakota\L2.mgf

Nominal mass (M_r): **65597**; Calculated pI value: **5.02**
 NCBI BLAST search of [GOX_ASPNG](#) against nr
 Unformatted [sequence string](#) for pasting into other applications

Taxonomy: [Aspergillus niger](#)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is
 Sequence Coverage: **40%**

Matched peptides shown in **Bold Red**

```

1 MQTLVSSSLV VSLAAALPHY IRSNGIEASL LTPDKDVSGR TVDYIIAGGG
51 LTGLTTAARL TENPNISVLV IESGSYESDR GPIIEDLNAY GDIFGSSVDH
101 AYETVELATN NQTALIRSGN GLGGSTLVNG GTWTRPHKAQ VDSWETVFGN
151 EGWNWDNVAA YSLQAERARA PNAKQIAAGH YFNASCHGVN GTVHAGPRDT
201 GGDYSPIVKA LMSAVEDRGV PTKKDFGCGD PHGVSMFPNT LHEDQVRSDA
251 AREWLLPNYQ RPNLQVLTGQ YVGKVLLSQN GTTPRAVGVE FGTHKGNTHN
301 VYAKHEVLLA AGSAVSPTIL EYSGIGMKSI LEPLGIDTVV DLPVGLNLQD
351 QTTATVRSRI TSAGAGQGQA AWFATFNETF GDYSEKAHEL LNTKLEQWAE
401 EAVARGGFHN TTALLIQYEN YRDWIVNHNV AYSELFLDTA GVASFDVWDL
451 LPPTRGYVHI LDKDPYLHFF AYDPQYFLNE LDLLGQAAAT QLARNISNSG
501 AMQTYFAGET IPGDNLAYDA DLSAWTEYIP YHFRPNYHGV GTCSMPKEM
551 GGVVDNAARV YGVQGLRVID GSIPPTQMS HVMTVFYAMA LKISDAILED
601 YASMQ

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Chapter 5

Evaluation of natural depolymerases isolated from bacteriophages and bacteria on disrupting the biofilm matrix – A proof of concept

ABSTRACT

The performance of whey concentration membranes is compromised by the formation of biofilms, which are very difficult to clean. Biofilms are formed by microbial cells attached to the surface and embedded in exopolymeric substances (EPS) that form the matrix of the biofilm. A series of preliminary studies were conducted to evaluate the potential of natural depolymerases, obtained from bacteriophages and from bacterial species with ability to degrade microbial exopolymeric substances, to remove biofilm. In a first study, a bacteriophage infective for the EPS producing lactic acid bacteria *Lactococcus lactis* ssp. *cremoris* [JFR(+)] was isolated from a dairy plant environment by swabbing. Our hypothesis is the bacteriophage must produce enzymes to break down the capsule to be able to attack and recognize their membrane receptors. Therefore we attempted to concentrate and purify extracellular depolymerases from phage suspension. Phage particles were isolated from plaques on M17 agar and propagated in liquid cultures of their host. Cultures were filtered through 0.22 µm filters to remove bacteria and phage particles were removed by high-speed centrifugation at 75,600xg for 60 min. Crude enzyme extracts from this supernatant were applied for 30 min in agitation to biofilms formed by the host strain JFR(+) on RO membrane pieces. Then membranes were rinsed three times with distilled water and the remaining attached cells were detached by mechanical homogenization with a stomacher and enumerated by serial dilution. No significant differences were observed between treated biofilms and the control. Results

might be due to a strong bond of the EPS degrading enzymes to phage particles, or to the fact that the EPS produced by the host strain does not participate in cell adhesion. In a second study, crude enzymes were obtained from the supernatant free of cells of JFR(+) grown in 10% WPC solutions during 3 days. The solutions, which were highly ropy at 24 h, lost ropiness completely by the end of the fermentation, so we hypothesized that EPS degrading enzymes were released after long fermentation due to cell lysis and prepared crude enzymes by centrifugation and ultrafiltration plus diafiltration. Total protein was quantified and enzyme activity of crude enzymes was evaluated by the Api-ZYM test and by determination of reducing sugars. The crude enzyme extracts, which showed a strong beta-galactosidase activity, were tested against biofilms formed on RO membranes by slime-producing *Bacillus* strains Bc and K1 for 30 min in agitation. We observed no significant reduction of viable counts in biofilms treated with JFR(+) crude enzymes, possibly for the lack of specificity of these enzymes to degrade the EPS produced by *Bacillus*, or to a poor stability of the enzymes. In a third study we obtained crude enzymes from supernatant of a culture of *Bacillus mojavensis* strain Bc in tryptic soy broth. This culture was positive for amylopectin degradation by the iodine method in agar plates, and was suspected to produce EPS degrading enzymes after observing reduction in viscosity of yogurt prepared with ropy cultures and inoculated with Bc. The crude enzyme extract reduced the viable counts of ST3534 in biofilms formed on RO membranes by about $0.2 \log \text{ cfu/cm}^2$, and was not considered statistically significant ($P>0.05$). The relatively poor performance of the crude enzyme was probably due to the lack of specificity of the enzymes against the EPS produced by ST3534. Results of this investigation show how enzyme specificity may be an issue in developing an effective

enzymatic cleaning treatment to disrupt the biofilm matrix, and suggest that a mixture of enzymes with different hydrolytic capabilities against different polysaccharides and protein polymers must be used in combination with other cleaning agents.

