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A Natural Antimicrobial from *Bacillus Subtilis* as a Biosanitizer for Resilient Membrane Biofilms

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A NATURAL ANTIMICROBIAL FROM *BACILLUS SUBTILIS* AS A BIOSANITIZER FOR RESILIENT MEMBRANE BIOFILMS

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THESIS ACCEPTANCE PAGE

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This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis 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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For my father who inspired my pursuits of scientific research, my mother and my elder sisters, Surya and Shabda for always being there for me.

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ABBREVIATIONS

ATCC: American type culture collection

CFE: Cell-free extract

CIP: Clean-in-place

EPS: Extracellular polymeric substances

MALDI-TOF: Matrix-assisted laser desorption/ionization time of flight

MIC: Minimum inhibitory concentration

RO: Reverse osmosis

rpoB sequencing: RNA polymerase beta-subunit encoding gene (rpoB) sequencing

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ABSTRACT

A NATURAL ANTIMICROBIAL FROM *BACILLUS SUBTILIS* AS A BIOSANITIZER FOR RESILIENT MEMBRANE BIOFILMS

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The microbial attachment and colonization on separation membranes lead to biofilm formation. Some selective isolates within the biofilm constitutive microflora acquire resistance and emerge predominant over prolonged use of the membrane. Thus, proving the cleaning and sanitization protocols to be ineffective in adequately removing resilient biofilms. This subsequently leads to selecting microbial resistance within the constitutive microflora to almost all antimicrobial treatments and hence, creates a need to develop novel alternative strategies to control biofilm formation on membrane surfaces.

The first research project under this study was designed to understand the microbial interactions and emergence of predominance within biofilm constitutive microflora of RO membranes. The second study was similar to the first where biofilm microflora from UF membranes was isolated and identified. The third study was conducted to investigate the factors influencing predominance within the biofilm constitutive microflora. This study included the antimicrobial activity of *Bacillus subtilis* isolate (within the membrane biofilm microflora) as a potential cause of its predominance. The fourth study was conducted as a proof of concept to evaluate the effectiveness of the antimicrobial substance from *B. subtilis* for cleaning membrane biofilms, under in vitro conditions.

In the first study, we isolated and identified six microorganisms from an 18-month-old RO membrane as *Bacillus licheniformis, Exiguobacterium aurantiacum, Acinetobacter*

radioresistens, Bacillus subtilis, and one unidentified species each of *Exiguobacterium* and *Bacillus*. The competitive exclusion study helped to establish the emergence of predominance using a co-culture technique. *Bacillus subtilis* emerged predominant with a mean log count of 7.22 ± 0.22 CFU/ mL. The predominance of *B. subtilis* was also validated using the process of natural selection in a multi-species growth environment. In the second study, eight microorganisms were identified from two UF membranes, sourced from two different locations. The species identified belonged to genus *Exiguobacterium, Enterococcus, Rehnella, Klebsiella, Citrobacter,* and *Bacillus*. It is important to note here that this was only a preliminary study to compare the biofilm microflora of the UF membrane with RO, and further studies are required to draw any conclusions. In the third study, antimicrobial activity as a potential cause of the predominance of *B. subtilis* was investigated. For which, 12 h freeze-dried cell-free extract (CFE) of *B. subtilis* was observed to exhibit zones of inhibition when screened against the test strain, *Micrococcus luteus*. Further, the antimicrobial activity when tested against other constitutive microflora of the membrane biofilm, and some common foodborne pathogens, revealed a broad-spectrum of inhibition against gram-positive and gram-negative bacteria. The antimicrobial substance was found to be proteinaceous, having total crude protein 51% (wt/wt), and its amino acid profiling revealed its major constituent to be glutamic acid (11.30% wt/wt). In the final steps of the study, in-vitro biofilms were developed (6.68 ± 0.12 CFU/ $inch^2$) using the isolated *B. subtilis*. These biofilms were exposed to a commercial cleaning and sanitation protocol. After 4 steps of the process, the residual biofilm counts were reduced to 2.18 ± 0.54 logs CFU/ $inch^2$. These counts got further reduced to 1.20 ± 0.09 logs CFU/ $inch^2$ on using the *B. subtilis* antimicrobial substance in place of the commercial sanitizer

during the cleaning process. The study thus provides a proof of concept for the higher efficacy of the natural antimicrobial substance released by *B. subtilis* in cleaning resilient biofilms and shows a promise for the future development of a biosanitizer.

CHAPTER 1

LITERATURE REVIEW

Significance of emergence of predominance within the constitutive microflora of RO-membrane biofilm and its implications

1.1. The process of Membrane fouling on dairy membrane

Filtration membranes have been extensively used in the dairy industry. During the filtration process as the product comes in contact with the membrane, it initiates bacterial colonization on the membrane surfaces (Marka and Anand, 2018). The RO membranes used in dairy liquids provide a large surface area for the potential for the bacteria to colonize (Flint et al., 2020). These are also referred to as bacterial biofilms which lead to biofouling. Biofouling is one of the biggest challenges faced by the dairy industry which limits the membrane performance due to a severe decline in the flux rate. These biofilms are difficult to clean because of the multilayer spiral wound structure of the membranes. Many a time even the chemical cleaning protocols prove to be ineffective, leading to frequent premature replacement of membranes (Anand et al., 2014). Membrane replacement cost constitutes 25-40% of the total cost of the membrane plants. That could vary from $10 -$15 million depending upon the type of membrane used (D'souza and Mawson, 2005).

Membrane fouling is affected by the hydrodynamics of the membrane filtration process, the interactions between the membrane and foulants in the feed stream, and between the fouling layer and foulants (D'souza and Mawson, 2005). The composition of the dairy feed is complex which consisting of different concentrations of proteins, carbohydrates (e.g., lactose), lipids (e.g., phospholipids), minerals, nonprotein N

compounds, citric and lactic acids, and microorganisms (Marka and Anand, 2018). Depending upon the substrate deposition, the membrane fouling can be categorized into different types including particulate-based, crystallization-based, chemical binding-based, and the most widespread is the adhesion of a variety of microorganism to the membrane surface. This type of fouling is known as biofouling (James et al., 2003). The presence of nutrients in the feed act as a medium for microorganism to grow and attach to the membrane surface more firmly. The solid-liquid interfaces provide an ideal environment for the microbial attachment and formation of biofilm (Sharma and Anand, 2002). The temperature of the feed also plays a critical role in the formation and growth rate of bacterial communities to form firm biofilms on the membrane surface (Chamberland et al., 2019). Biofouling adversely affects the structural integrity of the membrane, leading the system failure. Therefore increasing the operational and maintenance cost (Marka and Anand, 2018).

Studies have also reported the presence of foodborne pathogens and spoilage microorganisms as a part of biofilm microflora on a variety of surfaces used in food processing environments. There is a diverse range of factors that can influence bacterial colonization on food and dairy processing surfaces. Major factors include the type of microorganism and their growth stage, surface properties of the bacteria and their substrate, the microbial and nutritional quality of the feed stream, and pH of feed (Tang et al., 2009). Studies have also reported stronger attachment ability of the spores than vegetative cells, due to hydrophobicity and hair-like structures on the cell surface (Ronner et al., 1990; Husmark and Ronner, 1992; Kumar and Anand, 1998). Spore formers make firmer biofilms which also possess resistance to the cleaning regime (Avadhanula, 2011).

In the dairy industry, the biofilms formed on equipment surfaces rise safety and quality concerns and serves as a constant source of contamination of the final product with both spoilage and pathogenic bacteria which are difficult to remove even with existing cleaning procedures (Fadila, 2016). The bacterial attachment to the surface is different in biofilms than in their planktonic counterparts.

The biofilms could be single-species or multispecies communities enclosed in a protected layer of EPS and attached to the substratum (Hassan et al., 2010). The biofilms on the surface show different stages of development including reversible, irreversible, formation of microcolonies, development of mature biofilm with 3-dimensional structure, cell detachment, and dispersion (Anand et al., 2014). The reversible biofilms can be removed with ease from the surface by rinsing. Whereas, the irreversible biofilm offers greater resistance to the clean-in-place procedures. Once the irreversible biofilms are formed, strong forces are required for their removal from the surface of the membrane. The biofilm formation is a slow process, still, it accumulates a few millimeters thick layer in just a few days. As the biofilms mature and start to accumulate other components from the feed, these biofilms grow larger and finally, get detached through the process of rinsing and shearing (Marka and Anand, 2018). The dislodged cells are distributed to new locations, where they start to develop into new biofilms. It is observed that the cells in biofilms are more resistant to the cleaning processes than their planktonic state (Anand and Singh, 2013).

The biofilms have been observed to develop a hill-and-valley type of structure, where the hill represents mushroom-like appearance and the valley comprises dense matrices of EPS with embedded bacterial cells (Hassan et al., 2010). The mushroom looks

like a 3-dimensional structure, possibly due to excessive secretion of EPS (Hassan et al., 2010; Anand et al., 2014).

1.2. Constitutive microflora reported on RO-membranes

The biofilm microflora can serve as a reservoir for different types of microflora causing contamination of the product (Avadhanula, 2011). The biofilm microflora may consist of viable or nonviable, single-species, or multispecies communities of microorganisms embedded in EPS (Anand et al., 2014). Various studies have demonstrated the occurrence of multispecies biofilms on the RO membrane (Tang et al., 2009; Anand et al., 2012; Anand et al., 2014). Studies have also demonstrated the predominance of gram-positive microorganisms or thermo-resistant species such as spore-forming *Bacillus* species on filtration membranes used to process whey (Schreiber, 2001; Friedrich and Lenke, 2006). On the contrary, Tang et al. (2009), reported a higher proportion of gram-negative bacteria including coliforms as a part of biofilm microflora. Such results could be obtained in case of water contamination or general plant hygiene problems.

Previous studies have reported the presence of *Enterococcus, Staphylococcus, Micrococcus, Lactococcus, Lactobacillus, Arthrobacter, Bacillus, Streptomyces, Corynebacterium, Bacillus, Klebsiella, Aeromonas, Methylobacterium, Cronobacter, Pseudomonas, Escherichia coli, Streptococcus, Chryseobacterium* and bacteria from *Acinetobactia, Firmicutes* and *Proteobacteria* phylum as constitutive microflora found on dairy separation membranes (Tang et al., 2009; Avadhanula, 2011; Anand and Singh, 2013; Chamberland et al., 2017).

Tang et al. (2009) also reported *Klebsiella* spp. and *Bacillus* spp. to be dominant on membranes used in the dairy industry. Several other studies have reported the persistence

of *Bacillus* spp. on dairy filtration membrane and its resistance to the disinfectants and chemical cleaners used in the C.I.P. procedures (Sharma and Anand, 2002; Anand and Singh, 2013; Marka and Anand, 2018). Also, studies have reported the ability of persistent strains to produce significantly more biofilms than the nonpersistent strains (Alvarez-Ordóñez et al., 2019).

Studies on the change of biofilm microflora over prolonged use of membrane depicted the aerobic spore formers and lactic acid bacteria to be always a part of the constitutive microflora. Whereas, the occasional presence of other organisms like coliforms, *Pseudomonas* spp., and *S. aureus* were reported. This could be due to cross-contamination during the whey concentration (Avadhanula, 2011).

Presence of pathogens such as *Listeria monocytogenes*, *Yersinia enterocolitica, Campylobacter jejuni*, *Salmonella* spp., *Staphylococcus* spp., *Bacillus cereus* and even *Escherichia coli* 0157:H7 (Avadhanula, 2011; Yuan et al., 2019; Flint et al., 2020) have been reported to be present in the membrane biofilms. These foodborne pathogens serve as a constant source of contamination and rise product quality and safety concerns. Studies have also reported an increase in the attachment and survival of pathogenic bacteria in the presence of other bacterial species (Flint et al., 2020). A previous study on biofilm microflora reported that *B. cereus* constituted more than 12% of the biofilm microbial community (Sharma and Anand, 2002).

1.3. The emergence of predominance within the biofilm over prolonged use of membranes

Separation membranes are now increasingly used in the dairy and food processing industry (Anand et al., 2012) for diverse applications such as whey protein concentration, milk protein standardization, desalination, wastewater treatment, etc. (Daufin et al., 2001;

Turan et al., 2002). Despite numerous benefits, membrane processing has a major limitation of biofouling due to bacterial biofilm formation on the membrane surfaces (Suwarno et al., 2012). Multispecies biofilms are formed when the feed containing microorganisms comes in contact and initiate colonization on the membrane surface (Anand et al., 2012).

Multispecies biofilms are considered to be more complex and appear to form thicker and more stable biofilms, which may be due to the release by various bacterial species of extracellular polymeric substances (EPSs) (Hassan et al., 2010; Anand et al., 2014). The EPS secreted by multiple species in a biofilm matrix act as a shield and helps to develop resilient biofilms that are very difficult to clean (Anand et al., 2014). Even the cleaning and sanitization protocols prove to be ineffective for removing such resilient biofilms. Such biofouling not only results in a reduced flux rate but also serves as a constant source of contamination. Hence, leading to premature replacement of separation membranes and economic losses faced by the dairy industry (Marchand et al., 2012). The membrane replacement cost is high and could constitute around 25-40% cost of the membrane plant (Souza and Mawson, 2007).

Some organisms present within the biofilm microflora show predominance over others, over the prolonged use of membranes (Anand and Singh, 2013). Some of the other studies have also depicted the emergence of single species predominance in biofilms (Flint et al., 2009; Nadell et al., 2016). Competition among microorganisms in a shared environment occurs in all but the simplest ecosystems (Fredrickson and Stephanopoulos, 2017). The populations of microorganisms within the common setting outcompete one

microbial population over the other when the competition is focused on a single resource, hence, emerging as predominant in a constitutive microflora.

1.4. Factors influencing the emergence of predominance

The emergence of predominance could be associated with several factors including competition for the nutrients, faster-growing rate of one microorganism over the others, production of metabolites by the cells, production of bacteriocin, secretion of broad and narrow spectrum toxins with coupled privatized antitoxin, etc. (Bowden and Hamilton, 1989; Amézquita and Brashears, 2002; Nadell et al., 2016). Other decisive factors which can potentially lead to the prevalence of certain microbial species over the others within mixed-species biofilms are the surface charge, cell chaining, inoculum composition, oxygen availability or release of certain matrix protein-like TasA which provides structural integrity to *Bacillus subtilis* biofilms, etc. (Van Merode et al., 2007; Chen, 2019). According to Van Merode et al. (2007), the surface charge can influence the prevalence of one microorganism within a mixed-species biofilm. Besides, studies have demonstrated the importance of dual-species biofilm in which one bacterial strain stimulates the attachment of other bacterial strains (Bradshaw et al., 1996; Tait and Sutherland, 2002; Van Merode et al., 2007). That can protect other strain against the disinfectants and hence, leading to its predominance within a mixed species growth. Also, it is common to observe the occurrence of bacteriocin activity in the presence of closely related strains as a competitive trait (Tait and Sutherland, 2002).

The extracellular polymeric substances (EPS) is a major component in biofilm formation. It helps in cell-to-cell attachment and adhesion of bacterial biofilm to the membrane surface (Anand et al., 2014). The dairy biofilms are predominantly composed

of EPS and milk residues, mostly proteins, and calcium phosphate (Simões et al., 2010). The multispecies biofilms are known to be more complex and tend to form thicker and more stable biofilms, which could be due to the release of extracellular polymeric substances (EPS) by various bacterial species. (Hassan et al., 2010; Anand et al., 2014). Also, relatively higher production of EPS has been associated with cells within the biofilm as compared to the planktonic cells. The EPS secreted by multiple species in a biofilm matrix act as a shield and helps to develop resilient biofilms that are very difficult to clean (Anand et al., 2014). It was established that the rugosity of colony and pellicle morphology was directly correlated with the enhanced production of an EPS. That is known to develop additional resistance to chlorine, hence, demonstrating a protective function of the EPS against biofilm embedded cells (Hobley et al., 2015). Therefore, embedded biofilm microflora in a polysaccharide matrix can thus survive and cause human infections and may even be antibiotic-resistant (Anand et al., 2014).

The presence of mucoid colonies has been associated with the predominance of certain strains over others that do not produce mucoid colonies (Stratton et al., 1986). Also, a colony is considered hyper-mucoviscosity with a string of > 5mm. This could be one of the potential contributing factors for the predominance one strain over the other in a biofilm matrix.

Another factor that could be responsible for the predominance of one species or the other is the action of natural selection. When various species and strains come in contact with each other, it is by default that the competitive phenotype will predominate. This competition has led to the development of numerous strategic strategies, ranging from rapid growth and resource acquisition to the use of adhesion and matrix processing to capture

nutrient-rich biofilm locations. One of the most common ways of intermicrobial competition is to secrete wide and narrow-spectrum toxins in combination with privatized antitoxins that prevent self-poisoning (Nadell et al., 2016).

The release of narrow or broad-spectrum toxins by a strain is predicted to be most favored when the competition is confined, and the completing cell lineages are fairly well mixed in the space. The response of the toxin varies within different communities. The strength of each toxin secreting strain might be too low for an effective attack if the population mixture is too high. Whereas the toxic secreting strain may not even function when these populations are isolated. Studies have therefore stated that if the cell lines are well mixed, the sensitive cells inside the same biofilm may become outcompeted by the toxin-secreting species (Schluter et al., 2015; Nadell et al., 2016).