INTRODUCTION

During the whey processing by membrane filtration, membrane selectivity is affected by fouling or the buildup of adsorbed macromolecules, gels and deposited particles on or in the membrane surface, which cause concentration polarization. To control fouling and reduce the problem of pore blocking, filtration is run at increased cross-flow velocities. Biofouling, or the undesired accumulation of microorganisms on the membrane surface is a critical issue in membrane filtration that limits process performance, and increases cost. The long periods between successive cleaning cycles (Muthukumaran et al., 2005) are in favor of biofilm formation, which may serve as a continuous contamination source for food spoilage bacteria and pathogens in the finished product (Herzberg and Elimelech, 2007, Tang et al., 2009). Biofilms are multicellular communities encased in a self-produced polymeric matrix (EPS) (Chmielewski and Frank, 2003), and therefore EPS is thought to act as the cementing agent that holds cells together and contribute to the biofilm stability. Previously we observed that an EPS producing strain of *S. thermophilus* produced more biofilm than its EPS(-) genetic variant. The opposite was observed in *Lactococcus lactis* ssp. *cremoris*. Therefore, we concluded that the extent of biofilm and its structure would be affected by the type of EPS produced. Certain EPS producing starters can also support biofilm formation by non-EPS producing bacteria or harbor them (Hassan et al., 2004). Since some EPS are likely to play a major role in formation and stability of biofilm, it would be beneficial to

apply EPS degrading enzymes as a mechanism to destabilize this matrix that can help in biofilm removal. Therefore we hypothesized that depolymerase enzymes or EPS degrading enzymes which catalyze the decomposition of the EPS into smaller molecules can disrupt the biofilm matrix and help disintegrate the biofilm structure.

Bacteriophages are viruses that infect bacteria, and there have been reports of some bacteriophages that are able to colonize and infect EPS producing bacteria, by producing depolymerases that degrade the capsular polysaccharides in order to attach to the cell and recognize their membrane receptors (Drulis-Kawa et al., 2012). Phage-borne polysaccharide depolymerases in combination with phage-induced cell lysis have shown efficacy in reducing biofilms (Hughes et al., 1998). Engineered enzymatic bacteriophage have been investigated in dispersing biofilms of *E. coli* using enzyme DspB from *Actinobacillus actinomycetemcomitans* which selectively hydrolyzes beta-1,6-N-acetyl-D-glucosamine (a crucial adhesin necessary for biofilm formation and integrity in *Staphylococcus aureus* and *E. coli* k-12)(Itoh et al., 2005, Lu and Collins, 2007).

Some bacterial strains are known to produce depolymerases (Pason et al., 2006). One way to determine the efficacy of these enzymes in degrading bacterial EPS would be by texture analysis. The hypothesis was that production of depolymerases in the milk would degrade the complex EPS produced by the LAB culture into smaller simpler molecules and therefore, the texture of the fermented product would be affected. The genus *Bacillus*, and specially the model organism *B. subtilis* secretome have been studied in detail and many extracellular proteins have been described (Tjalsma et al., 2004). Some strains of *B. subtilis* produce enzymes that degrade a variety of molecules such as lipids, glutathione, phytic acid and extracellular nucleic acids under low nitrogen

conditions (Priest, 1977, Tjalsma et al., 2004). In addition, other polysaccharide-degrading enzymes produced by *Bacillus* such as levanase or amylase are well known (Saito, 1973, Kunst et al., 1977, Ray, 2000, Deutch, 2002). α -Amylase from *Bacillus licheniformis* ATCC 9945 was able to produce the hydrolysis of 91% of a 30% starch suspension after 24 h (Sokarda Slavic et al., 2015).

Some EPS producing bacteria produce enzymes that degrade their own EPS. In our laboratory we observed that the highly ropy LAB strain JFR(+) produces very viscous fermented dairy products, that are stable at refrigeration temperature, but those products loosed ropiness if fermentation was prolonged beyond 24h. This loss of ropiness after long fermentation was observed in JFR(+) cultures in agar and in liquid media as well. This phenomenon was described previously and it seems to be temperature dependent so by hence, it is likely that this culture possesses the ability to biodegrade its own EPS.

The objectives of the this study are to evaluate the potential of application of several depolymerases obtained from i) bacteriophages infecting EPS producing cultures, ii) from EPS producing culture *Lactococcus lactis* ssp. cremoris strain JFR(+) which is able to degrade their own EPS after long fermentation, and iii) *Bacillus mojavensis* strain Bc with the ability to degrade polysaccharides; to remove biofilms by degrading the EPS and disrupting the biofilm matrix.

MATERIAL AND METHODS

Isolation of bacteriophages infecting EPS degrading bacteria

Bacteriophages were isolated from cheese whey. For phage isolation, we collected cheese whey weekly at the pilot plant of South Dakota State University from a cheddar

cheese bath after cooking. In addition, samples for phage isolation were taken by swabbing various zones from dairy plant environment and swabs were soaked in whey. Whey was filtered with Whatman paper followed by microfiltration with 0.22 μ m filter to remove bacteria (at this point was considered a potential phage suspension). Nine mL of sterile M17 broth containing 0.5% lactose and 10mM of Calcium chloride in a culture tube was inoculated at 0.2% with the same cheese starter culture (DVS 970, Chr Hansen) that was used to make the cheese. The tube was incubated for 30 min at 30°C in a water bath. Then, 2 mL of filtered phage suspension was added under aseptic conditions and the tube was incubated for additional 4 h at the same temperature to encourage lysis (Auaud and Raya, 2001). Tube was centrifuged at 5,000 xg for 15 min at 4°C. The supernatant containing the phage lysates was filtered with 0.22 μ m filter and stored at 4°C. One hundred μ l of an overnight culture of the lactic culture tested for phage susceptibility [EPS(+) *L. lactis subsp. cremoris* JFR(+) or its EPS(-) mutant strain JFR(-), and the EPS(+) *S. thermophilus* ST3534 and its EPS(-) mutant ST5842] was mixed in triplicate microtubes with 100 μ l of the phage suspension that had been propagated in DVS 970 and 20 μ l of calcium chloride 100 mM. This tube was incubated at 30°C for 30 min in a water bath and then, mixed with 3 mL of soft agar and poured on M17 plates. The plates were incubated at 30°C overnight. After various weeks of whey sampling, several plaques of lysis were observed in both JFR(+) and JFR(-) cell lawns. These plaques were picked with a sterile micropipette tip and transferred to a tube containing 50 mL of sterile M17 broth and inoculated with JFR(+) or JFR(-) overnight cultures for phage propagation as described above.

Partial purification of EPS degrading enzymes from bacteriophages

The soft agar layer containing phage plaques and its enzymes was scratched with a sterile loop and transferred to a sterile tube containing 50 mL of fresh M17 broth, mixed, and then filtered through a 0.22 μ m filter to separate bacteria from phage and enzymes. The phage suspension was then transferred to a Beckman tube and ultra-centrifuged at 75,600 xg for 60 min on a JA2550 rotor at 4°C to separate phage particles from soluble enzymes. The pH of supernatant was adjusted from 4-4.4 to 7 with NaOH 1N. Five mL of the phage-free suspension were used to infect cells to ensure phage absence and the rest was stored at -70°C. Two mL of phage free suspension were added to 43 mL of M17 broth and inoculated with 100 μ l of an overnight culture of JFR(+) or JFR(-) respectively and incubated for 3 h at 30°C. Five mL of Calcium chloride 100mM were added and tubes incubated overnight at 30°C. A control of bacterial growth with no phage was also included at the time.

Application of crude enzymes from bacteriophages

An in vitro study was performed in our lab to test the efficacy of crude bacteriophage enzymes in the treatment of biofilm formed by EPS producer lactic acid bacteria on reverse osmosis membranes from dairy industry. For a preliminary trial with crude enzymes, biofilm was formed in a microtiter plate assay as previously described (Zmantar et al., 2010).

Crude enzyme solutions were used to clean biofilm formed on RO membranes. Ten pieces of double membranes (2x2 cm²) glued together exposing the retentate side, were used to form biofilm with JFR(+). A membrane pretreatment consisting of daily passages in sterile distilled water for 5 days followed by sanitation with 0.5% H₂O₂, and then 3 rinsing steps with sterile distilled water, preceded the inoculation with 50 mL of

M17 broth (0.5% lactose) at 10^8 cfu/mL in a petri dish and the plate containing the membranes was incubated at 30°C during 24 h. Then membranes were rinsed three times in 100 mL of distilled water to remove loosely attached bacteria and transferred to three separate petri dishes in groups of three for depolymerase treatments. One plate contained 15 mL of 1:4 parts of the crude enzyme solution from JFR(+) infected culture, a second plate contained 15 mL of 1:4 parts of the crude enzyme solution from JFR(-) infected culture and another plate containing PBS as negative control. One membrane was incubated in sterile broth for sterility control.

Application of crude enzymes from EPS producing culture after long fermentation

In our laboratory, we observed that cultures of an EPS producing strain of *L. lactis* subsp. *cremoris* [JFR(+)] that produced high ropiness in milk, whey and M17 broth, lost ropiness after long incubation times (greater than 24 h). The loss of ropiness was hypothesized to be due to a shortening of the long chains of EPS, likely due to enzyme degradation. The ropy character of the medium fermented for 8-24 h was kept constant at 4°C at least for weeks. The influence of temperature on the EPS degradation was an indication of enzymatic activity. Ropiness and viscosity of a JFR(+) culture in a 10% WPC solution after long incubation was assessed. A 10% solution of WPC was heated at 95°C for 15 min and cooled at 30°C. The solution was inoculated with JFR(+) at 1%, incubated at 30°C for 3 days using two fermentation pH profiles for enzyme production. First, fermentation was carried in 50 mL tubes, and pH was allowed to drop freely via auto acidification during 24h before been neutralized with NaOH 5N. In a follow up experiment, fermentation was performed in a Bioflo bioreactor in which 4 L of 10% solution of WPC sterilized by autoclaving was maintained at constant pH of 7 with

NaOH 5N and constant stirring.

In both types of fermentations, the samples were aseptically withdrawn daily and ropiness was determined visually (Folkenberg et al., 2006, Mende et al., 2012) after fermentation by pulling a spoon full from the sample beaker and classifying the length of the thread on a five-point scale, meaning (-) for non-ropy up to (++++) for very ropy samples. In addition, viscosity was measured at different time intervals using a Stresstech viscoanalyzer (ATS rheosystems, State College, PA) under shear stress control using a shear rate ramp from 0.1 to 300 s⁻¹. Viscosity was expressed in mPa·s.

The Api-Zym test was used on the cell-free supernatant of crude enzyme preparations of JFR(+) cultures after long incubation to identify enzymatic activities. The Api-Zym test is a commercial semi quantitative test by Biomerieux (Marcy-l'Étoile, France), that is designed to analyze and identify enzymatic activities on cell extracts, either intracellular, cell bound or extracellular. The kit is able to detect the following activities: Alkaline and acid phosphatases, butyrate esterase, caprylate esterase, lipase, myristate lipase, leucine, valine and cystine aminopeptidases, trypsin, chymotrypsin, phosphoamidase, alpha-galactosidase, beta-galactosidase, beta-glucuronidase, alpha-glucosidase, beta-glucosidase, beta-glucosaminidase, beta-mannosidase, and alpha-fucosidase.

The supernatant of JFR(+) grown in 10% WPC after long incubation (3 days) was partially concentrated by ultrafiltration (UF) plus diafiltration using AMICON Ultra-15 regenerated cellulose centrifugal filters of cutoff 10KDa (Millipore, Molsheim, Francia) and used as the crude enzyme for biofilm removal. The EPS degrading activity was studied by the detection of released reducing sugars with a spectrometer Cary 50 Bio spectrophotometer (Varian, Palo alto, CA) using 3,5-dinitrosalicylic acid (DNS) (Miller,

1959) and Xanthan gum 0.1% as substrate after 30 min of incubation at 37°C and the specific activity was corrected to the protein content. The protein content was determined by the method of Bradford (Bradford, 1976) using the Bio-Rad protein assay and a standard curve of bovine serum protein (Sigma-Aldrich, San Luis, MO).

Single species biofilms formed as described above by static conditions on RO membranes using slime producing strains *Bacillus mojavensis* strain Bc and *B. licheniformis* strain K1 were treated separately with 20 mL of PBS (control) or the same volume of PBS containing 0.1 mL or 0.2 mL of the crude extracts for 30 min at 37°C in a shaking incubator at 100 rpm. The number of attached cells was enumerated after rinsing the membranes and detaching cells with a stomacher at 230 rpm for 2 min. Cells were suspended in PBS and serially diluted and plated in tryptic soy agar (TSA) plates for enumeration of viable cells. Plates were incubated at 37°C for 24 h.