Quorum sensing was also observed for controlling competitive traits like bacteriocin release. Stress reactions to nutrient limitation and cell damage also upregulate the bacteriocins and antibiotics. The toxin secreting strains are known to inhibit the growth of the sensitive strains at or under localized conditions at a sufficiently high density of cells. Another mechanism for detecting the competitor's presence is because of the stresses they create when they are in close proximity. Such 'competition sensing' may manifest as a response to nutrient limitation or cell damage, perhaps more reliably (Cornforth and Foster, 2013; Nadell et al., 2016).

Therefore, it is important to understand the emergence of predominance within membrane biofilms. This can provide necessary information to bridge the gap between the development of mature biofilms on prolong use of membranes, and the potential of selective species to generate resistance which leads to the predominance of one

microorganism over the others in a biofilm microflora. This information would be vital to identify predominating species within biofilm microflora and to create new clean-in-place strategies to eliminate resistant biofilm microflora to extend the service life of filtration membranes in the dairy industry.

1.5. Implications of the emergence of predominance

Several microorganisms tend to attach to the surface of the membrane and initiate bacterial colonization on the membrane surfaces (Anand et al., 2012). These are also referred to as biofilms which consist of complex communities of microorganisms (Tait and Sutherland, 2002). These biofilms are very difficult to clean and have been reported to develop resistance to chemical processes as compared to planktonic counterparts (Anand et al., 2014; Sayem et al., 2018). Proving the cleaning and sanitization protocols to be ineffective and result in the formation of resilient multispecies biofilms (Stoodley et al., 2002). When the biofilm microflora is subjected to disinfectants, the sensitive cells die but resilient cells develop bacterial resistance against such chemical cleaner. Thus, creating the demand to discover new eco-friendly natural antimicrobials for effective membrane cleaning. A study on the biofilm microflora has reported developing resistance within selective isolates over prolonged use of membranes and their potential to generate predominance in the biofilm matrix (Anand and Singh, 2013). The microbial resistance makes the CIP cleaning protocols unsuccessful by reducing the efficiency of almost all antimicrobial treatments. Besides, these strains rapidly generate additional resistance against new synthetic derivatives as these have already acquired resistance to the parent agents (Sumi et al., 2015).

Under optimal conditions, the filtration membranes should be prevented from forming biofilms than later addressing biofilm issues. Unfortunately, currently, there is no technique available for preventing biofilms to form on the membrane surfaces during processing. Therefore, dairy industries have adapted Clean-in-place regimes for the removal of biofilms. To ensure efficient removal of foulants from membrane surfaces, proper cleaning regimes should be selected that do not promote microbial growth (Simões et al., 2010). The standard CIP procedure used for cleaning RO membranes consists of five stages, which include an alkaline wash, an acid wash, an alkaline wash, an enzyme wash and a final sanitizer wash (Tamime, 2009). The membranes are flushed with water in between different stages to remove adhered residues and biofilm debris (Garcia-Fernandez, 2016). Usually, not all stages of the CIP procedure are carried out at the end of each processing cycle. The most commonly used CIP procedure includes an alkaline wash, an acid wash, and a final alkaline wash to restore membrane pH with intermediate water flushing (Tang et al., 2010). Sanitization wash after cleaning is very important to kill the remaining microorganisms. It is noteworthy that the CIP procedure can remove 90% of microorganisms from the surface but cannot be relied on to sterilize them. Leading to the recolonization of bacteria on the membrane surfaces, which given time, water, and nutrients form stronger biofilms become more resilient to chemical cleaning protocols (Simões et al., 2010). Various studies have demonstrated the resilience of biofilm-embedded bacteria to the cleaning protocols over their planktonic counterparts (Shi and Zhu, 2009; Araújo et al., 2011; Anand and Singh, 2013). Thus, proving that the cleaning and sanitization protocols tend to be ineffective in adequately removing resilient biofilms (Anand et al., 2012), which raise quality and safety concerns in the final product. Also,

chemicals not being environmentally friendly contribute to the generation of non-biodegradable waste and having a harsh effect the membrane integrity (Regula et al., 2014). A previous study from our lab demonstrated the development of resistance by selective isolates within the resilient biofilms over prolonged use of membranes and the potential of such isolates to acquire predominance over the biofilm microflora (Anand and Singh, 2013). Further reducing the efficiency of the chemical cleaners and subsequently generating microbial resistance against almost all antimicrobial treatments. Also, these isolates generate additional resistance against the new synthetic derivatives as these have already developed resistance to the parent agents (Sumi et al., 2015). Consequently, creating opportunities to develop novel alternative strategies to combat biofilm formation on membrane surfaces.

Biofilms growing on the membranes have been documented to be a problem, resulting in membrane blockage, product contamination, and reduced membrane life due to the microbial activity on the membrane material (Tang et al., 2010). Studies have stated that after cleaning, the thermophilic biofilms that remain on the equipment surface, where the cells remain covered by foulant that persists after incomplete cleaning. It was also observed that binary species biofilms may survive sanitation regimes and may constitute reservoirs of contamination of the product resulting in spoilage and/or food safety hazards (Flint et al., 2020).

1.6. Futuristic sanitizer and biosanitizer

The biofilm microflora is known to develop resistance to the chemical cleaners over prolonged use of membrane and eventually develop resistance to their deviates. This makes the CIP procedures ineffective in cleaning membrane biofilms. Hence, creating a need to

develop natural antimicrobial preparation to inhibit the growth of biofilms on membrane surfaces. Also, the efforts need to be directed towards finding sanitizer that does not promote the resistance of biofilm embedded bacteria.

Among the possibilities, antimicrobial molecules of microbial origin (bacteriocins), provide a promising alternative for a biofilm control strategy. Bacteriocins are known to inhibit the growth of various organisms including pathogens (Ahmad et al., 2017). These substances have a different mode of action including disrupting the integrity of the cell wall, which facilitates pore formation or forms channels to inhibit protein or nucleic acid synthesis (Shelburne et al., 2007; (Sumi et al., 2015). Therefore, natural antimicrobials from microbial origin offer a promise as they would not contribute towards bio-burden accumulation. Several studies have reported a board-spectrum of inhibition of *Bacillus* bacteriocins. Also, some representatives of *Bacillus* spp., such as *Bacillus subtilis* and *Bacillus licheniformis* are categorized as 'generally recommended as safe' (GRAS) bacteria (Teo and Tan, 2005). Moreover, studies have reported the self-inhibition of the producing strain at a certain concentration and contact time (Shelburne et al., 2007; Altuntas, 2013).

Previous studies have also demonstrated the ability of many bacteria to form biofilms through a mechanism called quorum sensing which makes it an attractive target for their control. Giving rise to the possibility that quorum sensing inhibition may represent a natural, widespread, antimicrobial strategy with a significant impact on biofilm formation. Decent knowledge of cell-to-cell signaling phenomenon of bacteria can be used to flight biofilm formation issues on the filtration membrane, by the identification of products that can act as quorum sensing antagonists. This property can help enhance the

life of membranes during the filtration process by effective removal of resilient biofilms from the filtration membranes (Simões et al., 2010).

CONCLUSIONS

The membrane processes are now widely used in the dairy industry. The long filtration runs contribute to the deposition of different types of substrate come from the feed stream on to the membrane surface. When the microorganism coming from the feed encounters the membrane surface, they initiate bacterial attachment and colonization. The blocking of membrane pores due to biofilm formation is known as Biofouling. This is one of the biggest challenges faced by the dairy industry. The biofilm microflora develops resistance to the cleaning procedures. This makes the C.I.P. cleaning protocols ineffective, severely declines the flux rate. Hence, leading to premature replacement of biofilms. This creates a need to develop an alternative, which could be natural, eco-friendly, and biodegradable. The selective isolates from the biofilm microflora emerge predominant over prolonged use of membranes. The emergence of predominance could be associated with several factors. Out of which antimicrobial release by that selective strain would be associated with the emergence of predominance. Being naturally produced from the microbial origin, it provides a promising alternative for a biofilm control strategy. Also, the release of antimicrobial by microbial origin could be linked with the quorum signaling. This intervention strategy can implement to develop a bio-sanitizer exhibiting antimicrobial activity using quorum inhibitors for the prevention of biofilm formation on membranes.

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CHAPTER 2

A competitive exclusion study demonstrates the emergence of Bacillus subtilis as a predominant constitutive microorganism of a whey RO membrane biofilm-matrix

ABSTRACT

Microbial attachment and colonization on separation membranes lead to biofilm formation. Some isolates within the biofilm microflora acquire greater resistance to the chemical cleaning protocols on prolonged use of membranes. It is thus likely that the constitutive microflora might compete with each other, and result in certain species emerging as predominant, especially within older biofilms. To understand the microbial interactions within biofilms, the emergence of predominance was studied in the current investigation. An 18-month-old reverse osmosis membrane was procured from a whey processing plant. The membrane pieces (1 x 1 $inch^2$) were neutralized by dipping in Letheen broth. The resuscitation step was done in Tryptic Soy Broth (TSB) at 37°C, followed by plating on Tryptic Soy Agar (TSA) to recover the constitutive microflora. Distinct colonies of isolates were further identified using MALDI-TOF as *Bacillus licheniformis, Exiguobacterium aurantiacum, Acinetobacter radioresistens, Bacillus subtilis* ('rpoB' sequencing), and one unidentified species each of *Exiguobacterium* and *Bacillus*. Further, the competitive exclusion study helped to establish the emergence of predominance using a co-culturing technique. Fifteen combinations (of two isolates each) were prepared from the isolates. For which, pure cultures of the respective isolates were spiked in the ratio of 1:1 in TSB, and incubated at 37°C for 24 h, followed by plating on TSA. The enumerated colonies were distinguished based on colony morphology, gram staining, and MALDI-TOF to identify the type of the isolate. Plate counts of *Bacillus subtilis* emerged as predominant with a

mean log counts of 7.22±0.22 CFU/ mL. The predominance of *B. subtilis* was also validated using the process of natural selection in a multi-species growth environment. In this case, the TSB broth culture with overnight-incubated membrane piece (with mixed-species biofilm) at 37℃ for 12 h was inoculated in fresh TSB and incubated for the second cycle. Overall, five such sequential broth-culture incubation cycles were carried out, followed by pour plating on TSA plates, at the end of each cycle. The isolates obtained were identified based on colony morphology, gram staining, and MALDI-TOF identification. The fifth subsequent transfer depicted the presence of only one isolate *B. subtilis* on plating, thereby validating its predominance.

Keywords: RO membrane, constitutive microflora, competitive exclusion

INTRODUCTION

Separation membranes are now increasingly used in the dairy and food processing industry (Anand et al., 2012) for diverse applications such as whey protein concentration, milk protein standardization, desalination, wastewater treatment, etc. (Daufin et al., 2001; Turan et al., 2002). Despite numerous benefits, membrane processing has a major limitation of biofouling due to bacterial biofilm formation on the membrane surfaces (Suwarno et al., 2012). Multispecies biofilms are formed when the feed containing microorganisms comes in contact and initiate colonization on the membrane surface (Anand et al., 2012). The multispecies biofilms are known to be more complex and tend to form thicker and more stable biofilms, which could be due to the release of extracellular polymeric substances (EPS) by various bacterial species. (Hassan et al., 2010; Anand et al., 2014). The EPS secreted by multiple species in a biofilm matrix act as a shield and helps to develop resilient biofilms that are very difficult to clean (Anand et al., 2014). Even the cleaning and sanitization protocols prove to be ineffective for removing such resilient biofilms. Such biofouling not only results in a reduced flux rate but also serves as a constant source of contamination. Hence, leading to premature replacement of separation membranes and economic losses faced by the dairy industry (Marchand et al., 2012). The membrane replacement cost is high and could constitute around 25-40% cost of the membrane plant (Souza and Mawson, 2007). Previous studies have reported the presence of *Enterococcus, Staphylococcus, Micrococcus, Streptomyces, Corynebacterium, Bacillus, Klebsiella, Aeromonas, Pseudomonas, Escherichia coli, Streptococcus, Chryseobacterium* and bacteria from *Acinetobactia, Firmicutes* and *Proteobacteria* phylum as constitutive

microflora found on dairy separation membranes (Avadhanula, 2011; Chamberland et al., 2017).

Some organisms present within the biofilm microflora show predominance over others over the prolonged use of membranes (Anand and Singh, 2013). Some of the other studies have also depicted the emergence of single species predominance in biofilms (Flint et al., 2009; Nadell et al., 2016). Competition among microorganisms in a shared environment occurs in all but the simplest ecosystems (Fredrickson and Stephanopoulos, 2017). The populations of microorganisms within the common setting outcompete one microbial population over the other, when the competition is focused on a single resource, hence, emerging as predominant in a constitutive microflora. Several factors can be responsible for emergence of predominance such as competition for the nutrients, faster-growing rate of one microorganism over the others, production of metabolites by the cells, production of bacteriocin, secretion of broad and narrow spectrum toxins with coupled privatized antitoxin, etc. (Bowden and Hamilton, 1989; Amézquita and Brashears, 2002; Nadell et al., 2016). The competitive exclusion is a unique interaction between species, which leads to the predominance along with various degrees of coexistence (DeBach, 1966; Weiner et al., 2017). This is mainly dependent on three factors including the relative growth rate of the microorganisms present in a mixed species, a ratio of the inoculum, and an effective range of toxin (Weber et al., 2014). Studies also support that the coexistence is eventually lost as a result of selective sweeps in growing microbial colonies (Karahan, 2012). Therefore, it is important to understand the emergence of predominance within membrane biofilms. This could provide necessary information to bridge the gap between the development of mature biofilms on prolonged use of membranes, and potential for

selective species to generate resistances leading to the predominance of one microorganism over others within a biofilm matrix. This information would be useful to create targeted clean-in-place strategies to eliminate the resistant biofilm microflora and extend the service life of filtration membranes in the dairy industry, in addition to reducing cross contamination.

MATERIAL AND METHODS

Procurement of a used RO-membrane

An 18 months old reverse osmosis whey concentration membrane was procured from a whey processing commercial plant located in the mid-west region. The used membrane was drawn after the completion of the respective clean-in-place (CIP) cycle. The whey filtration process in that plant was a continuous run used to filter 350 gallons of cheese whey per min at about 60-65°F (15-19°C). The routine CIP protocol involved the combination of alkaline, acid and enzyme wash at the end of each cycle. The membrane was sealed aseptically in a plastic wrap before bringing it to our lab for analysis purposes.

Isolation and identification of membrane constitutive microflora

Isolation through Enrichment Process

The constitutive microflora was isolated by following the protocol described by Anand and Singh (2013) with slight modifications. A cross-sectional piece was aseptically cut out from an eighteen-month-old membrane with the help of a reciprocating saw (DWE 304, DEWALT Industrial Tool Co., Towson, MD, USA). Aseptic conditions were maintained by sterilizing the blade of the electric saw and wiping the surrounding area before cutting the membrane with 70% ethanol. Three membrane pieces (1×1-inch^2) were cut with a pair of sterile scissors and neutralized using Letheen Broth (Puritan™ ESK™

Sampling Kits with Pre-filled Letheen Broth). The composition of Letheen Broth is designed to support the growth of a variety of microorganisms, while lecithin helps to neutralize quaternary ammonium compounds, and the presence of Tween 80 enables it to neutralize the phenolic disinfectants and hexachlorophene present in the disinfectant (Base, 1948; Letheen Media. Instructions for use). Three neutralized membrane pieces were resuscitated by suspending each membrane piece in a bottle containing 200 ml Tryptic Soy Broth (TSB) (Bacto^{TM}, MD, USA) and incubating for 12 h at 37°C (Figure 2. 1). The TSB, being a non-specific medium, was used for the resuscitation process to provide a suitable environment for a variety of the stressed intact microorganisms to grow. After resuscitation, the bottles were taken out from the incubator and the broth was properly mixed by shaking the bottle containing the suspended membrane piece for 25 times in a 1-foot arc within 7 seconds before plating (BAM, 1969). Serial dilutions were pour-plated on TSA and incubated at 37°C for 24 h to enumerate the constitutive microflora.

Identification of isolates

Distinct colonies obtained on the plates prepared via enrichment process were distinguished based on colony morphology and gram staining. Finally, the distinct isolated colonies were identified using Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) at the Veterinary Science Department, South Dakota State University, Brookings. The MALDI-TOF resulted in the identification of most of the isolates except three isolates, which were only identified up to the genus level. Out of which one *Bacillus* isolate, later also identified as the predominant species of the constitutive microflora, was identified up to its species level using ‘rpoB’ sequencing (Department of Food Science, Cornell University).

Co-culture Growth for studying Competitive Exclusion

The competitive exclusion study helped to establish the predominance using the co-culture technique. Six isolates identified using colony morphology, gram staining, and MALDI-TOF were named as VQ1, VQ2, VQ3, VQ4, VQ5, and VQ6. The list of the isolates is presented in Table 2. 2.

All the isolates were individually grown in TSB at 37℃ for 24 h and the plate count exhibited an average mean count of 7 log CFU/ mL. For the competitive exclusion studies, using the co-culture technique, six isolates were paired into fifteen possible combinations of two isolates each (Table 2. 1). For each of these combinations, the overnight grown isolates were suspended in the ratio of 1:1 in Tryptic Soy Broth (TSB), and the co-cultures were incubated at 37℃ for 24 h. Serial dilutions were prepared, and plating was carried out on Tryptic Soy Agar (TSA). As explained before, the isolates on the plates were distinguished based on colony morphology, gram staining, and MALDI-TOF. Finally, the plate counts were compared to establish the predominance of one culture over the other in the co-culture growth.