Application of crude enzymes from amylolytic bacteria to biofilms formed on RO membranes

A slime-producing strain of *Bacillus mojavensis* Bc isolated from a dairy powder was streaked on basic agar containing amylopectin to evaluate possible production of extracellular enzymes for polysaccharide degradation by the formation of clear zones surrounding colonies. The basic medium contained only amylopectin as the sole carbon source and was prepared with 2g of (NH₄)₂SO₄, 100mg of KCl, 500mg of K₂HPO₄, 500mg of MgSO₄·7H₂O, 2g of amylopectin (carbon source) and 15g of agar in 1 liter of distilled water, and was autoclaved for 20 min at 121°C (Shawky Gebreil, 2011). Amylopectin degradation was revealed by the iodine method (Allen, 1918), in which a clear zone around the cells appears after plates were floated in a saturated iodine solution

[0.005% (wt/vol) of I₂ and 0.05% (wt/vol) of KI]. In addition, Bc colonies were streaked on agar plates containing the EPS produced by ST3534. These EPS containing plates were prepared by adding 1.5% agar to a M17 liquid overnight culture of ST3534 that was previously adjusted to pH 7 with NaOH 1N. Clear zones were observed under the uv light after staining EPS for 15 min with 10 mL of the fluorescent dye Calcofluor White M2R (Sigma-Aldrich, San Luis, MO) prepared at 200 mg/mL in a dark room. This dye binds polysaccharides with beta-1->4 or beta-1->3 linkages (Wood, 1980).

This culture inoculated at 1000 cfu/mL was observed to reduce viscosity of a fermented 10% solution of WPC30 inoculated with ropy yogurt cultures (YCX-11, YFL-702 and YoFlex-premium) after 4h of incubation when compared with control (without *Bacillus*) using Stresstech rheometer (ATS rheosystems, State College, PA) at 100 s⁻¹.

The strain Bc was used to obtain a crude preparation of extracellular enzymes and the crude preparation was applied to biofilms formed by the EPS producing *Streptococcus thermophilus* strain ST3534. A flask containing 500 mL of TSB was inoculated with 1 mL of an overnight culture of Bc in TSB, and incubated at 37°C during 24 h. Then 250 mL of the culture were transferred to a sterile flask (treatment A). The cells of an overnight culture of ST3534 in 10 mL of M17 broth were harvested by centrifugation (5,000xg), washed with PBS, and dissolved in 1 mL of PBS, which was aseptically added to 250 mL of the overnight culture of Bc and was incubated for 30 min at 37°C to stimulate the production of EPS degrading enzymes (treatment B). Fifteen mL of the supernatants free of cells obtained by centrifugation at 5,000 xg and microfiltration (0.22µm) were concentrated by ultrafiltration (UF) plus diafiltration using AMICON Ultra-15 regenerated cellulose centrifugal filters of cutoff 10KDa (Millipore, Molsheim,

Francia) and centrifuging to remove small molecules such as salts, sugars, amino acids and minerals which to avoid the presence of nutrients that can enhance microbial growth and were used as crude enzymes. The same ultracentrifugation procedure was applied to a solution of TSB and this was applied to the biofilms as control.

Biofilms formed as described above by static conditions on RO membranes using ST3534 were treated with a 0.5% (vol/vol) crude enzyme extract for 30 min at 37°C in a shaking incubator at 100 rpm. The number of attached cells was enumerated after rinsing the membranes and detaching cells with a stomacher at 230 rpm for 2 min. Cells were suspended in PBS and serially diluted and plated in M17 agar plates for enumeration of viable cells. Plates were incubated at 37°C for 24 h.

Statistical analysis

All experiments were performed in three replicates. Statistical analysis was completed using computer program SAS 9.3 TS LEVEL 1M0 W32_7PRO platform. The pair-wise comparisons between treatments using a Student's T-test were performed to examine whether pair means were significantly different. ANOVA test was performed to determine significant differences between more than two means. The significance level was set at 5%.

RESULTS AND DISCUSSION

Bacteriophage crude-enzymes

Plaque assay revealed numerous plaques of lysis. JFR(-) showed greater phage sensitivity than JFR(+), which might be due to the lack of interference of the EPS layer with phage attachment to cell surface phage receptors. The ability of the phage to infect JFR(+) was thought to be due to the production of phage depolymerases that degrade the

EPS layer and allow the phage to encounter its receptors.

To evaluate the action of crude enzymes from a bacteriophage isolated from a dairy plant environment the host strain JFR(+) was used for biofilm formation. This is because although depolymerases are likely to have broader activity than their parent bacteriophages among closely related bacteria, different species of bacteria produce different polysaccharides components, and therefore a depolymerase that digest the EPS produced by one bacteria may not be active against that produced by other species (Harper et al., 2014).

The results of biofilm formation by JFR(+) and removal using the phage-free enzyme suspension thawed at room temperature yielded no biofilm reduction compared with control on microtiter plates (data not presented). Crude phage enzyme solutions were then evaluated with biofilm formed on RO membranes (Table 1). The viable cell count analysis revealed a difference of 0.5 log cfu/cm² between number of attached cells of JFR after treatment with control versus JFR(-) derived crude phage enzymes, however, the difference was not statistically significant ($P>0.05$). It was concluded that the crude enzyme preparation was not effective in disrupting biofilms of JFR(+) under the conditions of the experiment. It is possible that the bacteriophage isolated that infect JFR do not produce EPS degrading enzymes, even though it was hypothesized that this would be a requirement for the recognition and binding during the infective process of a phage active against cells that are surrounded by capsular polysaccharides (Poranen and Domanska, 2008), such as JFR(+). It is likely that the enzymes are linked to the phage particles. Partial purification of enzymes from phages were previously tested for activity against slime polysaccharide produced by cultures of *P. aeruginosa* infected with bacteriophage particles, although it was concluded that depolymerase activity was strongly bond to the phage particle (Bartell and Orr, 1969). It is also possible that a EPS

degradation of JFR did not reduced biofilm formation as this EPS was found to interfere negatively with bacterial adhesion (Chapter 2). Additional experiments with other EPS producing organisms and further enzyme separation steps are necessary. Supplementary test are also required to evaluate bacteriophage enzyme location and stability.