The emergence of Predominance in a Multispecies Growth Environment

The results of the co-culture growth were further validated using the process of natural selection in a multispecies growth environment. For natural selection study, 1×1 $inch^2$ piece of 18-month -old RO membrane was suspended in Letheen broth to neutralize any chemical residue, then the membrane piece was suspended in TSB and incubated at 37℃ for 12 h (cycle 1 incubation). The sub-culturing of the broth was followed by 5 cycles of subsequent transfers (Figure 2. 2). Simultaneously, serial dilutions were prepared at the

end of each cycle and plated on TSA using the pour plate technique. The enumerated organisms were identified based on colony morphology, gram staining, and MALDI-TOF.

Statistical Analysis

All the experiments were carried out in duplicates and the data were calculated for means values and standard deviations.

RESULTS AND DISCUSSION

Biofilm Constitutive Microflora on an 18-month-old Membrane

The culturing techniques used to isolate constitutive microflora revealed the presence of gram-positive as well as gram-negative bacteria. The distinct isolates obtained using the enrichment process were distinguished based on colony morphology and gram staining. Individually streaked distinct isolates were then outsourced for MALDI-TOF identification. From which, six isolates were identified as *Bacillus licheniformis, Exiguobacterium aurantiacum, Acinetobacter radioresistens,* and one unidentified species of *Exiguobacterium* and two species of *Bacillus*. Out of the unidentified species, one *Bacillus* strain depicting predominant characteristics (explained below in co-culture experiment), was outsourced for 'rpoB' sequencing, and was identified as *Bacillus subtilis*. For the ease of working, these microorganisms were assigned isolate codes, which are depicted in Table 2. 2, along with their colony morphology, gram stain and species name based on MALDI-TOF identification or rpoB sequencing. A similar type of biofilm constitutive microflora was previously also reported on spiral wound membranes, where species associated with *Actinobacteria* and *Bacillus* genera were observed (Chamberland et al., 2017b). Some other studies (Avadhanula, 2011; Tang et al., 2009) on whey RO biofilms have reported the presence of *Bacillus* as a part of biofilm microflora but didn't

report the presence of some other types of microflora found in the current study. This could be due to variations like feed or operational parameters, as these factors are reported to greatly affect the diversity of bacteria colonizing membranes (Chamberland et al., 2017a).

The cell morphology was observed under a light microscope with 1000X magnification power (Table 2. 3) to develop a better understanding of the common type of cellular morphology responsible for forming resilient biofilms. The microscopic observations from different isolates revealed the presence of rod-shaped cells with varied rod length. The gram staining revealed the presence of gram-positive as well as gram-negative bacteria with 50% of the isolates depicted sporulating behavior. Several studies have shown that bacterial spores are much more resilient than vegetative cells (Brown, 2000; MacLean et al., 2013). Therefore, the spore formers might be responsible for forming resilient biofilms that cannot be effectively cleaned with the help of existing CIP chemicals.

Emergence of Predominance

To establish the emergence of predominance within the biofilm microflora of the 18-month-old membrane used in our study, the interaction between 15 possible co-cultures was investigated. Each co-culture combination consisted of 2 isolates and expressed different colony morphology on TSA plates after the co-culture interaction. The isolates obtained on the plates were distinguished based on colony morphology, gram staining, and MALDI-TOF identification. The plate count of different isolates in the co-cultures was recorded and the $\log_{10}$ counts of each co-culture combination were compared. In all the co-culture combinations, *B. subtilis* emerged as predominant with a mean log counts of 7.22±0.22 CFU/ mL (Table 2. 4). It was interesting to note that another isolate, *B. licheniformis,* expressed equally in a co-culture with *B. subtilis* and did not get completely

inhibited. Whereas, other isolates did not show any presence under the co-culture state. The data for all co-culture combination has been depicted in Table 2. 4.

Similarly, previous studies have reported the predominance of spore-forming *Bacillus* spp. in membrane biofilms (Avadhanula, 2011; Anand et al., 2014). This could be associated with commonly present gram-positive microorganisms or thermo-resistant spore-forming *Bacillus* spp. in whey starter population (Tang et al., 2009). The co-culture study in which two isolates were suspended at a time also depicted similar results where *B. subtilis* emerged predominant within the constitutive microflora. The presence of *B. licheniformis* in a co-culture with *B. subtilis* could also be associated with the concept of coexistence (Borenstein et al., 2013; Abrudan et al., 2015). Some studies have demonstrated the presence of *B. subtilis* and *B. licheniformis* together in various raw milk samples and Midwest dairy farms (Brown, 2000; Buehner, 2014).

A string test was conducted for *B. subtilis* and *B. licheniformis* isolates to compare the two isolates for their competing attributes. The presence of mucoid colonies has been associated with predominance of certain strains over others that do not produce mucoid colonies (Stratton, 1983). The test was found to be positive for *B. subtilis* only. In addition, it was noteworthy that hyper-mucoviscosity was observed with a string of > 5mm (Figure 2. 3). This could be one of the potential contributing factors for the predominance of *B. subtilis* in a co-culture growth. Studies have also reported prolonged use of membrane and specific operational parameters used during membrane filtration to be responsible for the emergence of predominance within different bacterial populations in biofilms (Anand and Singh, 2013; Sánchez, 2018). Also, Anand and Singh (2013) reported that selective

bacterial species that developed resistance to the chemical cleaning protocols over prolonged use of membrane acquires predominance in the biofilm microflora.

Validation of Predominance using Mixed-species Growth

A validation study was also conducted to establish the predominance of *B. subtilis* by 'natural selection', using a non-selective nutrient broth. This experiment allowed the multispecies microflora to interact and demonstrate the emergence of predominance, similarly to the one demonstrated in the co-culture growth using combinations of two isolates each. The first cycle of incubation of membrane pieces in TSB yielded all the six isolates previously identified. As the cycles of TSB transfers and subsequent incubations progressed, the types of isolates started to decrease, and finally, only one isolate *B. subtilis* was enumerated at the end of the fifth transfer. Therefore, it can be concluded that *B. subtilis* emerged predominant within both co-culture and multispecies growth conditions. Previous studies have reported that the natural selection process in a well-mixed accelerates the transition process by recurring life cycles (Korolev et al., 2012). A similar approach was adopted in this study where subsequent transfers were used to continue the process, which resulted in the emergence of the predominance of one isolate over the others. Also, it is important to note that there is a possibility for the isolates in the microbial suspension to coexist by simply maintaining a balance between growth rates and effective toxicity (Weber et al., 2014).

CONCLUSIONS

Based on the results obtained, it can be concluded that the isolates within the whey RO biofilm microflora demonstrated the emergence of the predominance of *B. subtilis* amongst all the isolates. This study thus provides important information related to the

constitutive microflora present within an 18-month-old RO-membrane and identification of *B. subtilis* as the predominant species within the biofilm microflora using co-culture and mixed-species growth.

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TABLES AND FIGURES

Table 2. 1 Fifteen combinations of the co-cultures used to demonstrate predominance using a competitive exclusion study.

Fifteen co-culture combinations using six isolates identified from the constitutive microflora of an old RO-membrane				
VQ1-VQ2	VQ2-VQ3	VQ3-VQ4	VQ4-VQ5	VQ5-VQ6
VQ1-VQ3	VQ2-VQ4	VQ3-VQ5	VQ4-VQ6	
VQ1-VQ4	VQ2-VQ5	VQ3-VQ6		
VQ1-VQ5	VQ2-VQ6			
VQ1-VQ6				

Table 2. 2 Identification of constitutive microflora based on colony morphology, gram staining, and MALDI-TOF

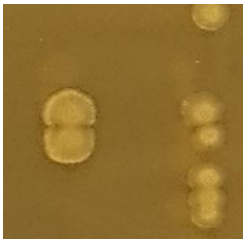
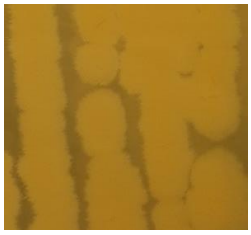
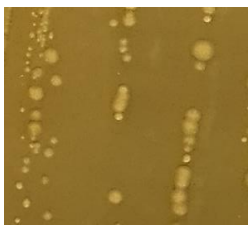
Isolate codes	Description of Colony Morphology	Picture of Colony Morphology	MALDI-TOF Identification/ rpoB sequencing
VQ1	Irregular, undulated and opaque		*Bacillus subtilis*
VQ2	Round, raised and translucent		*Exiguobacterium sp.*
VQ3	Irregular, rough and opaque		*Bacillus licheniformis*
VQ4	Irregular, lobate and opaque		*Bacillus sp.*
VQ5	Round, entire and opaque		*Exiguobacterium aurantiacum*
VQ6	Small colonies, round and opaque		*Acinetobacter radioresistens*

Table 2. 3 Linking colony morphology of an isolate with its cell morphology

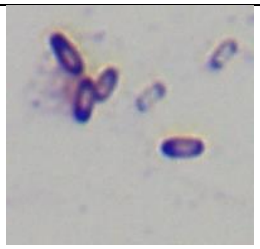
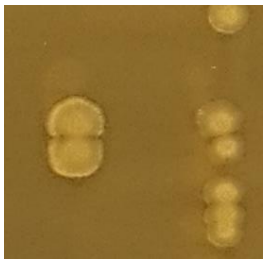
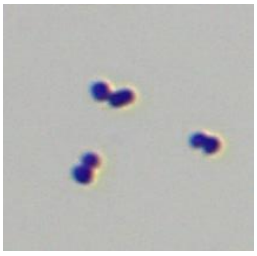
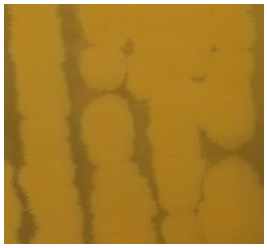
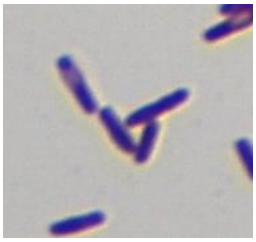
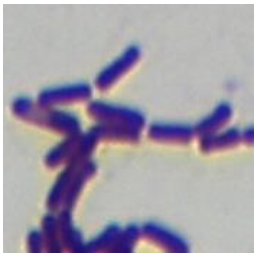
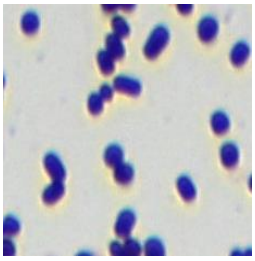
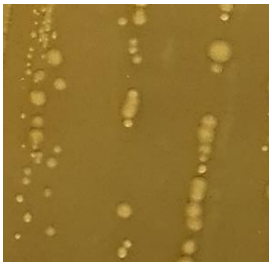
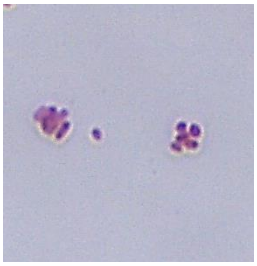
Isolate codes	Colony Morphology	Cell Morphology	Microscopic observation (100X) on cell morphology
VQ1			Gram-positive, coccobacillus, central endospore (spore former), and cells were found individually and in small clumps.
VQ2			Gram-positive, short rods (coccobacillus), non-spore forming, and cells were found in pairs.
VQ3			Gram-positive, long-rod shaped and subterminal endospore (spore former).
VQ4			Gram-positive, slightly curved rods, streptobacilli and spore former.
VQ5			Gram-positive, short rods (coccobacillus), non-spore-forming and cells were found individually, in pairs and clusters.
VQ6			Gram-negative, short rods (coccobacillus), non-spore-forming and cells were found in clusters.

Table 2. 4 Log counts of culture combinations under co-culture growth conditions

Co-culture Combinations	**Log count (CFU/ mL) after 24 h incubation**	
	First organism in the combination	**Second organism in the combination**
VQ1-VQ2	7.72±0.66	-
VQ1-VQ3	6.83±0.67	7.49±1.14
VQ1-VQ4	7.23±0.25	-
VQ1-VQ5	7.53±0.59	-
VQ1-VQ6	7.27±0.48	-
VQ2-VQ3	7.20±0.39	7.57±1.20
VQ2-VQ4	5.33±0.47	7.45±0.65
VQ2-VQ5	7.26±0.29	-
VQ2-VQ6	6.75±0.55	-
VQ3-VQ4	7.30±1.16	6.94±0.35
VQ3-VQ5	7.69±1.32	6.45±0.58
VQ3-VQ6	7.81±1.27	-
VQ4-VQ5	7.14±0.50	-
VQ4-VQ6	7.30±0.32	-
VQ5-VQ6	-	6.97±0.40

(VQ1) *Bacillus subtilis*, (VQ2) *Exiguobacterium sp.*, (VQ3) *Bacillus licheniformis*, (VQ4) *Bacillus sp.*, (VQ5) *Exiguobacterium aurantiacum*, and (VQ6) *Acinetobacter radioresistens*.

*(-) Not detected

Figure 2. 1 Pictorial representation of experimental design used for isolate constitutive microflora via resuscitation.

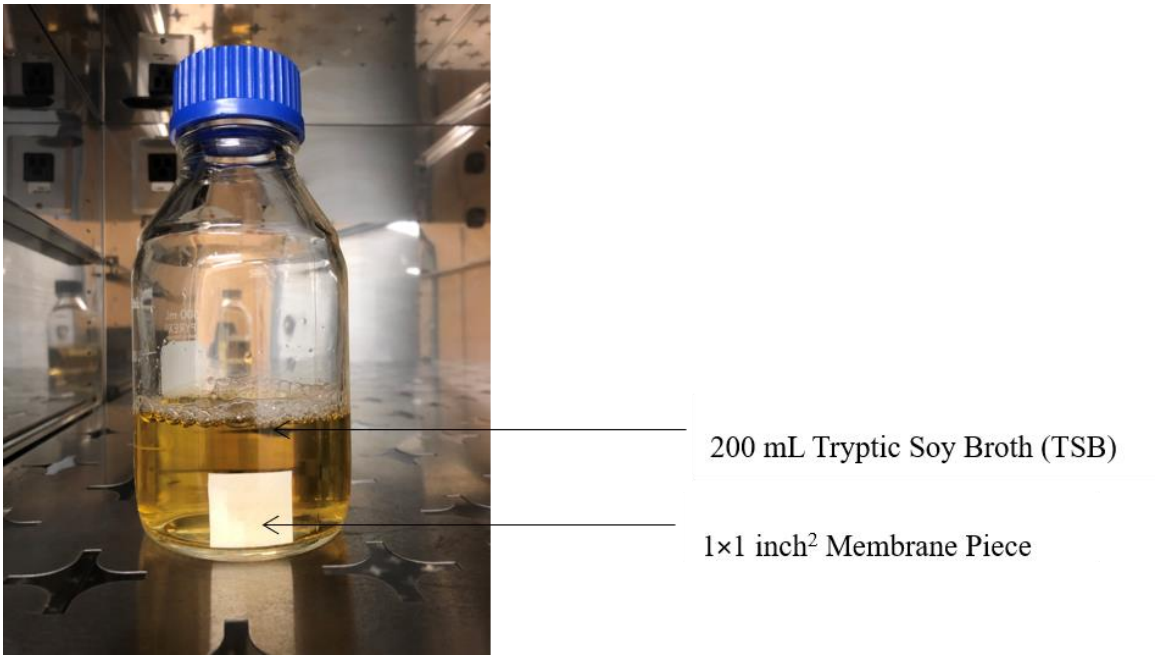


Figure 2. 2 Flow chart of 5 cycles of subsequent transfers to study emergence of predominance in a multispecies growth environment