Further attempts to produce crude phage enzymes failed due to the lack of plaques on JFR(+) or JFR(-) cultures. These results indicate possibly a very poor stability of the phage under storage at 4°C and -70°C. A focused study of the conditions that increase phage viability (use of solvents or additives) leading to its ability to infect the host cells would be necessary in future. Another possibility is that the culture might have developed resistance to phage infection. These difficulties, along with the long time required for new phage screening, directed the further scope of our research towards the evaluation of depolymerases from bacterial origin.

Depolymerases from EPS producing culture that degrades its own EPS

Without any pH control, at 24 h of incubation, the WPC solution was highly ropy and viscous (++++), but after 24 h viscosity decreased progressively (+++), until all ropiness disappeared after 3 days (-). Similarly, ropiness decreased under conditions of constant pH control in a Bioflo bioreactor and constant stirring (Figure 1), viscosity was maximum at 7 h of incubation ($7.6 \pm 1.4 \text{cP}$), decreasing after 24h ($4.8 \pm 1.2 \text{cP}$) until minimum viscosity was reached at 48h of incubation ($2.4 \pm 0.6 \text{cP}$) and solution became very thin at 72 h ($1.75 \pm 0.32 \text{cP}$). After three days of incubation, when ropiness completely disappeared, medium was centrifuged to remove cells and aggregated proteins, and crude enzyme extracts were prepared from the supernatant. The crude enzymes from JFR(+) tested contained 156-188 μg of protein/mL and a estimated specific activity of 9.9U/mg

of protein, which was reduced to 5.19 U/mg after concentration and desalting.

EPS producing cultures have a significant effect on the physicochemical properties of dairy products, and one of the most noticeable changes is the increase in viscosity. The molecular weight of the exopolysaccharides has been correlated with the thickening properties of the cultures (Behare et al., 2010). The EPS of *L. lactis* ssp. *cremoris* strain JFR(+) has been extensively characterized, and the presence of a 4-glucan and a rhamnose polymers has been detected (Pottier, 2012). The reduction of ropiness in the WPC medium observed during this work was likely to be produced by EPS degradation into smaller molecules.

Multiple enzymatic activity was detected on the crude extracts by API-ZYM (Biomérieux) including intense leucine arylamidase, acid phosphatase, and β -galactosidase activity, and moderate esterase lipase, naphthol phosphohydrolase activity (Figure 2). Supernatant of overnight M17 cultures of JFR(+) free of cells were negative for β -galactosidase, indicating an increased permeability after long incubation possibly by means of cell lysis, consistent with an observed reduction in viable counts. The EPS degradation was not observed to be a result of pH reduction by lactic acid production as the pH was controlled in the fermenter, but possibly due to the release of intracellular enzymes of lysed cells after long fermentation. This hypothesis was supported by the presence of β -galactosidase in the cell-free crude enzyme extracts, and its absence in the cell-free supernatant of fresh cultures.

It is possible that the depolymerization of EPS from JFR(+) is due to the action of β -galactosidase released into the medium or that the enzyme responsible of degrading EPS is other enzyme alone, or a combination of multiple enzymes that act successively.

Moving forward, extensive purification and protein characterization is necessary to determine this. Commercially available beta-galactosidases typically are derived from different sources including *Aspergillus* (no significant similarity with *Lactococcus* enzyme at DNA level), *Escherichia coli* (60% similarity), *Bacteroides fragilis* or *Xanthomonas*. It could be possible to produce β -galactosidase from JFR commercially, since the DNA sequence of β -galactosidase from *Lactococcus* has been elucidated and the gene has been cloned, and over expressed in *E. coli* (Vincent et al., 2013). Others have described depolymerizing activity of β -galactosidase from different sources towards EPS (van Casteren et al., 2000), however, the application of this enzyme from *Lactococcus* in biofilms have not been studied.

Results from the evaluation of crude enzymes from JFR(+) showed no significant reduction in viable counts of cells in biofilms formed with Bc or K1 (Table 2). The EPS of Bc and K1 was studied (Chapter 3) and it was found that was mainly composed of poly glutamic acid (PGA), and since glutamic acid is an amino acid, proteases would be needed to hydrolyze this polymer. Since several proteinases have been described in *L. lactis* ssp. *cremoris* (Bruinenberg et al., 1992, Meijer et al., 1996) the crude enzyme of JFR possibly would include diverse proteolytic enzymes however, the PGA seem to be quite resistant to degradation by JFR crude enzymes. PGA is a homo poly amino acid formed by glutamate units linked together by γ -amide linkages and not α -peptide bonds, a fact that was revealing as γ -amide bonds are remarkably stable to the action of proteases (Seebach et al., 2004). Another factor that might have influenced the activity of the enzymes in the biofilm trials is the presence of whey proteins in the medium used to obtain the crude enzymes. Even though a great portion of denatured proteins was

removed by centrifugation, the solution was still dense and the high protein content greatly interfered with the purification process. Concentration of proteins by UF reduced the activity of the enzymes, which could also indicate that the presence of other proteins was interfering negatively with the EPS degrading enzymes.

Application of crude enzymes from amylolytic *Bacillus* strain to biofilms formed on RO membranes

Production of polysaccharide degrading extracellular enzymes by *B. mojavensis* strain Bc was revealed in agar plates containing amylopectin and stained by the iodine method, and in M17 plates containing a EPS(+) culture ST3534 and stained with Calcofluor (Figure 3). In addition, we also detected a reduction of viscosity on 10% solutions of WPC30 fermented with ropy cultures (data not presented here), and inoculated with strain Bc at 10^3 cfu/mL. We hypothesized that viscosity reduction could be due to enzymatic degradation of EPS by Bc.