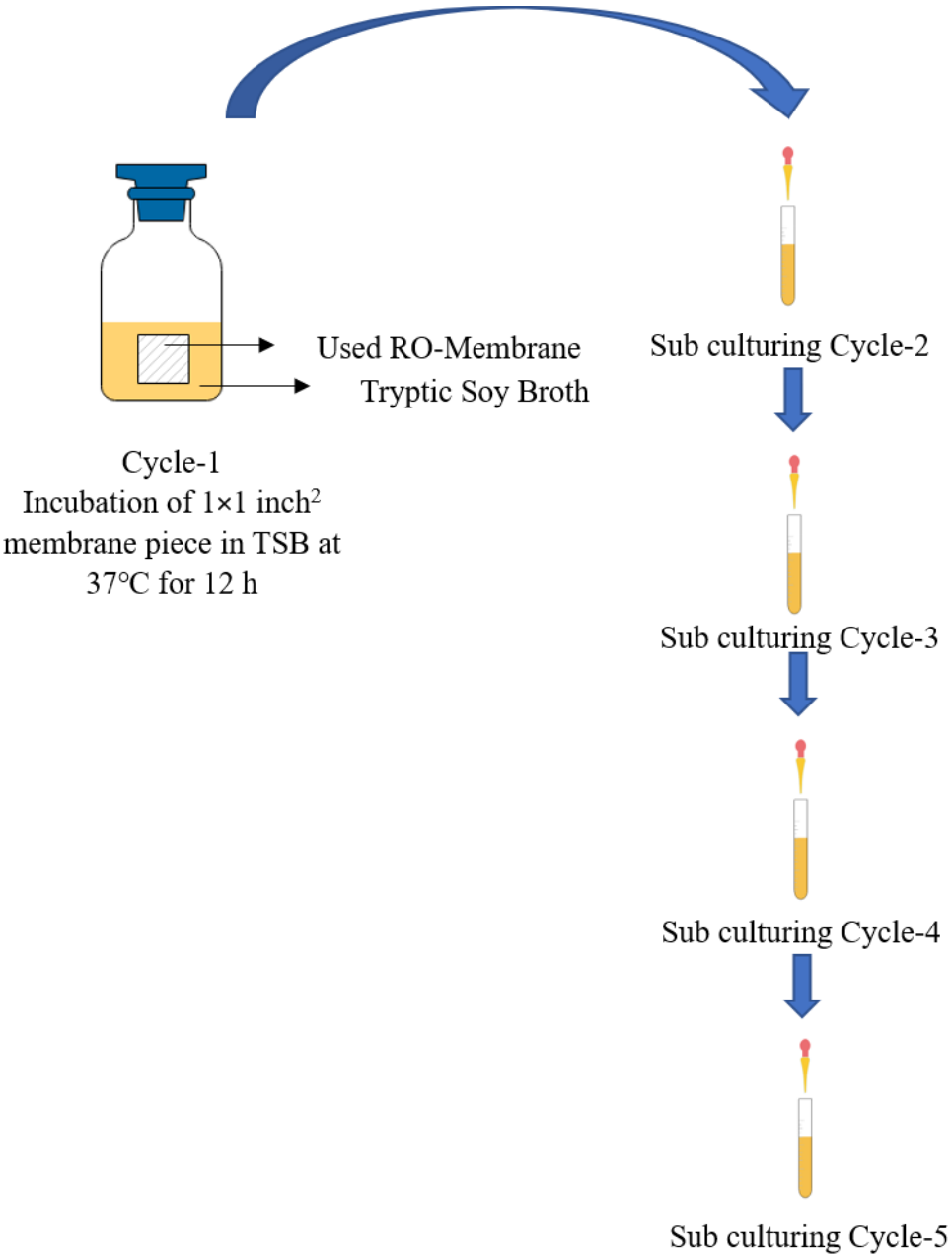
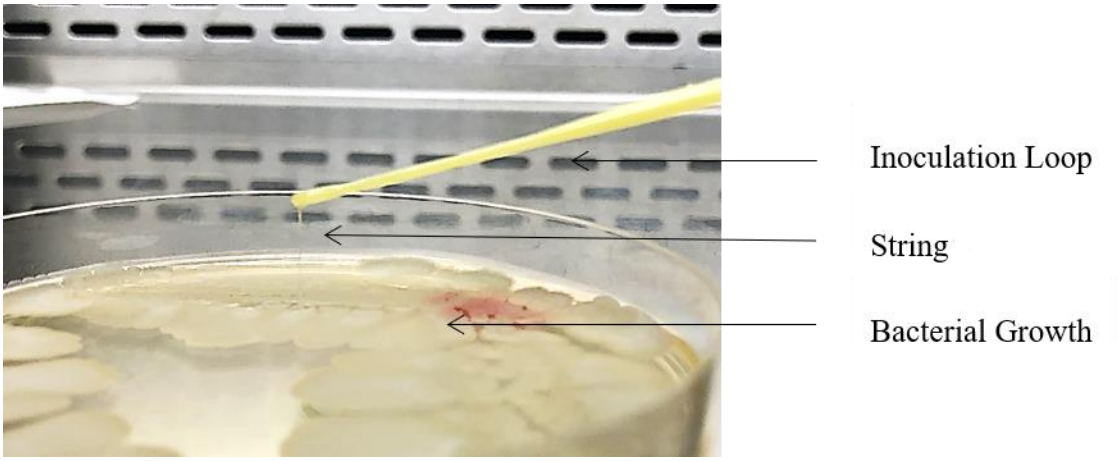


Figure 2. 3 Bacillus subtilis was found positive for string test and gave a hyper mucoid appearance (string size > 5mm) when formed a string



CHAPTER 3

Isolation and identification of the constitutive microflora from UF membrane biofilm-matrix

ABSTRACT

Biofilm formation on dairy separation membranes is one of the most critical issues. As the multispecies biofilms formed on the membrane surface matures, the biofilm microflora generates resistance to the chemical cleaners over prolonged use of membranes. Also, selective resilient strains emerge predominant making the cleaning and sanitization processes ineffective in cleaning separation membranes. This results in a severe decline in flux rate and rising product cross-contamination issues. Therefore, the type of biofilm constitutive microflora was investigated in the present study as it would be vital information to create targeted clean-in-place strategies to eliminate the resistant biofilm microflora**.** Two UF membranes were procured from two different locations. Out of which, a 15-month-old UF membrane was procured from a whey processing commercial plant located in the mid-west region used to concentrate whey. Another UF membrane was procured from the southwest region from a milk processing plant. The UF membrane was 6-8-month-old used for skim milk processing. A cross-sectional piece was aseptically cut out from the UF membranes separately with the help of a reciprocating saw. Aseptic conditions were maintained by sterilizing the blade of the electric saw and wiping the surrounding area with 70% ethanol before cutting the membrane. The membrane pieces (1 x 1 $inch^2$) were neutralized by dipping in Letheen broth. The resuscitation step was done in Tryptic Soy Broth (TSB) at 37°C, followed by plating on Tryptic Soy Agar (TSA) to recover the constitutive microflora. Distinct colonies of isolates were further identified

using MALDI-TOF. All the experiments were carried out in triplicates with a replicate of three. A total of 8 isolates were identified from both membranes based on colony morphology and gram staining. Distinct colonies from the 15-month-old UF-membrane were identified as *Enterococcus faecalis, Rahnella aquatilis,* and two unidentified species of *Exiguobacterium* and 6-8-month-old UF membrane revealed the presence of *Klebsiella oxytoca*, *Citrobacter freundii, Bacillus licheniformis,* and one unidentified species of *Enterococcus* using MALDI-TOF identification. Therefore, these isolates can be further studied to establish any emergence of predominance within biofilm microflora and creating a targeted cleaning procedure to remove resilient biofilms from the surface of separation membranes.

Keywords: Biofilm microflora, resilient, MALDI-TOF

INTRODUCTION

Fouling of filtration membranes remains one of the major issues affecting the efficiency of filtration processes in the dairy industry. Fouling begins when the feed constituting of several components comes in contact with the membrane surface (Chamberland et al., 2017a). Which includes but is not limited to proteins, minerals, casein micelles, and microflora within the feed. Multispecies biofilms are formed when the feed containing microorganisms comes in contact and initiate colonization on the membrane surface (Verma et al., 2020). The multispecies biofilms are known to be more complex and tend to form thicker and more stable biofilms, which could be due to the release of extracellular polymeric substances (EPS) by various bacterial species (Hassan et al., 2010; Anand et al., 2014).

As the biofilms formed on the membrane surface mature, the resilient strains become difficult to be cleaned as they generate chemical resistance over prolonged use of a membrane. Also, the selective resilient strains acquire predominance within the biofilm microflora (Anand and Singh, 2013). Even the cleaning and sanitization protocols prove to be ineffective for removing such resilient biofilms. These resilient biofilms not only result in a reduced flux rate but also serves as a constant source of contamination. Hence, leading to premature replacement of separation membranes and economic losses faced by the dairy industry (Anand et al., 2012; Marchand et al., 2012).

Previous studies have reported the presence of *klebsiella oxytoca*, *Enterobacter* sp., *Lactococcus lactis* ssp., *Bacillus* sp., and *Chryseobacterium* sp. and bacteria from *Acinetobactia, Firmicutes,* and *Proteobacteria* phylum on dairy UF separation membranes (Tang et al., 2009; Chamberland et al., 2017).

Some organisms present within the biofilm microflora show predominance over others over the prolonged use of membranes (Anand and Singh, 2013). Some of the other studies have also depicted the emergence of single species predominance in biofilms (Flint et al., 2009; Nadell et al., 2016).

Therefore, it is important to understand the composition of biofilm on the surface of separation membranes and the emergence of predominance within membrane biofilms. It is important to note here that this is only a preliminary study to compare the biofilm microflora of the UF membrane with RO, and further studies are required to draw any conclusions. This could provide necessary information to bridge a gap between limited data available concerning the composition of biofilms found at the surface of filtration membranes and its relation with the long-term failure of numerous strategies developed to control biofilm formation on filtration membranes (Chamberland et al., 2017b). This information would be useful to create targeted clean-in-place strategies to eliminate the resistant biofilm microflora and extend the service life of filtration membranes in the dairy industry, in addition to reducing cross-contamination.

MATERIAL AND METHODS

Procurement of a used UF-membrane

Two UF membranes were procured from two different locations. Out of which, a 15-month-old UF membrane was procured from a whey processing commercial plant located in the mid-west region used to concentrate whey. The used membrane was drawn after the completion of the respective clean-in-place (CIP) cycle which was carried out at 75-115°F (23.89-46.11°C). Another UF membrane was procured from the southwest region from a milk processing plant. The UF membrane was 6-8-month-old used for skim milk

processing. The used membrane was drawn after the completion of the respective clean-in-place (CIP) cycle which was carried out at 115-120°F (46.11-48.89°C). The ultrafiltration process in that plant was a continuous run for 24-36 h at about 36-38°F (2.22-3.33°C). The routine CIP protocol involved the combination of alkaline, acid, and enzyme wash at the end of each cycle. Both membranes were sealed aseptically in a plastic wrap before bringing it to our lab for analysis purposes.

Isolation of membrane constitutive microflora through the enrichment process

The constitutive microflora was isolated by following the protocol described by Anand and Singh (2013) with slight modifications. A cross-sectional piece was aseptically cut out from the UF membranes separately with the help of a reciprocating saw (DWE 304, DEWALT Industrial Tool Co., Towson, MD, USA). Aseptic conditions were maintained by sterilizing the blade of the electric saw and wiping the surrounding area with 70% ethanol before cutting the membrane. Three membrane pieces (1×1-inch^2) were cut with a pair of sterile scissors from each UF membrane and the pieces were neutralized using Letheen Broth (Puritan™ ESK™ Sampling Kits with Pre-filled Letheen Broth). The composition of Letheen Broth is designed to support the growth of a variety of microorganisms, while lecithin helps to neutralize quaternary ammonium compounds, and the presence of Tween 80 enables it to neutralize the phenolic disinfectants and hexachlorophene present in the disinfectant (Base, 1948; Letheen Media. Instructions for use). Three neutralized membrane pieces were resuscitated by suspending each membrane piece in a bottle containing 200 ml Tryptic Soy Broth (TSB) (Bacto^{TM}, MD, USA) and incubating for 12 h at 37°C. The TSB, being a non-specific medium, was used for the resuscitation process to provide a suitable environment for a variety of the stressed intact

microorganisms to grow. After resuscitation, the bottles were taken out from the incubator and the broth was properly mixed by shaking the bottle containing the suspended membrane piece for 25 times in a 1-foot arc within 7 seconds before plating (BAM, 1969). Serial dilutions were pour-plated on TSA and incubated at 37°C for 24 h to enumerate the constitutive microflora.

Identification of membrane constitutive microflora

Distinct colonies obtained on the plates prepared via enrichment process were distinguished based on colony morphology and gram staining. Finally, the distinct isolated colonies were identified using Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) at the Veterinary Science Department, South Dakota State University, Brookings. The MALDI-TOF resulted in the identification of most of the isolates except few isolates, which were only identified up to the genus level.

RESULTS AND DISCUSSION

Biofilm Constitutive Microflora from old UF-Membrane

Constitutive microflora of a 15-month-old UF-membrane

The culturing techniques used to isolate constitutive microflora revealed the presence of gram-positive as well as gram-negative bacteria. The distinct isolates obtained using the enrichment process were distinguished based on colony morphology and gram staining. Individually streaked distinct isolates were then outsourced for MALDI-TOF identification. From which, four isolates were identified as *Enterococcus faecalis*, *Rahnella aquatilis*, and two unidentified species of *Exiguobacterium*. These microorganisms were assigned isolate codes, which are depicted in Table 3. 1, along with their colony morphology, and species name based on MALDI-TOF identification.

The cell morphology was observed under a microscope with 100X magnification power (Table 3. 2) to develop a better understanding of the common type of cell structures responsible for forming resilient biofilms. The microscopic observations from different isolates revealed the presence of rod and cocci-shaped cells. The gram staining revealed the presence of gram-positive and gram-negative bacteria.

Constitutive microflora of a 6-8-month-old UF-membrane

Four isolates were identified using MALDI-TOF as *Klebsiella oxytoca*, *Citrobacter freundii*, *Bacillus licheniformis,* and one unidentified species of *Enterococcus* from a 6-8-month-old UF membrane used for skim milk processing. These microorganisms were assigned isolate codes, which are depicted in Table 3. 3, along with their colony morphology and species name based on MALDI-TOF identification.

Also, the cell morphology was studied to understand the common type of cell structures responsible for forming resilient biofilms. The microscopic observations from different isolates revealed the presence of rod and cocci-shaped cells (Table 3. 4). The presence of spore former was also observed. The ram stain revealed the presence of gram-positive as well as gram-negative bacteria within the biofilm constitutive microflora.

Previous studies have isolated various kinds of bacteria from dairy UF membranes which demonstrated the presence of *klebsiella oxytoca*, *Enterobacter* sp., *Lactococcus lactis* ssp., *Bacillus* sp., and *Chryseobacterium* sp. and bacteria from *Acinetobactia, Firmicutes,* and *Proteobacteria* phylum were also found as constitutive microflora on dairy separation membranes (Tang et al., 2009; Chamberland et al., 2017a). Likewise, this study revealed the presence of similar biofilm microflora including *Klebsiella oxytoca, Bacillus sp., Enterococcus sp.*, etc. Also, the presence of some other types of microflora reported in

previous studies was not found in the current study. This could be due to variations like feed or operational parameters, as these factors are reported to greatly affect the diversity of bacteria colonizing membranes (Chamberland et al., 2017a). Studies have also reported a strong ability of the *Klebsiella* strains to attach to the surfaces (Tang et al., 2009).

Whereas, studies have reported the predominance of spore-forming *Bacillus* spp. in the RO membranes which might be responsible for generating more resilient biofilms (Avadhanula, 2011). Also, our previous study on the RO-membrane revealed the presence of three spore-forming *Bacillus* spp. including *Bacillus subtilis* and *B. licheniformis* as a part of the biofilm microflora from an 18-month-old RO-membrane (Verma et al., 2020). It is noteworthy that the presence of gram-positive as well as gram-negative bacteria was observed in both UF and RO membrane which could be the result of post-pasteurization cross-contamination.

CONCLUSIONS

This study revealed the presence of eight microorganisms from two different UF membranes used for whey concentration and skim milk processing. The species identified belonged to genus *Exiguobacterium, Enterococcus, Rehnella, Klebsiella, Citrobacter,* and *Bacillus*. The presence of these bacteria has also been reported in previous studies. The information obtained helped us to understand the variability within the biofilm constitutive microflora of UF-membrane used in the dairy industry. The data generated should be used in developing evidence on the emergence of predominance within the constitutive microflora using competitive exclusion studies as carried out in the previous chapter. It is important to note here that this is only a preliminary study to compare the biofilm

microflora of the UF membrane with RO, and further studies are required to draw any conclusions.

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wound membranes used in the dairy industry. Dairy Sci. Technol. 96:827–843. doi:10.1007/s13594-016-0305-2.

Flint, S.H., P.J. Bremer, and J.D. Brooks. 2009. Biofilms in dairy manufacturing plant - description , current concerns and methods of control BIOFILMS IN DAIRY MANUFACTURING PLANT- 7014. doi:10.1080/08927019709378321.