The crude enzyme extract obtained from Bc contained 186 ± 16 $\mu\text{g/mL}$ of protein. The results of application of crude enzymes from Bc on biofilms formed by EPS producing *S. thermophilus* strain ST3534 showed a reduction of only 0.2 log cfu/cm² of viable counts ($P>0.05$) (Table 3). Crude extracts of extracellular α -amylase from *B. subtilis* have been previously tested against pathogenic bacteria and have been reported to cause biofilms reductions of 51.81-73.07% (Kalpana et al., 2012). The poor reduction of viable counts of biofilms by crude enzyme extracts from Bc might be improved with a purification of the crude enzyme preparation, however, more work needs to be done to obtain information about the present enzymes, such as determining the size of the enzymes and or its net charge that can help in the purification process. In addition, it is

possible that more than one enzyme participate in the degradation of the EPS, making the purification process an arduous task that would require long time. The failure of Bc crude enzymes in disrupting most of the biofilms formed by EPS producing LAB strain is likely because of a different slime composition, suggesting again, a strong substrate specificity of the EPS degrading enzymes that could potentially limit its applicability in a multispecies biofilm. This can be explained by lack of specificity of the depolymerases from this culture, and the reduced viscosity could be due to whey protein degradation. The clear zones around Bc colonies in agar plates prepared with EPS producing ST3534 could also be produced by proteolytic activity, since the EPS was not opaque to show a difference, and the fluorescent dye used might have stained the cell wall of yeasts extracts that were included in the broth, or to the agar polysaccharide itself. However, since the crude enzyme was concentrated by UF, this might have caused the loss of certain cofactors necessary for the activity of the EPS degrading enzymes of Bc. Future enzyme purification and stabilization work needs to be conducted to confirm this hypothesis.

CONCLUSIONS

Crude enzyme extracts from the supernatant of JFR(+) cultures infected with a bacteriophage strain isolated from a dairy plant did not produce any reduction of biofilm formed by cultures of JFR(+) under the experimental conditions. This was possibly because of an unsuccessful enzyme separation due to the EPS degrading enzymes being associated to the phage particles.

Highly ropy cultures of JFR(+) in 10% WPC lost ropiness completely after prolonged incubation of solutions at pH 7. These enzymes showed high β -galactosidase

activity but were not active against slime of biofilms formed by *Bacillus*. Similarly, the crude enzymes obtained from a highly proteolytic *Bacillus* strain (Bc) that produced amylopectin degrading enzymes, did not produce sufficient biofilm reduction of polysaccharide-producing *S. thermophilus* (ST3534). A combination of enzymes and other cleaning agents might be necessary to achieve acceptable levels of biofilm reduction, especially in the presence of EPS producing cultures.

EPS degrading enzymes from natural sources, including bacteriophage and bacteria, may have potential in biofilm matrix disruption applications. Our preliminary work using crude enzymes against biofilms formed by single species of EPS producing strains suggests a strong specificity or incompatibility with the EPS components tested, and generally a poor stability of these enzymes, which will have to be addressed prior to the development of such a strategy. Our results indicated that efficacy was dependent on the type of the EPS of the biofilm, and therefore crude enzymes obtained from single specie are anticipated to have limited efficacy against multispecies biofilms, due to heterogeneity of the structural components that make the EPS. Future studies need to be conducted using a mixture of broad-spectrum proteolytic enzymes in combination with enzymes specific against the EPS produced by the most adhesive microorganisms of the biofilm of interest.

Chapter 5. Tables and Figures.

Figure 1. Ropiness test of 10% WPC after long fermentation with JFR at constant pH of 7.

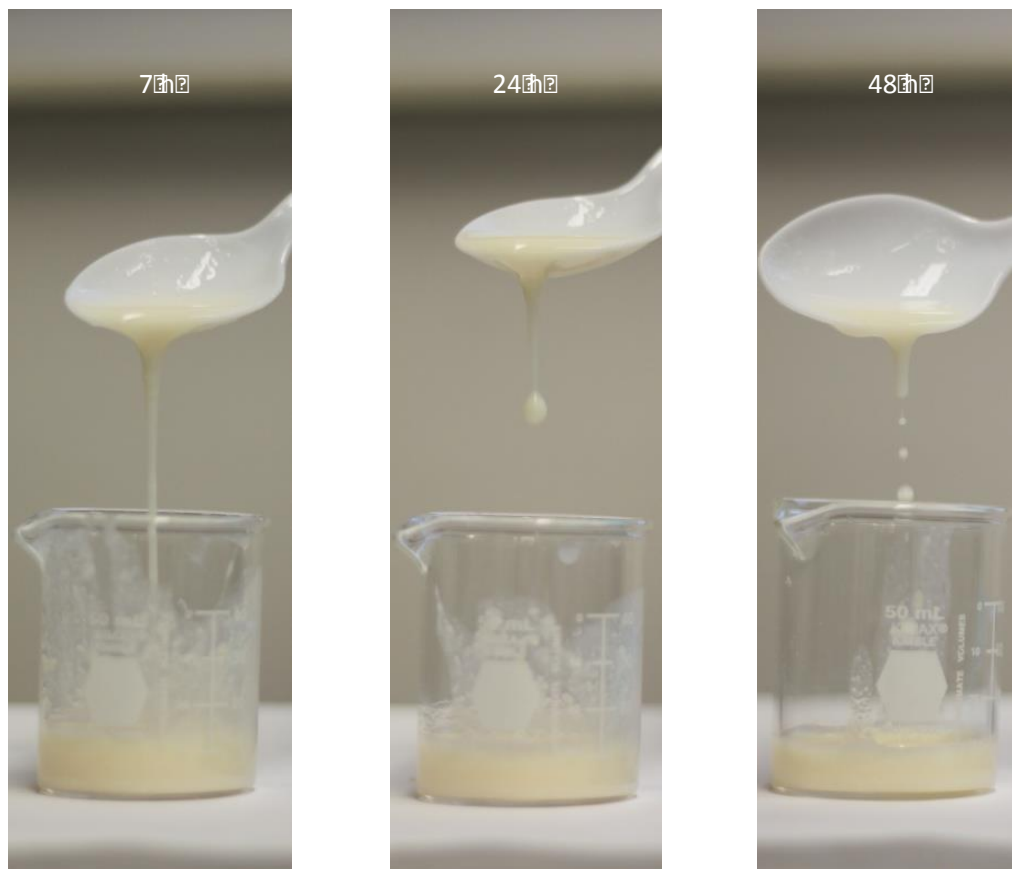


Figure 2. Determination of enzymatic activity of JFR crude enzyme extract by the API-ZYM test. The colorimetric reactions reveal intense leucine arylamidase (6), acid phosphatase (11), beta-galactosidase (14), and moderate esterase lipase (4) and naphthol phosphohydrolase (12).