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TABLES AND FIGURES

Table 3. 1 Identification of constitutive microflora of a 15-month-old UF membrane used for concentrating whey-based on colony morphology and MALDI-TOF

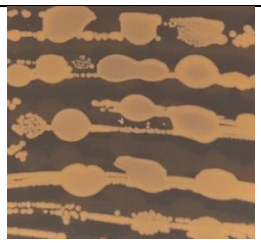
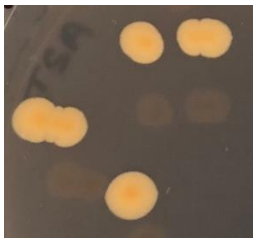
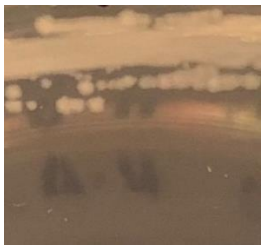
Isolate codes	**Description of Colony Morphology**	**Picture of Colony Morphology**	**MALDI-TOF Identification/ rpoB sequencing**
UF1	Circular, fused, dull and translucent		*Exiguobacterium sp.*
UF2	Round, fused, pigmented, and raised		*Exiguobacterium sp.*
UF3	Small colonies, round and opaque		*Enterococcus Faecalis*
UF4	Irregular, undulated margins and opaque		*Rahnella aquatilis*

Table 3. 2 Linking colony morphology of an isolate with its cell morphology from microorganisms isolated from a 15-month-old UF membrane

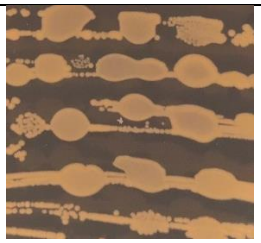
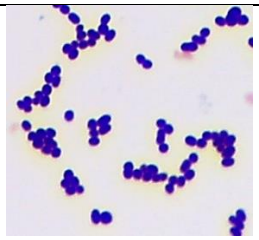
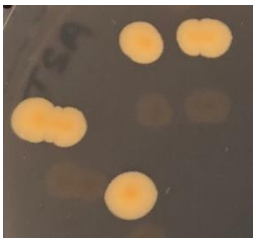
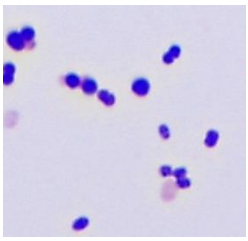
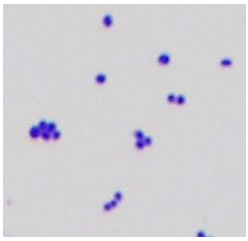
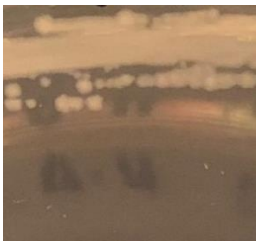
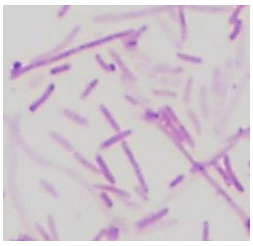
Isolate codes	Colony Morphology	Cell Morphology	Microscopic observation (100X) on cell morphology
UF1			Gram-positive, bacillus, non-spore forming, and cells were found in cluster.
UF2			Gram-positive, short rods, non-spore forming, and cells were found in pairs.
UF3			Gram-positive, cocci-shaped, and cells found in pairs and short chains.
UF4			Gram-negative and rod-shaped bacteria.

Table 3. 3 Identification of constitutive microflora of a 6-8-month-old UF membrane used for skim-milk processing based on colony morphology and MALDI-TOF

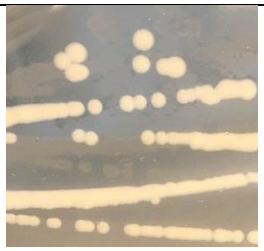
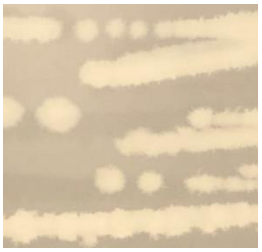
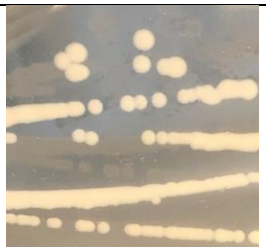
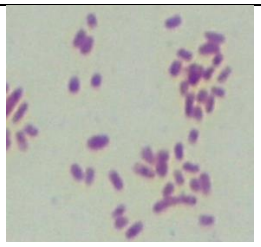
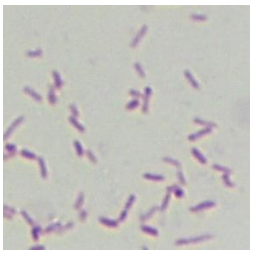
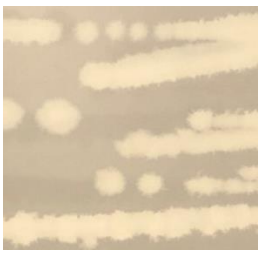
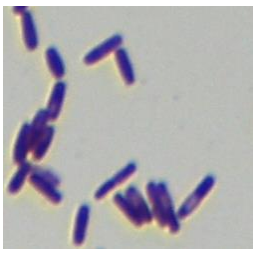
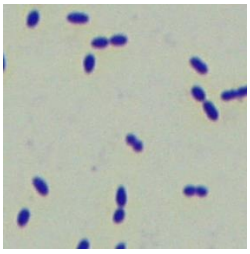
Isolate codes	**Description of Colony Morphology**	**Picture of Colony Morphology**	**MALDI-TOF Identification/ rpoB sequencing**
FL1	Circular, entire margins and opaque		*Klebsiella oxytoca*
FL2	Entire margin, smooth, low and convex		*Citrobacter freundii*
FL3	Irregular, rough and opaque		*Bacillus licheniformis*
FL4	Small colonies, round and opaque		*Enterococcus sp.*

Table 3. 4 Linking colony morphology of an isolate with its cell morphology from microorganisms isolated from a 6-8-month-old UF membrane

Isolate codes	Colony Morphology	Cell Morphology	Microscopic observation (100X) on cell morphology
FL1			Gram-negative and rod-shaped bacteria.
FL2			Gram-negative, rod-shaped bacteria and cells were found individually, and no pairing was observed.
FL3			Gram-positive, long-rod shaped and subterminal endospore (spore former).
FL4			Gram-positive and cocci-shaped bacteria and cells were found in pairs.

CHAPTER 4

Antimicrobial activity may lead to the predominance of Bacillus subtilis within the constitutive microflora of a whey RO membrane biofilm.

ABSTRACT

Current cleaning and sanitation protocols may not be adequately effective in cleaning separation membranes and result in the formation of resilient multispecies biofilms. The matured biofilms may result in a bacterial predominance with resilient strains, on prolonged use of membranes. In our previous study, we isolated organisms such as *Bacillus subtilis, Bacillus licheniformis, Exiguobacterium aurantiacum,* and *Acinetobacter radioresist*ance from an 18-month-old reverse osmosis membrane. The competitive exclusion studies revealed the predominance of *B. subtilis* within the membrane biofilm microflora. This study investigated the antimicrobial activity of the *B. subtilis* isolate as a potential cause of its predominance. The culture isolate was propagated in tryptic soy broth (TSB) at 37°C, and micro-filtered to prepare cell-free extracts (CFEs) at 8, 10, 12, 14, 16 and 18 h intervals. The CFEs were freeze-dried and suspended in minimum quantities of HPLC grade water to prepare concentrated solutions. The antimicrobial activities of CFEs were tested using the agar-well assay against the biofilm constitutive microflora and some common food pathogens. The experiments were conducted in triplicates and means were compared for significant differences using a general linear mixed model procedure of SAS. The results indicated the highest antimicrobial activity of 12 h CFE of *B. subtilis* against other constitutive microflora such as *Exiguobacterium* sp., *E. auranticum,* and *A. radioresistens, Listeria monocytogenes*, *Bacillus cereus,* and *Salmonella enteritidis* with average inhibition zone sizes of 16.5 ± 0.00, 16.25 ± 0.66, 20.6 ± 0.00, 18.0 ± 1.00 , 13.83

± 0.58 and 9.00 ± 1.32 mm, respectively. On treating with proteinase K, the CFE completely lost its antimicrobial activity, establishing it to be a proteinaceous compound. The amino acid profiling revealed the total crude protein in CFE to be 51% (wt/wt) having its major constituent to be glutamic acid (11.30% wt/wt). The freeze-dried CFE was thermally stable on exposure to the common temperature used for sanitizer applications (23.8°C for 5 and 10 min) and over a pH range of 3.0-6.3. The results indicate the potential role of the antimicrobial compound produced by *B. subtilis* as a cause of its predominance within the biofilm constitutive microflora.

Keywords: Biofilms, Antimicrobial Activity, Predominance

INTRODUCTION

Several microorganisms tend to attach to the surface of the membrane and initiate bacterial colonization on the membrane surfaces (Anand et al., 2012). These are also referred to as biofilms that consist of complex communities of microorganisms (Tait and Sutherland, 2002). These biofilms are hard to clean and have been reported to develop resistance to chemical processes as compared to planktonic counterparts (Anand et al., 2014; Sayem et al., 2018). Proving the cleaning and sanitization protocols to be ineffective and result in the formation of resilient multispecies biofilms (Stoodley et al., 2002). When the biofilm microflora is subjected to disinfectants, the sensitive cells die, but resilient cells develop bacterial resistance against such chemical cleaners. This emphasizes the need to discover new eco-friendly natural antimicrobials for effective membrane cleaning and prevention of resistance development in the biofilm constitutive microflora. Our previous study on the resilient biofilm microflora has reported developing resistance by selective isolates over prolonged use of membranes and their potential to gain predominance in the biofilm matrix (Anand and Singh, 2013). The microbial resistance development makes the CIP cleaning protocols unsuccessful by reducing the efficiency of almost all antimicrobial treatments. Besides, these strains rapidly generate additional resistance against new synthetic derivatives as these have already acquired resistance to the parent agents (Sumi et al., 2015). This has encouraged the researchers to focus on novel alternative strategies to control biofilm formation. In recent years, various approaches have been followed to eliminate biofilm development. Some studies have focused to prevent the adhesion of microbes and others on inhibition of biofilms by killing the microorganisms attached to the contact surfaces (Thallinger et al., 2013). The emergence of predominance could be

associated with several factors including competition for the nutrients, faster-growing rate of one microorganism over the others, production of metabolites by the cells, production of bacteriocins, secretion of broad and narrow spectrum toxins with coupled privatized antitoxin, etc. (Bowden and Hamilton, 1989; Amézquita and Brashears, 2002; Nadell et al., 2016). Other decisive factor that can potentially lead to the prevalence of certain microbial species over the others within mixed-species biofilms are the surface charge, cell chaining, inoculum composition, oxygen availability or release of certain matrix protein-like 'TasA', which provide structural integrity to *Bacillus subtilis* biofilms, etc. (Van Merode et al., 2007; Chen, 2019). According to Van Merode et al. (2007), the surface charge can influence the prevalence of one microorganism within a mixed-species biofilm. Besides, studies have demonstrated the importance of multi-species biofilms, in which, one bacterial strain may stimulate the attachment of other bacterial strains (Bradshaw et al., 1996; Tait and Sutherland, 2002; Van Merode et al., 2007). Under these conditions, a strain may protect the other against disinfectants, and hence, lead to its predominance within a mixed species growth. Also, it is possible to observe the occurrence of bacteriocin activity in the presence of closely related strains as a competitive trait (Tait and Sutherland, 2002). Thus, the use of natural antimicrobial peptides has the potential to eliminate the issue of ineffectiveness, due to the generation of antimicrobial resistance (Lisboa et al., 2006a, Rotem and Mor, 2009). These substances mainly form channels or interact directly with the cell membrane and cause membrane disruption leading to bacterial cell death (Shelburne et al., 2007; Sumi et al., 2015). The release of bacteriocin is however, likely to be also influenced by environmental factors and selective forces (Bucci et al., 2011). This thus provides an edge to such substances to prevent biofilm formation over the

conventional chemical cleaners. Previous studies have reported that some antimicrobial substances are produced by the genus *Bacillus* (Lisboa et al., 2006a; Abriouel et al., 2011). Such *Bacillus* bacteriocins have gained attention due to their broad-spectrum inhibition and have shown antagonistic effect against fungi, bacteria, and insects (Hammami et al., 2009). Studies have supported that such bacteriocins inhibit the growth of closely related species (Lisboa et al., 2006b; Nadell et al., 2016), and self-inhibition of the producing strain at a certain level of bacteriocin production (Balakrishnan et al., 2002; Altuntas, 2013). Our study is one such attempt where we screened the predominant *Bacillus subtilis* isolate for natural antimicrobials that might have inhibited the growth of the other constitutive microflora of an 18-month-old RO membrane biofilm. The *B. subtilis* strain used in this study was originally isolated in the previous study conducted in our lab, along with other constitutive microflora (Verma et al., 2020).

MATERIAL AND METHODS

Bacterial isolates, growth conditions, and culture media

Bacillus subtilis, used in this study, was originally isolated during a previous study conducted in our laboratory using an 18-month-old RO membrane obtained from a whey processing commercial plant (Verma et al., 2020). The *B. subtilis* strain was observed to be predominant within the RO membrane-biofilm constitutive microflora.

The other isolates obtained from the biofilm microflora of that membrane that were used in this study included *Exiguobacterium* sp., *Bacillus licheniformis, Bacillus* sp., *Exiguobacterium aurantiacum,* and *Acinetobacter radioresistance.* All these isolates were stored in cryovials at −75°C in a deep freezer (Nuarie, Plymouth, MN). The culture isolates were activated in the Tryptic Soy Broth (TSB) (Bacto™, MD, USA) by inoculating a single

bead from the stock cryovials and incubating at 37℃ for overnight. *Micrococcus luteus* (ATCC 10240) was used as the indicator strain due to its high sensitivity (Oscáriz et al., 1999), for initial screening antimicrobial activity of the CFE of *B. subtilis*, using agar well assay. The culture was grown using Nutrient broth (Difco™, MD, USA) for 72 h at 30°C. Other pathogenic cultures including *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 6538 and *Listeria monocytogenes* ATCC 19115 were purchased from Microbiologics, and *Escherichia coli* ATCC 35041 and *Salmonella enteritidis* ATCC 13076 were purchased from American Type Culture Collection (ATCC). All the pathogenic cultures were grown in specific media and for optimum time-temperature conditions recommended by ATCC.

Antimicrobial activity of the *B. subtilis* Cell-free extract (CFE)

Preparation of the freeze-dried CFE

The predominant *B. subtilis* isolate from the RO membrane biofilm microflora was selected for the antimicrobial study. To evaluate the release of an antimicrobial substance, 1 mL activated culture of *B. subtilis* was inoculated into 100 mL Tryptic Soy Broth (TSB), and incubated for 12 h at 37℃. The culture growth was centrifuged at 6,000 g (7,200 rpm) at 4℃ for 15 min (Camargo et al., 2003), and micro-filtered using 0.22 µm Millipore filter (Stericup Quick Release, EMD Millipore Corporation, Billerica, MA, USA) to prepare the cell-free extract (CFE). The CFE was kept at -75℃ in a deep-freezer (Nuaire, Plymouth, MN) for overnight, and subsequently freeze-dried (LABCONCO Corporation, Kansas City, Missouri), by setting up the freeze-drier at 0.498 mBar vacuum pressure with the collector temperature at -50±2℃.

Screening for the antimicrobial activity of freeze-dried CFE against the test strain M. luteus

The antimicrobial activity of the CFE was screened using the in-vitro agar well assay method described by Balouiri et al. (2016). The freeze-dried CFE of *B. subtilis*, containing 51% protein content, was reconstituted by adding 0.7620 g in 650 µl of sterile HPLC grade water. Petri plates were poured with 13 mL of the Tryptic Soy Agar (TSA), pre-inoculated with 500 µl of the activated test culture, *M. luteus* to achieve 7-8 logs CFU/ mL. To perform the agar well assay, four holes, of 6 mm each, were punched in the solidified medium, using a sterile cork-borer, and 20 µl of the reconstituted CFE was put into each of the wells. Three wells were used to put the sample (reconstituted CFE), while sterile HPLC grade water was put into the fourth well as a control. The plates were incubated at 37℃, and intermittently examined, for the zones of inhibitions, up to 12 hours

Optimizing the release of antimicrobial substance by B. subtilis during culture incubation

The samples drawn at different stages of the growth curve of *B. subtilis* were examined to identify the incubation duration necessary for the maximum antimicrobial activity. The *B. subtilis* overnight growth was inoculated in tryptic soy broth (TSB) to achieve low inoculation levels of about 2 $\log_{10}$ CFU/ mL, and the culture was incubated in a shaking incubator at 37℃ for 72 h. To examine the growth, the culture absorbance was measured at 600 nm at regular intervals (Kim et al., 2011; Sharma et al., 2018). The culture dilutions were also plated to relate the absorbance to the viable counts at a particular time interval. The micro-filtered CFEs were prepared at 8, 10, 12, 14, 16 and 18 h intervals, were freeze-dried, and tested for antimicrobial activity as explained earlier. The incubation

period was correlated with the antimicrobial activity of CFEs to ascertain the incubation duration needed for maximizing the antimicrobial activity.

Characterization of the freeze-dried CFE

Enzymatic digestion to confirm the proteinaceous nature of the antimicrobial substance

The 12 h CFE, showing maximum antimicrobial activity, was treated with different enzymes as per their respective reaction times (Table 4. 1) to determine the nature of substance responsible for bacterial growth inhibition. To maintain similar protein concentration before digestion, the enzyme was added in a way that did not alter the volume to weight proportion for reconstituting CFE (650 µL HPLC grade water for 0.7620 g of freeze-dried CFE). The pH of the mixture and incubation temperature were adjusted to those optimal for the respective enzyme activities, followed by agar-well assay to compare the zones of inhibition. All experiments were conducted three times, with samples tested in the replicates of 3 for each trial, against one of the sensitive strains of the constitutive microflora of the 18-month-old RO membrane (*Exiguobacteria* sp. (VQ2).

Crude protein estimation and amino acid profiling of the freeze-dried CFE

The crude protein content of the freeze-dried CFE (prepared using lyophilization) was analyzed using a protein analyzer (FLASH 1112 series EA, Thermo Finnigan) by following the modified Dumas method (AACC, 11^{th} edition). In this method, approximately 100 mg of the sample was weighed (using Mettler Toledo AT21 Comparator weighing balance) and kept in the analyzer. The protein analyzer connected to a detector represented the nitrogen percent (%) that was used to calculate the protein content of each sample by multiplying with 6.25 (as a conversion factor). All the samples

were analyzed in duplicates. The freeze-dried CFE was analyzed for amino acid profiling and to validate the protein content, estimated using protein analyzer (outsourced to Dr. Thomas P. Mawhinney Experiment Station Chem Labs (ESCL), University of Missouri. Columbia, MO).

Stability of the antimicrobial activity of the freeze-dried CFE to temperature and pH

The ability of the CFEs to retain the antimicrobial activity at the commonly used sanitization temperature, in a typical CIP protocol, was tested by exposing the reconstituted freeze-dried CFE to 24℃ for 5 and 10 min. This time-temperature combination was similar to the sanitizer step for cleaning of RO membranes using a commercial sanitizer, Oxonia (EcoLab Inc., St. Paul, Minnesota, U.S.A.). After the heat treatment, 20 µl of the CFE was tested antimicrobial activity using the agar well assay, as described earlier in methodology, against *M. luteus*.

To study the antimicrobial stability of the CFE to pH changes, the pH of the reconstituted CFEs was adjusted to 3.0, 4.0, and 5.0 using a 1N HCL solution. The samples were held at room temperature for 1 min (Sutyak et al., 2008) and tested for their antimicrobial activity with agar-well assay against *M. luteus,* as explained above.

Minimum Inhibitory Concentration (MIC) of the antimicrobial substance in the freeze-dried CFE

The freeze-dried CFE was evaluated for the MIC, which is the lowest concentration of the antimicrobial compound required to prevent growth of the test strain (Rota et al., 2008). To evaluate the MIC, the gradient test described by Syal et al. (2017), with slight modification was followed. Activated *M. luteus,* with an inoculum size of 10^7-10^8 log CFU/ mL, were seeded in the agar and 20 µL of the CFE with varying concentrations (100-10%)

was suspended in 6 mm wells. The plates were incubated at 37℃ and zones of inhibition were observed for 12 h at different time intervals (6, 8 and 12 h).

Inhibition of the constitutive microflora of the RO membrane biofilm and extended spectrum using freeze-dried CFE

The inhibition spectrum of the antimicrobial substance produced by *B. subtilis* was tested against the constitutive microflora of the RO membrane biofilm and common food-borne pathogens using the in-vitro agar well assay method (Balouiri et al., 2016). A similar experimental design was followed, as mentioned above, for screening the antimicrobial activity using the indicator organism *M. luteus*. After adding the reconstituted CFE in the wells, the plates were incubated at 37℃. The zones of inhibition were observed at 6, 8 and 12 h of incubation.

Statistical Analysis

Each experiment was conducted three times, with samples in replicates of 3 and means were compared for significant differences at $P < 0.05$ for the zones of inhibition using a general linear mixed model (GLMM) procedure of SAS.

RESULTS AND DISCUSSION

Antimicrobial activity of the CFE of *B. subtilis* and its optimization

To investigate the release of an antimicrobial compound by *B. subtilis*, the reconstituted freeze-dried CFE of *B. subtilis* (pH 6.4) was screened against the indicator strain, *Micrococcus luteus* using agar well assay. It is noteworthy that the zones of inhibition were observed at 6 h incubation at 37℃. Therefore, revealing its antimicrobial ability against the test strain (Figure 4. 1).

Optimizing the release of the antimicrobial substance using growth of B. subtilis

The growth curve of *B. subtilis* helped in optimizing maximum zones of inhibition as studies have demonstrated that the antimicrobial substance is produced in a growth-associated manner (Hammami et al., 2009). The results obtained demonstrated a similar trend when the freeze-dried CFEs prepared at 8, 10, 12, 14, 16 and 18 h were tested for their antimicrobial activity. Based on the zones of inhibition, 12 h CFE depicted maximum antimicrobial activity against *M. luteus* (Figure 4. 1). The eight-hour CFE did not exhibit any antimicrobial activity. It was interesting to note that a previous study (Bhuvaneswari et al., 2015) an isolate of *B. subtilis* exhibited the maximum release of antimicrobial activity at 24 h, pH 7.0 with an incubation temperature of 37℃. Another study (Hussain, 2017) supported the maximum antimicrobial release by *B. subtilis* at neutral pH (7.0) and 37℃ incubation for 36 h. Also, Khochamit et al. (2015) showed a drop in inhibition after a certain time of bacterial growth. The growth curve study also helped to understand the cultivation time of *B. subtilis*. It is noteworthy to observe that *B. subtilis* didn't reach the death phase even at 72 h. Hammami et al., (2009) depicted similar results where *B. subtilis* was observed in the stationary phase at 72 h when the time course of *B. subtilis* was studied. This could be due to the sporulating nature of the culture.

Maximizing the zone of inhibition size on test plates

The 12 h CFE optimized for maximum release of antimicrobial was selected to optimize the size of inhibition zones on agar-well assay plates. For which, the test plates containing 20 µL of reconstituted freeze-dried CFE in the wells were incubated at 37℃, and zones of inhibition were measured at 6, 8 and 12 h. Means of the zones of inhibition from different time intervals (h) were compared. The maximum zones of inhibition were

obtained at 12 h incubation of growth plates (Table 4. 2). It is important to note that the zones of inhibition remained constant on growth plates after 12 h of incubation.

Characterization of the freeze-dried CFE

Effect of enzyme digestion on antimicrobial activity

Bacteriocin production, a trait by bacteria to secrete proteinaceous substances to suppress the growth of the competitors could be associated with antimicrobial activity (Bucci et al., 2011). Therefore, the objective to determine the sensitivity of freeze-dried CFE to proteolytic enzymes was to establish whether the antimicrobial production in *B. subtilis* is proteinaceous or not. The inhibition assay revealed complete loss of activity when the CFE was treated with proteinase k and the activity significantly decreased by pepsin, trypsin, and chymotrypsin, establishing the proteinaceous nature of the CFE of *B. subtilis* (refer Table 4. 1).

Studies related to loss of antimicrobial activity of *B. subtilis* have reported similar results where complete loss of activity was observed when the compound was treated with proteinase k (Wu et al., 2013) and reduction in inhibition zones were observed on treatment with pepsin and trypsin (Teo and Tan, 2005). Whereas, another study demonstrated complete loss of activity when treated with trypsin but little or no effect was observed when CFE of *B. subtilis* was treated with proteinase k (Compaoré et al., 2013). Therefore, the characteristics of this antimicrobial substance is different than the previously reported compounds, which suggest that this could be a new type of compound released by *B. subtilis* responsible for inhibition of other biofilm constitutive microorganisms.

Protein estimation

The protein analyzer determined the presence of 51% total crude protein content in 12 h freeze-dried CFE prepared from *B. subtilis*. This was further validated by outsourcing the freeze-dried 12 h CFE for amino acid profiling. This revealed the presence of 51% crude protein in CFE and provided the distribution of amino acids contributing to the total crude protein concentration (Figure 4. 2). From which, glutamic acid was found to be the major constituent of the amino acid present in the CFE with 11.30%. Previous studies have stated the importance of glutamic acid in exhibiting antimicrobial activity and have associated its presence with more effective bactericidal activity (Avigad, 1970; Lee et al., 2014; Ajayeoba et al., 2019).

The CFE antimicrobial stability

The thermal tolerance of the antimicrobial activity of CFE makes it suitable for a sanitizer application for membranes. Exposure to low pH had no apparent effect on the protein at any of the pH values ranging from 3.0-5.0. The purpose of testing the stability of the antimicrobial at sanitizer cleaning specification was to understand the usefulness of this antimicrobial to be incorporated as the sanitizer step in the regular RO membrane CIP cleaning regime.

Minimum-inhibitory Concentration of freeze-dried CFE

The minimum inhibition activity of antimicrobial substance release by *B. subtilis* was detected using agar-well assay against the indicator strain *M. luteus*. The results obtained revealed the MIC of reconstituted freeze-dried CFE to be as low as 10% (Figure 4. 3). The results thus suggest a potential to produce a cost effective biosanitizer for industry applications. Current studies in our lab are addressing this aspect.

Inhibition spectrum of freeze-dried CFE against the biofilm constitutive microflora and the extended spectrum

The antimicrobial activity of freeze-dried CFE was tested against the constitutive microflora of the RO membrane. The agar well assay showed that the reconstituted freeze-dried CFE of *B. subtilis* was active against most of the biofilm constitutive microflora. Clear zones of inhibitions were observed in the agar well assay for the constitutive microflora (Figure 4. 4). The antimicrobial activity of *B. subtilis* thus demonstrated its ability to inhibit a variety of bacteria. Another study (Sumi et al., 2015) has also reported the broad spectrum of inhibition of *B. subtilis* against gram-positive and gram-negative bacteria. On the other hand, several other studies on bacteriocins have demonstrated their ability to inhibit the growth of closely related species to the producing strain (Lisboa et al., 2006; Abriouel et al., 2011; Ansari et al., 2012).

The antimicrobial activity of freeze-dried CFE was also tested against some common food-borne pathogens. The agar-well assay demonstrated zones of inhibition against *Listeria monocytogenes*, *Bacillus cereus,* and *Salmonella enteritidis* (Figure 4. 5). The results of antimicrobial activity again proved its inhibition against gram-positive and gram-negative bacteria. Therefore, it demonstrated broad-spectrum of inhibition including food-borne pathogenic bacteria.

Another study (Sumi et al., 2015) has also reported the broad spectrum of inhibition of *B. subtilis* against gram-positive and gram-negative bacteria, including *Salmonella* and *B. cereus*. On the Contrary, some previous studies have revealed the inhibition of *B. cereus, L. monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* (Zheng and Slavik, 1999; Fernandes et al., 2007; Sumi et al., 2015), whereas the antimicrobial compound release by

B. subtilis in this study didn't exhibit inhibition against *S. aureus* and *E. coli*. Therefore, the characteristic of this antimicrobial compound is different than the existed compounds which suggest that this could be a new type of compound released by *B. subtilis* responsible for inhibition of various microorganisms.

CONCLUSIONS

To our knowledge, antimicrobial activity against membrane biofilm constitutive, by the antimicrobial substance produced by a *B. subtilis* isolate of the membrane biofilm origin, has been reported for the first time. In conclusion, the present study revealed that the *B. subtilis* of membrane biofilm origin is a promising source of an antimicrobial substance that could be associated with its predominance within the biofilm constitutive microflora. Further studies are being conducted in our lab to study the active site responsible of antimicrobial activity and the effect of protein folding on the inhibition spectrum.

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TABLES AND FIGURES

Table 4. 1 Effect of enzymatic digestion on antimicrobial activity of CFE of *Bacillus subtilis.*

Enzyme	**Enzyme diluents**	**Enzyme concentration (mg mL^{-1})**	**Incubation time (hours)**	**Zones of inhibition (mm)***
Proteinase k	dd water	10	6	No zone detected
Pepsin	10 mmol l^{-1} HCL	10	1	13.16 ± 0.05
Trypsin	1 mmol l^{-1} HCL	20	1	12.74 ± 0.01
Chymotrypsin	1 mmol l^{-1} HCL	10	1	13.50 ± 0.10

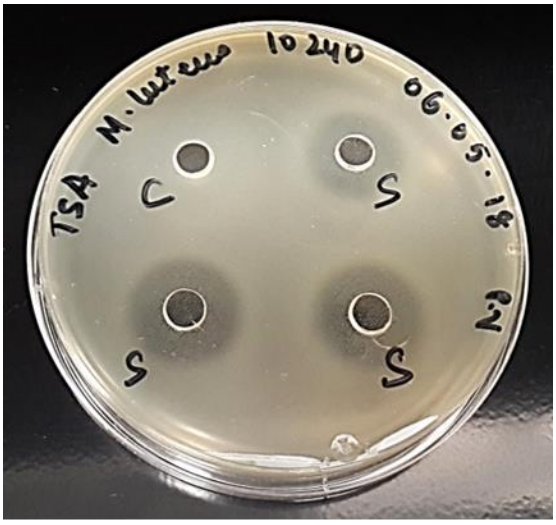
*Zone of inhibition includes well size (6mm)
Note: Average zone of inhibition obtained from untreated reconstituted cell-free extract (CFE) against VQ2 (*Exiguobacterium* sp.) is 16.50 ± 0.00 mm (including 6 mm well size).

Table 4. 2 Maximum zones of inhibition (18.33±0.00 mm) were observed on agar-well assay plates at 37℃/ 12 h against *Micrococcus luteus*

Incubation time (hour)	**Zones of inhibition (mm)***
12	18.33 ± 0.00
8	17.78 ± 0.25
6	15.61 ± 1.58

*Average zones of inhibition include 6 mm of well size

Figure 4. 1 Clear zones of inhibition obtained by reconstituted freeze-dried CFE of Bacillus subtilis against Micrococcus luteus.



Micrococcus luteus

(18.33 ± 0.00 mm)

Note:
1. Average zones of inhibition include 6 mm of well size
2. The notation ‘C’ represents the control (HPLC grade water); ‘S’ the reconstituted CFE sample

Figure 4. 2 Amino Acid Profile revealed glutamic acid to be the major constituent (11.30%) of the freeze-dried CFE.

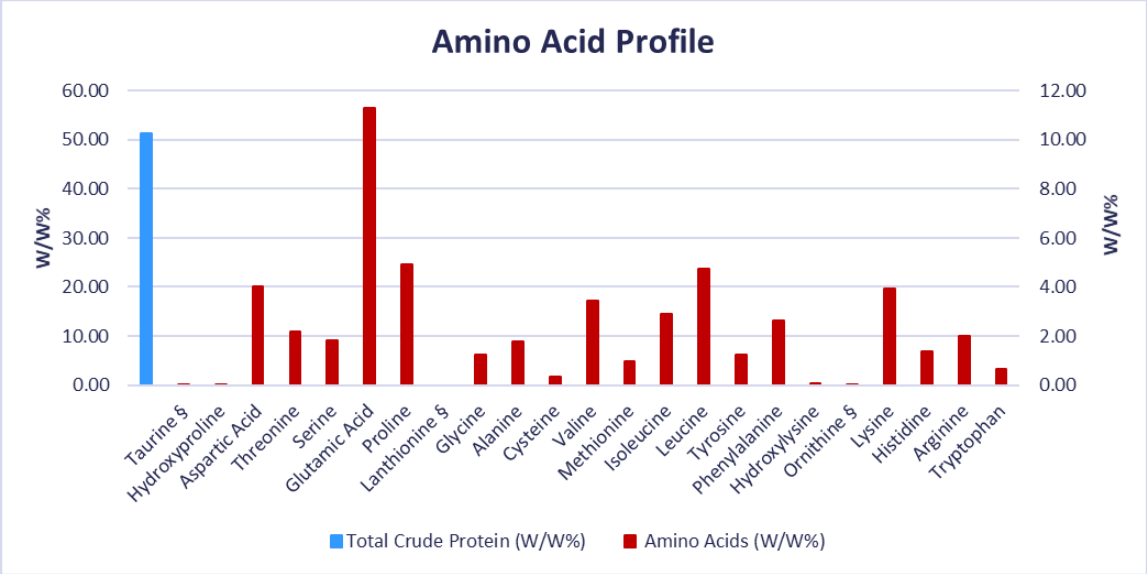
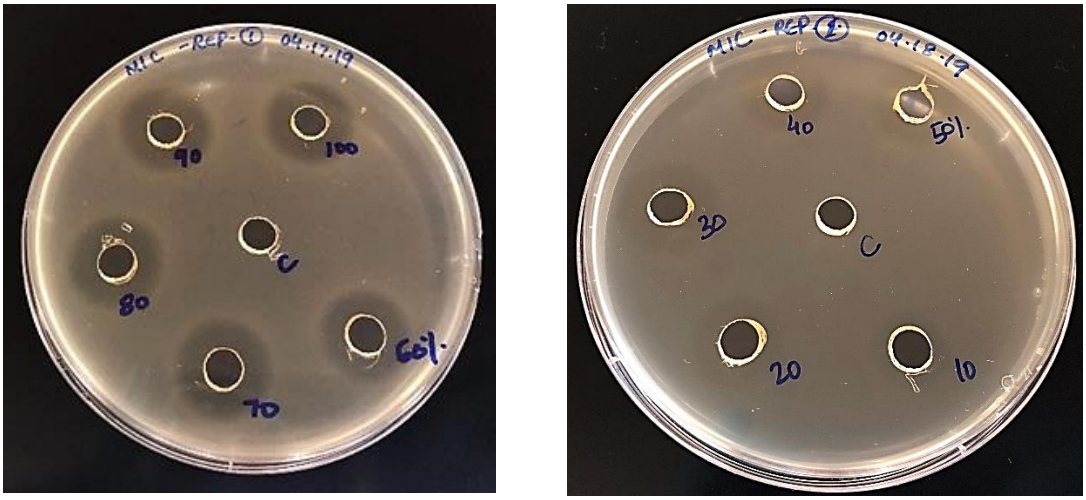
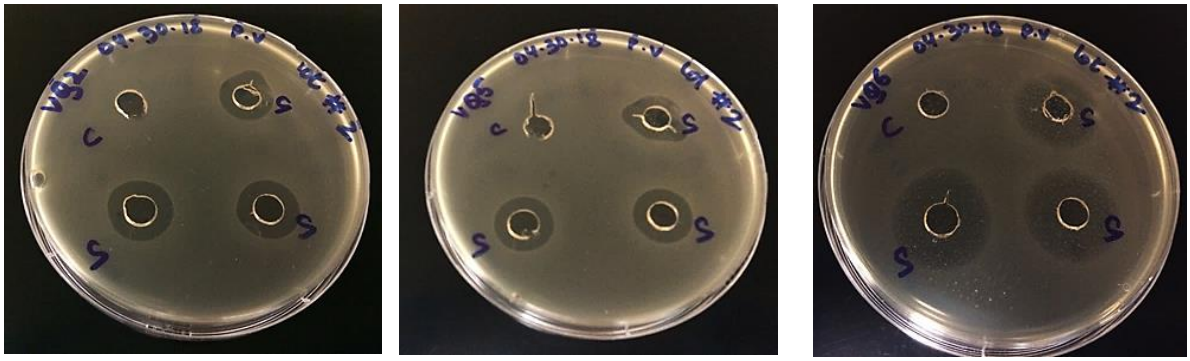


Figure 4. 3 The minimum inhibitory concentration of reconstituted freeze-dried CFE (10 to 100% w/v).