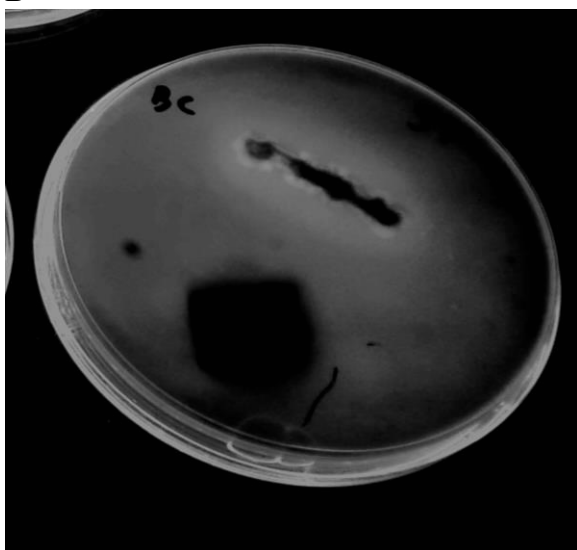


Figure 3. Clear zones surrounding *Bacillus* colonies streaked on basic agar containing amylopectin (A) and M17 agar containing EPS from a culture of ST3534 (B stained with Calcofluor and observed under UV light, C, unstained) indicating possible polysaccharide degradation produced by extracellular enzymes.

A



B



C

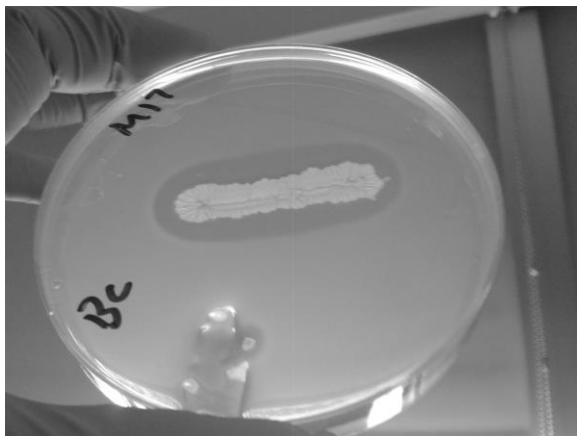


Table 1. Application of crude enzyme solution of phage on biofilms formed by JFR.

Repeat	Phage extract			Difference (log cfu/cm ²)	
	Control (log cfu/cm ²)	JFR(+) Phage extract (log cfu/cm ²)	JFR(-) Phage extract	Control- JFR(+)	Control- JFR(-)
1	7.12	7.06	6.98	0.06	0.14
2	7.14	7.34	6.39	-0.20	0.75
3	7.20	7.18	6.87	0.02	1.11
Average	7.15 ^a	7.21 ^a	6.69 ^a	-0.06	0.46
SD	0.04	0.14	0.45	0.03	0.10

Superscripts with the same letter in a row are not significantly different.

Table 2. Counts of attached cells of *Bacillus* Bc and K1 on biofilms treated with increasing concentrations of crude enzyme solution of JFR(+).

Treatment	Strain			
	Bc		K1	
	Counts	SD	Counts	SD
Control	4.08 ^a	0.29	4.85 ^a	0.91
E1	3.85 ^a	0.19	4.73 ^a	0.10
E2	3.73 ^a	0.04	4.15 ^a	0.43

Counts are means (Log cfu/cm²) of three replicates. SD: Standard deviation.

Control: PBS without enzymes. E1: 100 µl of crude enzyme were applied in 20 mL of PBS. E2: 200 µl of crude enzyme were applied in 20 mL of PBS.

Superscripts with the same letter in a column are not significantly different.

Table 3. Counts of attached cells of ST3534 on biofilms treated with crude enzyme solution of *Bacillus* Bc.

Treatment	Counts	SD
Control	4.61 ^a	0.16
Bc	4.40 ^a	0.28
Bc+ST	4.53 ^a	0.06

Counts are means (Log cfu/cm²) of three replicates. SD: Standard deviation. Control: ultra-filtered TSB fresh solution free of cultures and/or enzymes. Bc: Crude enzyme extract from the supernatant of a culture of *Bacillus mojavensis* strain Bc. Bc+ST: Crude enzyme extract from the supernatant of *Bacillus mojavensis* strain Bc that was challenged with ST3534.

Superscripts with the same letter in a column are not significantly different.

SUMMARY AND CONCLUSIONS

The formation of microbial biofilms in dairy processing equipment is an unavoidable phenomenon that continues to challenge manufacturers and presents quality and safety issues. Decades of extensive research on biofilms performed under specific conditions have been done, however often the conclusions cannot be generalized due to the heterogeneity of the different environments. Control strategies depend on the nature of the microorganisms on a particular environment and the peculiarity of the surfaces that hold the biofilms. The present research work offers new insights on the role of EPS in biofilm formation in dairy filtration systems by bacteria of interest such as cheese starters and spoiling bacteria. Contrary to what was generally believed, not all bacterial exopolymers are actually involved in the adhesion process as, while the results of this work validates the adhesive function of some types of EPS (in strains ST3534 and Bc) we also observed the opposite behavior of others (JFR and K1).

The EPS produced by *Streptococcus thermophilus* strain ST3534 significantly increased the biofilm formation on polyamide RO membranes. Moreover, ST3534 also enhanced biofilm formation by a β -hemolytic *Bacillus* strain (10/1) that had the potential to cause food poisoning. The EPS showed significant hydrophobicity for this strain as seen by contact angle and MATH measurements. Also, macroscopic cell aggregation and cell adhesion to polypropylene conical tubes were observed in liquid cultures, showing its affinity for hydrophobic surfaces.