The notation ‘C’ represents the control (HPLC grade water)

Figure 4. 4 Inhibition spectrum of freeze-dried CFE of Bacillus subtilis against the constitutive microflora of the RO membrane.



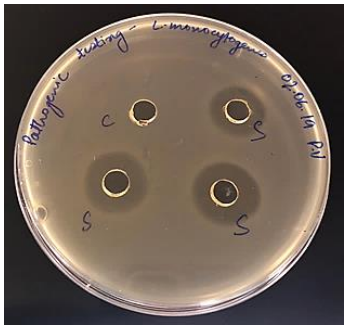
Exiguobacterium sp. (16.50 ± 0.00 mm)

Exiguobacterium aurantiacum (16.25 ± 0.66 mm)

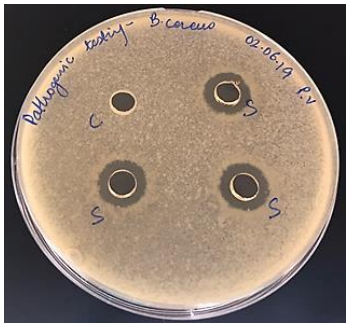
Acinetobacter radioresistens (20.60 ± 0.00 mm)

Notes:
1. Average zones of inhibition include 6 mm of well size
2. The notation ‘C’ represents the control (HPLC grade water); ‘S’ the reconstituted CFE sample

Figure 4. 5 Inhibition spectrum of freeze-dried CFE of Bacillus subtilis against some common food-borne pathogens.



Listeria monocytogenes
(18.00 ± 1.00 mm)



Bacillus cereus
(13.83 ± 0.58 mm)

Salmonella enteritidis
(9.00 ± 1.32 mm)

Notes:
1. The notation ‘C’ represents the control (HPLC grade water); ‘S’ the reconstituted CFE sample
2. From the food-borne pathogens, *Staphylococcus aureus* and *Escherichia coli* didn’t show any zones of inhibition.

CHAPTER 5

Evaluation of an antimicrobial from Bacillus subtilis with a commercial sanitizer for membrane biofilms – A proof of concept

ABSTRACT

Membrane biofilms are likely to develop resistance against the chemical cleaning protocols over prolonged use of membranes. Also, selective microorganisms within biofilms emerge resilient and acquire predominance over a period. In our previous study, we identified the predominance of *Bacillus subtilis* in an 18-month-old RO membrane from a cheese whey processing plant. The antimicrobial was established as one of the factors for its predominance and was tested for effectiveness to remove membrane biofilms. A dialyzed cell-free extract (CFE) of *B. subtilis* was prepared and concentrated using dialysis tubing to achieve a protein content of 97.01 mg/ mL. It was screened for antimicrobial activity against *Micrococcus luteus* using the spot-on-lawn method. After identifying its potential to inhibit the growth of the test strain, the inhibition spectrum of the dialyzed CFE was determined against its producing strain and the constitutive microflora of the biofilm matrix (isolated in our previous study). To evaluate the effectiveness of crude antimicrobial preparation for removing biofilm, sterile virgin RO membrane pieces (1×1 $inch^2$) were used to generate 72 h old in-vitro biofilms using a resilient *B. subtilis* strain. Filter-sterilized (passed through 0.22 µm Millipore filter) whey inoculated with 7 logs of *B. subtilis* was used as a menstruum to develop biofilms using a shaking incubator at 37°C for 72 h. The spent whey was replaced after every 8-9 h with fresh sterile whey and viability of the culture was checked by streaking. The membrane pieces with 72 h old biofilms were exposed to a common CIP protocol by replacing the commercial sanitizer step with the

dialyzed natural antimicrobial preparation. The swab technique was used to obtain viable counts of membrane biofilm. The antimicrobial screening and inhibition spectrum trials were conducted in triplicates with a replicate of 3. For the evaluation trial, Mean SPC of these treatments was compared using one-way analysis of variance (ANOVA) model in GLM procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) and results were presented in log reduction of Pre-CIP counts when membrane pieces were subjected to Pre-sanitizer and Post-antimicrobial treatment. The dialyzed CFE showed self-inhibition of the producing strain and all the isolates of the biofilm constitutive microflora. The in-vitro biofilm logs were 6.68 ± 0.12 CFU/ $inch^2$, after 4 steps industrial process the residual counts reduced to 2.18 ± 0.54 logs CFU/ $inch^2$ which further reduced to 1.20 ± 0.09 logs CFU/ $inch^2$. This shows significant decrease in growth of biofilm microflora. The use of natural antimicrobial is a way better approach which would not lead to antimicrobial resistance due to its mode of action. Thus, the study provides a proof of concept for the efficacy of the antimicrobial substance released by *B. subtilis*, which shows promise for future development of biosanitizers.

Keywords: *Bacillus subtilis*, Antimicrobial, Biosanitizer

INTRODUCTION

Under optimal conditions, the filtration membranes should be prevented from forming biofilms than later addressing biofilm issues. Unfortunately, currently, there is no technique available for preventing biofilms to form on the membrane surfaces during processing. Therefore, dairy industries have adapted Clean-in-place regimes for removal of biofilms. To ensure efficient removal of foulants from membrane surfaces, proper cleaning regimes should be selected that do not promote microbial growth (Simões et al., 2010). The standard CIP procedure used for cleaning RO membranes consists of five stages, which include an alkaline wash, an acid wash, an alkaline wash, an enzyme wash and a final sanitizer wash (Tamime, 2009). The membranes are flushed with water in between different stages to remove adhered residues and biofilm debris (Garcia-Fernandez, 2016). Usually, not all stages of the CIP procedure are carried out at the end of each processing cycle. The most commonly used CIP procedure includes an alkaline wash, an acid wash, and a final alkaline wash to restore membrane pH with intermediate water flushing (Tang et al., 2010). Sanitization wash after cleaning is very important to kill the remaining microorganisms. It is noteworthy that the CIP procedure can remove 90% of microorganisms from the surface but cannot be relied on to sterilize them. Leading to the recolonization of bacteria on the membrane surfaces, which given time, water and nutrients form stronger biofilms become more resilient to chemical cleaning protocols (Simões et al., 2010). Various studies have demonstrated the resilience of biofilm-embedded bacteria to the cleaning protocols over their planktonic counterparts (Shi and Zhu, 2009; Araújo et al., 2011; Anand and Singh, 2013). Thus, proving that the cleaning and sanitization protocols tend to be ineffective in adequately removing resilient biofilms (Anand et al.,

2012), which raise quality and safety concerns in the final product. Also, chemicals not being environmentally friendly contribute to the generation of non-biodegradable waste and having harsh effect the membrane integrity (Regula et al., 2014). A previous study from our lab, demonstrated the development of resistance by selective isolates within the resilient biofilms over prolonged use of membranes and potential of such isolates to acquire predominance over the biofilm microflora (Anand and Singh, 2013a). Further reducing the efficiency of the chemical cleaners and subsequently generating microbial resistance against almost all antimicrobial treatments. Also, these isolates generate additional resistance against the new synthetic derivatives as these have already developed resistance to the parent agents (Sumi et al., 2015). Consequently, creating opportunities to develop novel alternative strategies to combat biofilm formation on membrane surfaces. Among the possibilities, antimicrobial molecules of microbial origin (bacteriocins), provide a promising alternative for a biofilm control strategy. Bacteriocins are known to inhibit the growth of various organisms including pathogens (Ahmad et al., 2017). These substances have a different mode of action including disrupting the integrity of the cell wall, which facilitates pore formation or forms channels to inhibit protein or nucleic acid synthesis (Shelburne et al., 2007; Sumi et al., 2015a). Therefore, natural antimicrobials from microbial origin offer a promise as they would not contribute towards bio-burden accumulation. Several studies have reported a board-spectrum of inhibition of *Bacillus* bacteriocins. Also, some representatives of *Bacillus* spp., such as *Bacillus subtilis* and *Bacillus licheniformis* are categorized as ‘generally recommended as safe’ (GRAS) bacteria (Teo and Tan, 2005). Moreover, studies have reported the self-inhibition of the

producing strain at a certain concentration and contact time (Shelburne et al., 2007; Altuntas, 2013).

In our previous study (Verma and Anand, 2020), a natural antimicrobial produced by *Bacillus subtilis* was identified to inhibit the growth of most of the constitutive microflora isolated from an 18-month-old RO membrane. On characterization, the antimicrobial was found to be proteinaceous. In this study, the proteinaceous antimicrobial substance was tested for self-inhibition. Resilient 72 h old biofilm were generated using the predominating strain, *B. subtilis* from the constitutive microflora of 18-month-old RO membrane and efficiency of antimicrobial preparation was tested against the resilient biofilm under in vitro conditions. This information serves as a proof-of-concept that the natural antimicrobial could be used as a sanitizer step in place of the commercial sanitizers used in a typical CIP.

MATERIAL AND METHODS

Bacterial strains, growth conditions and culture media

Bacillus subtilis used throughout this study was originally isolated during a previous study conducted (Verma et al., 2020) in our laboratory, using an 18-month-old RO membrane obtained from a commercial cheese whey processing plant. The *B. subtilis* strain was found predominant within the RO membrane constitutive microflora.

The other isolates obtained from the biofilm microflora of that 18-month-old membrane, along with *B. subtilis*, have also been used in this study namely *Exiguobacterium* sp., *Bacillus licheniformis*, *Bacillus* sp., *Exiguobacterium aurantiacum*, and *Acinetobacter radioresistance*. The isolates were stored in cryovials at −75°C in the deep freezer (Nuarie, Plymouth, MN). The culture isolates were activated in Tryptic Soy

Broth (TSB) (BactoTM, MD, USA) by inoculating a single bead from the stock cryovials and incubating at 37℃ for overnight. *Micrococcus luteus* was used as a test strain for initial screening of the antimicrobial activity of the CFE of *B. subtilis* using the spot-on-lawn method. *Micrococcus luteus* ATCC 10240 was purchased from the American Type Culture Collection (ATCC) (Manassas, VA). The culture was grown using Nutrient broth (DifcoTM, MD, USA) for 72 h at 30°C.

Antimicrobial activity of the B. subtilis dialyzed Cell-free extract (CFE)

Preparation of the dialyzed CFE

The predominant *B. subtilis* isolate of the RO membrane biofilm microflora was used to prepare the dialyzed CFE. The screening of antimicrobial substance released by *B. subtilis* already provided positive antimicrobial activity results when used in the freeze-dried form (Verma and Anand, 2020).

The dialyzed CFE was prepared using SnakeSkin ™ Dialysis Tubing with 3,500 MW cut-off (Thermo Scientific, Rockford, IL). The *B. subtilis* culture grown for 12 h was centrifuged at 6,000 xg (7,200 rpm) at 4℃ for 15 min (Camargo et al., 2003), and micro-filtered using 0.22 µm Millipore filter (Stericup Quick Release, EMD Millipore Corporation, Billerica, MA, USA) to prepare cell-free extract (CFE). Freshly prepared CFE of *B. subtilis* was filled in the dialysis tubing and material was safely secured with the help of SnakeSkin ™ dialysis Tubing Clips (Thermo Scientific, Rockford, IL). The CFE was dialyzed using the concentrator powder (G-Biosciences, Gene Technology Inc., USA), commonly used for concentrating solutions by dialysis (Figure 5. 1). The concentrator powder rapidly absorbs water from the sample and dialyze the sample by reducing its volume. After generously adding the concentrator powder on to the dialysis tube containing

the sample, it was kept at 4℃. The dialysis tube was inspected visually during the holding duration, and the concentrator was removed once the desirable volume was achieved. To accelerate the process, the wet concentrator was removed according to the manufacturer's instructions and the dialysis tubing was kept again in the fresh concentrator powder until the desired reduction in volume was attained.

For the dialyzed CFE preparation (Figure 5. 2), the protein content was quantified to be 97.01 mg/ mL with the application of a direct A280 method using the NanoDrop (ND-2000) spectrophotometer (Thermo scientific) (Figure 5. 3). The A280 method for protein quantification was implemented as it gives direct measurement, it is quick and easy and due to the accessibility of smaller volumes of CFE after dialysis.

Screening for the antimicrobial activity of dialyzed CFE against the test strain M. luteus

The dialyzed CFE having a protein content of 97.01 mg/ mL was tested against the indicator strain, *Micrococcus luteus* ATCC 10240 due to its high sensitivity (Oscáriz et al., 1999). The antimicrobial activity of dialyzed CFE was tested using the spot-on-lawn method (Tsukatani et al., 2009), in which 3 µL of dialyzed CFE was spotted onto the top layer of 6 ml of the soft layer that was inoculated with 72 h grown *M. luteus* culture at a level of 10^7-10^8 CFU/ mL, overlaid on a regular pre-poured TSA plate. After spotting the dialyzed CFE, the plates were refrigerated for 30 min for the CFE to diffuse into the agar layer and then the plates were incubated at 37℃. The bacterial lawns were observed at 6, 8 and 12 h for any antimicrobial activity.

The inhibition spectrum and the Minimum Inhibition Concentration (MIC) of the dialyzed CFE

The antimicrobial activity was tested primarily against *B. subtilis* and the rest of the microorganisms isolated from the biofilm microflora. The inhibition spectrum of 12 h dialyzed CFE was analyzed using the spot-on-lawn method mentioned above. The bacterial lawns were observed at 6, 8 and 12 h for any antimicrobial effect.

Also, the antimicrobial activity of the dialyzed CFE was enhanced by concentrating the CFE to higher protein concentration for MIC screening purpose. The objective of enhancing the activity was to compare the MIC of dialyzed CFE v/s freeze-dried CFE of *B. subtilis* having a protein content of 175. 95 mg/ mL. The dialyzed CFE with 175.45 mg/ mL protein content (analyzed using NanoDrop 2000) was evaluated for its minimum inhibitory concentration (MIC). The MIC is the lowest concentration of the antimicrobial compound required to prevent bacterial growth (Rota et al., 2008). To evaluate the MIC, the gradient test described by Syal et al. (2017) with slight modification was followed. Petri plates were poured with 13 mL of the Tryptic Soy Agar (TSA), inoculated using activated *M. luteus* culture with an inoculum size of 10^7-10^8 log CFU/ mL. To perform the agar well assay, 6 holes of 6 mm each were punched in the solidified medium, using a sterile cork-borer, and 20 µL of the dialyzed CFE with varying concentration was put into each of the wells. Out of 6 wells, 1 well was used to add sterile HPLC grade water as a control. The plates were incubated at 37℃, and examined for the zones of inhibitions, intermittently up to 12 h.

Biofilm formation by the predominant Bacillus subtilis and effectiveness of natural antimicrobial to remove biofilm

Bacillus subtilis, the predominant isolate was propagated and used to develop 72 h old biofilm under lab conditions. The membrane pieces used to generate 72 h old biofilm were subjected to the clean-in-place protocol and natural antimicrobial preparation by replacing commercial sanitizer step to determine the effectiveness of the natural antimicrobial in removing resilient biofilms.

Procurement of New RO Membrane

A spiral wound new RO membrane constructed with a polypropylene outer shelf to minimize channeling & fluid by-pass was purchased from a membrane manufacturer (FilmTec™ Hypershell™ RO-8038). This membrane has a wide pH tolerance (2.00-10.00) and low thermal stability (50°C). The cross-sectional pieces of the membrane were cut with the help of a reciprocating saw (DEWALT DWE304, DEWALT Industrial Tool Co., Towson, MD) under sterile conditions. The cross-sectional pieces were dipped in soak solution (1% Ultrasil MP) and were stored in the R&D cold store of Davis Dairy plant in closed containers.

Sourcing whey for using as the suspension medium

Cheese whey was used as a suspension medium for the growth of *B. subtilis* to form biofilms. The cheese whey was obtained from a commercial cheese plant and was immediately stored in the deep freezer at -75°C until further use. The whey was thawed under refrigeration temperature at the time of the experiment and was filter-sterilized using a vacuum-driven disposable filtration system (Figure 5. 4) with a 0.22 µm pore size (Stericup, Millipore Corp., Billerica, MA) before use.