The EPS from *Lactococcus lactis* ssp. *cremoris* strain JFR showed anti-adherent properties. A significantly lower attachment and biofilm formation was observed for JFR(+) compared with its EPS(-) mutant. Moreover, when both pairs were separately co-

cultured with *Bacillus* spp. 10/1, less number of *Bacillus* cells were observed in the biofilms in the presence of JFR(+) than in the presence of JFR(-), which suggest its potential as biological anti-biofilm agent. This EPS producing LAB itself could prove suitable as a competitive exclusion microorganism to prevent biofilm formation by pathogenic bacteria. The anti-biofilm property was not related to antimicrobial activity, as demonstrated by the spot agar assay, but to the anti-adherent property of the EPS itself, more likely due to its extreme hydrophilicity as shown by contact angle and MATH. This hydrophilicity was also indicated by the low adherence of cells to hydrophobic polypropylene tubes even after centrifugation, which resulted in difficulties during the cell harvesting.

Surface charge alone did not explain the different biofilm behaviors observed between EPS(+) and EPS(-) pairs, and therefore may not be associated with the adhesive properties of the EPS producing bacteria, as both EPS(+) LAB organisms showed marked greater negative charge than their EPS(-) mutants. Hydrophobicity was observed to be associated with adhesion and it appeared to enhance the attachment and biofilm formation of LAB to RO membranes.

Treatment of planktonic cells with surfactants modified the surface hydrophobicity of the EPS(+) and EPS(-) LAB and spore forming bacteria to different degrees. Overall, a surfactant with very low HLB improved bacterial attachment and even reduced the anti-adherent behavior of the EPS produced by JFR(+), although under the conditions of the experiment, this anti-biofilm behavior was not completely abolished. In addition, using a surfactant with high HLB, we observed reduced attachment of strains producing biofilm-supporting EPS.

Based on these observations, we suggest the design of cleaning formulation that include high HLB surfactants to remove the more stubborn biofilms, which are likely to be hydrophobic. The search of strategies to modify membrane surfaces to intensify hydrophilicity, to prevent the attachment of cells and reduce risk of biofilm formation by the potential spoilage and disease-causing bacteria should also be prioritized.

A slight greater negative charge was observed on both EPS(-) *Bacillus* strains compared with their slime-positive parental strains. Once again, hydrophobicity was associated with adhesion of the slime producing *Bacillus* strains. Both strains tested (Bc and K1) produced PGA which has been previously associated with cell-aggregation, but K1 is likely to produce additional polymers based on the greater carbohydrate content of its pure slime material. The additional polymer may counteract the adhesive properties of PGA, judging by the lower attachment of K1 than its slime (-) mutant.

The slime of the *Bacillus* strains was analyzed, and both contained the PGA polymer, which is common in the genus *Bacillus*, and is formed by glutamate molecules linked by γ -amide bonds, which are known to be stable to the classical proteases. This means that the classical cleaning enzymes would not be useful in disrupting biofilms formed by this type of microorganisms, which are common in membrane biofilms in the dairy industry.

During the completion of early studies, we performed necessary tests such as spot agar assays and bacteriocin test to rule out any antimicrobial activity of certain cultures that were used in mixed species biofilms. For example, to test production of Nisin or Lacticin by JFR that could explain the reduced adhesion of *Bacillus* 10/1 to RO membranes in the presence of JFR(+). At one point, we observed antimicrobial activity in

one of our culture supernatants, but we could determine that this was due to Catalase (Cat), which was added with the intention of removing hydrogen peroxide. The inhibition of spores and their vegetative cells by Cat was previously not reported, and therefore, our investigation was redirected to test the antimicrobial activity of Cat on biofilms formed by EPS producing *Bacillus* spp. and other spore forming bacteria of interest in dairy industry. This also gave us the opportunity to evaluate the resistance of the slime to antimicrobial agents. Food grade Cat preparations produced significant growth inhibition of multiple spore-forming bacteria that cause spoilage of milk and dairy products, including spores and vegetative cells. The inhibitory activity was determined to be sporostatic.

The Cat preparations significantly reduced viable counts in biofilms of both slime and non-slime producing strains, while a 100% of cells in planktonic state were killed. The different hydrophobicity levels of the EPS producing *Bacillus* strains were associated with the resistance of their biofilms to cleaning with antimicrobial preparations of fungal Cat. Multi-species biofilms were more resistant to the Cat preparation than single-species biofilms, possibly due to the complex nature of their EPS. Commercial Cat preparations from fungal origin contain GOx, as demonstrated by agarose electrophoresis and protein sequencing, which contributes to the antimicrobial properties by production of hydrogen peroxide. Both enzymes are generally recognized as safe and food grade.

Crude enzyme extracts from a bacteriophage isolate that attack JFR(+) produced no significant reduction in the counts of biofilms formed by JFR, and a greater screening for bacteriophage isolates would be necessary to obtain depolymerases enzymes that are able to hydrolase EPS. An especial selection of depolymerases that degrade hydrophobic

EPS should be more interesting when the aim is to use them for the removal of biofilms. This work can be costly and time consuming, and requires a careful quality assurance procedure to ensure production of only the enzymes avoiding introducing active phages into the dairy plant.

Crude enzyme extracts from WPC solutions fermented with JFR(+) after long incubation showing ropiness disappearing were not capable of removing biofilms formed on RO membranes by single cultures of slime-producing *Bacillus* strains Bc and K1 respectively obtained from dairy products. The slime of these strains was mostly composed by PGA although there might be other polysaccharides present. This could be ascribed to the great resistance of γ -amide bond of PGA to the action of hydrolases.

Bacillus mojavensis strain Bc showed extracellular amyolytic activity in agar plates containing amylopectin as the sole carbon source, and reduced viscosity of WPC solutions fermented with ropy yogurt cultures. In addition, this organism showed marked proteolytic activity in milk. Crude enzyme extracts from TSB cultures fermented with the slime-producing strain Bc were not able to remove biofilms formed by the EPS producing strain ST3534 on RO membranes. This could be attributed to the low specificity of the EPS degrading enzymes against the hydrophobic EPS produced by ST3534.

Future studies are necessary to improve the efficacy of crude enzymes from EPS degrading bacteria in removing biofilms. Taking all the observations above into consideration, future work must be done in multiple species biofilms obtained from the field preferably with a cocktail of different hydrolytic enzymes, targeting the most resilient polysaccharide and proteins from the different microorganisms of interest. In

addition, better biofilm removal results should be expected in combination with other cleaning agents such as surfactants that can help in the solubilization of degraded molecules.

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