Development of B. subtilis Biofilm under lab conditions

In-vitro biofilm formation experiment was carried out in a replicate of three, where nine 1×1 inch^2 membrane pieces were used. The membrane rolls dipped in soak solution (1% Ultrasil MP) were cut in 1×1 inch^2 membrane pieces. These membrane pieces were sterilized using Oxonia (60-160 ppm) for a contact time of 15 min, followed by neutralizing in Letheen broth for 10 min and then two rinses in sterile distilled water (Figure 5. 5). This sterilization process was able to inactivate the background microflora. One sterilized membrane piece was then tested for any residual antimicrobial substance after Oxonia treatment, which could potentially inhibit the formation of biofilm during the later experimentation. For which, TSA was poured in the Petri dish with a top layer of seeded soft agar with *M. luteus*, the retentate side of the sterile membrane piece was brought in contact with the seeded agar for 5 min. The use of soft agar was critical as the rate of dispersion would be much higher as compared to TSA. The plates were incubated at 37℃ and were observed for any microbial growth after 12-24 h of incubation.

The other sterile membrane pieces were then placed in Petri dishes (60×15 mm) with the retentate side facing upwards. Each Petri dish contained one membrane piece (1×1 inch^2). Sterile filtered whey containing log 7 CFU/ mL *B. subtilis* inoculum was added in each Petri dish equally. The incubation was carried out in a shaking incubator at 37℃ for 72 h (Figure 5. 6). A zero-hour count was taken to evaluate the viability of the culture and total plate count in the whey. The spent whey was replaced after every 8-9 h with fresh filtered whey. The viability of the *Bacillus* inoculum was checked by streaking the spent whey collected (after every 8-10 h) each time from each plate separately. At the end of 72

h, viability was determined by plating different dilutions using spent whey from each Petri plate separately. The plates were incubated at 37℃ for 12-24 h.

The viable counts from the membrane biofilms were enumerated using the swab technique reported by Marka and Anand (2018). Biofilm swabbing was carried out on the retentate side of the membrane piece (Figure 5. 7), where a Letheen swab was used in 4 directions: top to bottom, left to right, diagonally upward and diagonally downwards. The Letheen swab was added to its tube containing 4 mL of letheen broth after swabbing. The tube containing the swab was vortexed for 60 s, then dilutions were prepared, plated on TSA (Tryptic Soy Agar) and incubated at 37℃ for 12-24 h. The counts were reported as colony-forming units per inch sq.

Evaluating the effectiveness of natural antimicrobial from B. subtilis in removing membrane biofilms

Biofilm formed for 72 h on the RO-membrane piece was subjected to standard 5-step CIP protocol followed at Davis Dairy Plant, South Dakota State University by replacing the sanitizer step with the natural dialyzed antimicrobial CFE preparation. All chemicals were obtained and used as per the cleaning process used for RO-membrane in the Davis Dairy Plant. The steps followed during the CIP have been depicted in Table 5. 1 along with the temperatures and pH maintained for each wash. The standard CIP approach involving 5-steps was selected for the evaluation study. However, the industries generally follow the 3-step (alkaline, acid and alkaline wash) approach with the incorporation of enzyme step, sanitizer step or combination of both in addition to the 3-step protocol after two to three processing cycles which mainly depends upon the decreased influx rate.

After 72 h biofilm development, the membrane pieces were carefully picked up with the help of sterile scissors. The loosely attached cells were rinsed with the distilled water with the help of a sterile dropper. Out of nine, the first set of 3 membrane pieces were kept aside for pre-treatment counts. The second set of membrane pieces were subjected to the standard CIP protocol except for the sanitizer step. Another set of 3 membrane pieces were subjected to complete 5-step CIP protocol by replacing the commercial sanitizer step with the dialyzed CFE preparation having 97.01 mg/ mL of protein content.

The stock CIP chemical solutions were prepared just before the experiment. After the CIP, the membrane pieces were soaked in letheen broth for 10 min before being swabbed for enumeration of the viable cells. After the treatment, the membrane pieces were enumerated for viable counts, as explained above.

Statistical analysis

The antimicrobial screening and inhibition spectrum trials were conducted in triplicates with three replicates. For the evaluation trial, data of standard plate counts (3 replicates) for three treatments (Pre-C.I.P., Pre-sanitizer, and Post-antimicrobial) was compared using one-way analysis of variance (ANOVA) model in GLM procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). The data were log-transformed to meet the normality assumption of ANOVA. Then, log-mean separation within treatments was conducted using a pairwise statistical comparison (i.e. Duncan method) at the $\alpha = 0.05$ level.

RESULTS AND DISCUSSION

Antimicrobial activity of dialyzed CFE

To investigate the release of an antimicrobial compound by *B. subtilis*, the dialyzed CFE having 97.01 mg/ mL protein content (pH 6.4) was screened against the indicator strain, *M. luteus* using the spot-on-lawn method. It is noteworthy that the zones of inhibition were observed at 6 h on incubation of plates at 37℃. Therefore, revealing its antimicrobial ability against the test strain. The antimicrobial activity kept on increasing till 12 h of incubation after which it remained constant. Figure 5. 8 depicts the inhibition of *M. luteus* when subjected to dialyzed antimicrobial preparation.

Inhibition spectrum of dialyzed CFE against the constitutive microflora

Dialyzed CFE having 97.01 mg/ mL protein content was tested against the constitutive microflora of an 18-month old RO-membrane using the spot-on-lawn method. Zones of inhibition were observed against the producing stain and as well as against all the constitutive microflora of RO-membrane biofilm. These results depict the self-inhibition of *B. subtilis* (the producing strain) and against all the isolates of the biofilm microflora (Figure 5. 9). Similar results have been reported in other studies where self-inhibition was observed by the producing strain (Balakrishnan et al., 2002; Karam et al., 2004; Altuntas, 2013).

It is noteworthy that the dialyzed CFE was able to inhibit closely related species and depicted broad-spectrum inhibition against gram-positive and gram-negative microorganisms. Several studies have proven the inhibition of closely related species by the antimicrobial agents by bacteria (Abriouel et al., 2011; Ansari et al., 2012; Sumi et al., 2015). This variability in the spectrum of inhibition of biofilm microflora could be a result

of alteration in protein folding due to the difference in the protein aggregation of the freeze-dried and dialyzed CFE (Costantino et al., 1998).

Minimum Inhibition Concentration (MIC) of the dialyzed CFE

To evaluate the MIC, the dialysed CFE was further concentrated to achieve protein content 175.45 mg/ mL. The intent was to compare the MIC of the dialyzed CFE with the freeze-dried CFE of *B. subtilis* where the MIC was observed at 10% concentration (Verma and Anand, 2020). The results obtained revealed the MIC of the dialyzed CFE having 175.01 mg/ mL protein content at as low as 20% (Figure 5. 10). Therefore, there is a need to further understand the influence of protein folding in different forms, their active sites and their impact on the inhibition spectrum.

Application of crude antimicrobial preparation (dialyzed CFE) from B. subtilis to biofilm formed on RO membrane

The purpose of sanitizers is to knock out only a small amount of remaining microbial viable cells after cleaning (Marka, 2014). The effectiveness of natural antimicrobial (protein content 97.01 mg/ mL) produced by *B. subtilis* was tested by replacing the commercial sanitizer wash from the existing CIP procedure (Table 5. 1) against biofilm embedded cells. The 72 h biofilm was generated using a single species culture of *B. subtilis*, the predominant species of the constitutive microflora.

Before this experiment, the antimicrobial residues of the sterilized membrane were evaluated by contact plate where *M. luteus* was used as a test strain to determine whether the Oxonia treated membrane contributes towards any hindrance for the growth of the microorganism or not. The results demonstrated negative for any antimicrobial potential of the treated membrane. Therefore, the growth of *B. subtilis* would not be affected by the

treated membrane pieces used in the experiment. These membrane pieces were sterilized to avoid any competition due to the presence of a mixed culture population and to avoid any discrepancy in the results. The Oxonia treatment used for the sterilization of the membrane pieces was able to remove the background microflora from the unused membrane except for random one or two counts on a few membrane pieces. The presence of one or two colonies on the membrane piece after sterilization treatment with hydrogen peroxide was also reported by Marka and Anand (2018).

The biofilm generated was analyzed and standard plate count (SPC) were obtained before any CIP procedure (Pre-CIP), after treating biofilm embedded cells with wash 1-wash 4 from the existing CIP procedure (Pre-sanitizer treatment) and after treating biofilm embedded cells with first four washes of the existing CIP procedure and replacing commercial sanitizer treatment with our dialyzed CFE antimicrobial preparation (Post-antimicrobial) in a replicate of 3. Mean SPC of these treatments were analyzed and results were presented in log reduction of Pre-CIP counts when membrane pieces were subjected to pre-sanitizer and Post-antimicrobial treatment. The in-vitro biofilm logs were 6.68 ± 0.12 CFU/ $inch^2$, after 4 steps industrial process the residual counts reduced to 2.18 ± 0.54 logs CFU/ $inch^2$ which further reduced to 1.20 ± 0.09 logs CFU/ $inch^2$ (Table 5. 2). This shows significant decrease in growth of biofilm microflora. The data were log-transformed to meet the normality assumption of ANOVA. Then, log-mean separation within treatments was conducted using pairwise statistical comparison. The results obtained depicted a significant difference between Pre-sanitizer and Post-antimicrobial treatment.

Another study from our lab depicted 0.64 to 1.52 log reduction from an initial count using different isolates when 12 h old biofilm was subjected to sanitizer step (Anand and

Singh, 2013b); Anand et al., 2014). Also, it demonstrated lower effectiveness of the sanitizer treatment to *Bacillus* spp. biofilms, which reduced the initial count by only 0.64 to 0.87 log. Similarly, the current study depicted 0.98 log reduction from the initial count after exposing the resilient *B. subtilis* 72 h old biofilm to the dialyzed antimicrobial preparation having 97.01 mg/ mL protein content. Therefore, proving the effectiveness of dialyzed CFE (natural antimicrobial) in removing membrane biofilms. The protein concentration can be optimized to obtain higher log reduction of biofilm-embedded cells using this antimicrobial preparation.

CONCLUSIONS

The biofilm microflora is known to develop resistance to the chemical cleaners over prolonged use of membrane and eventually develop resistance to their deviates. This makes the CIP procedures ineffective in cleaning membrane biofilms. Hence, creating a need to develop natural antimicrobial preparation to inhibit the growth of biofilms on membrane surfaces. This study aimed to prove the effectiveness of the dialyzed natural CFE antimicrobial produced by *B. subtilis* for removing biofilms by replacing the sanitizer wash step from the existing C.I.P. protocol. This crude preparation depicted a broad spectrum of inhibition against gram-positive and gram-negative bacteria, including the producing strain. On replacing the commercial sanitizer step with dialyzed antimicrobial preparation 0.98 log reduction was observed against resilient *B. subtilis* biofilm. Hence, serving as a proof-of-concept that this natural antimicrobial preparation is capable of removing resilient biofilms. Bacteriocins produced by *B. subtilis* being categorized as ‘generally recommended as safe’ (GRAS) show their ability to be developed into a novel biosanitizer by incorporating the antimicrobial release by *B.* subtilis strain isolated from an 18-month-

old RO membrane. Based on that, this study suggests that this natural antimicrobial compound should be further studied, purified and concentrated to achieve maximum reduction. More studies need to be carried out to understand the active sites responsible for inhibition, the effect of protein folding on the inhibition spectrum and formulating chemistry. Also, pilot-scale studies are required to entirely determine the exact industrial applicability of the dialyzed antimicrobial preparation.

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TABLES AND FIGURES

Table 5. 1 Washing steps for C.I.P of RO membranes

Wash	Treatment	Time (min)	Temperature [°C (°F)]	pH value
Wash 1	Caustic wash	20	47.7-50.0 (118-122)	10.8-11.2
		Rinse with warm water for 20 min		
Wash 2	Enzyme wash	40	44.0-47.0 (112-117)	9.0-10.0
		Rinse with warm water for 20 min		
Wash 3	Acid wash	20	46.0-49.0 (115-120)	1.8-2.5
		Rinse with warm water for 20 min		
Wash 4	Caustic wash	25	47.7-50.0 (118-122)	10.8-11.2
		Rinse with warm water for 20 min		
Wash 5*	Sanitizer wash	15	21-27 (70-80)	4.0-5.0
		Rinse with warm water for 25 min		

*Wash 5 (sanitizer wash) was replaced with dialyzed CFE prepared at pH 6.4

Table 5. 2 Survival count after treating 72 h old *B. subtilis* biofilm with different treatments

Treatments	**Survival Count at different Steps (Log CFU/ inch^2)**
Pre-CIP	6.68 ± 0.12
Pre-Sanitizer	2.18 ± 0.54
Post-Antimicrobial	1.20 ± 0.09

Figure 5. 1 Dialysis Tubing generously covered with concentrator powder and sample secured with the help of clips.

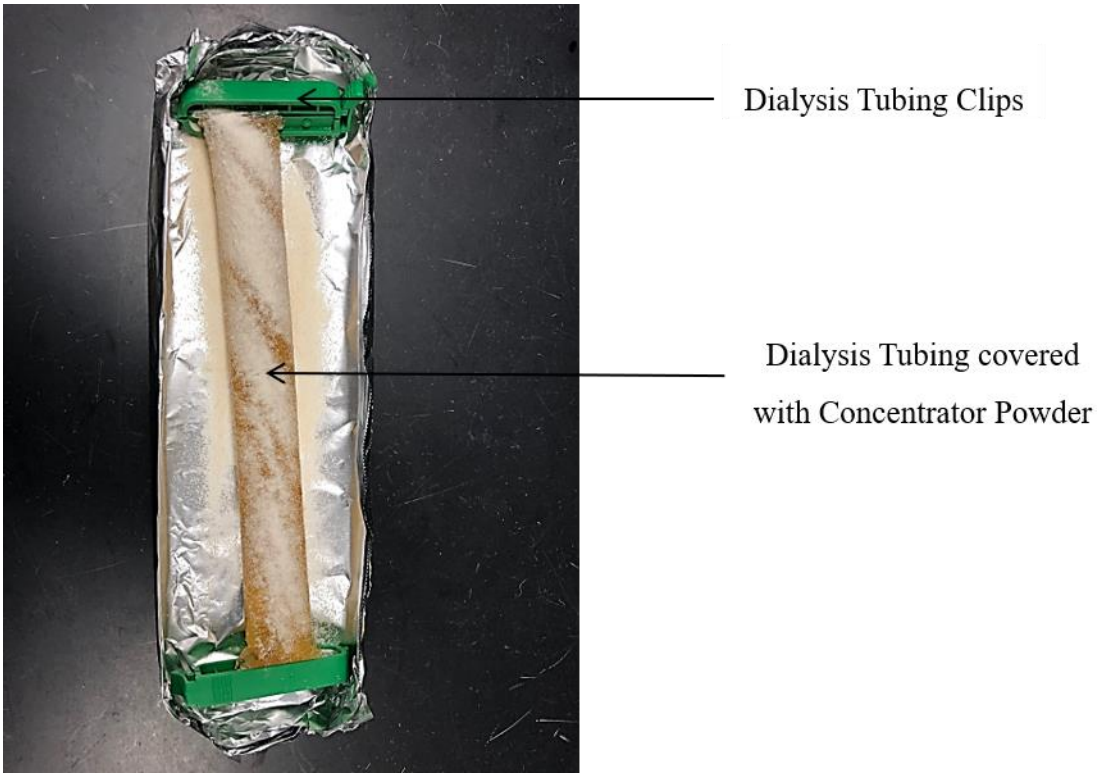


Figure 5. 2 Sixty (60) mL of CFE reduced to 1 mL using dialysis tubing in 6 days

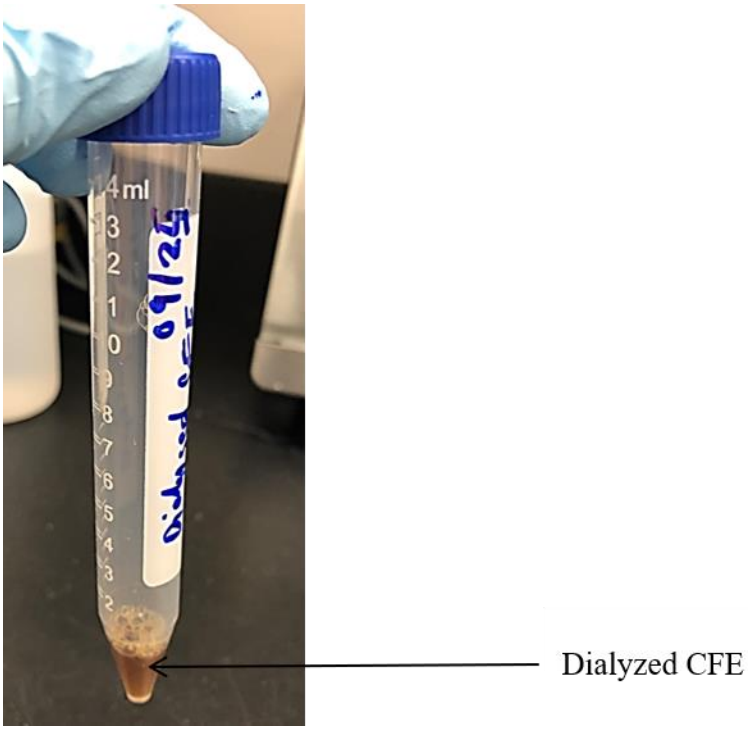


Figure 5. 3 Nanodrop 2000 spectrophotometer used for estimating protein content of the dialyzed CFE with the help of NanoDrop2000 software

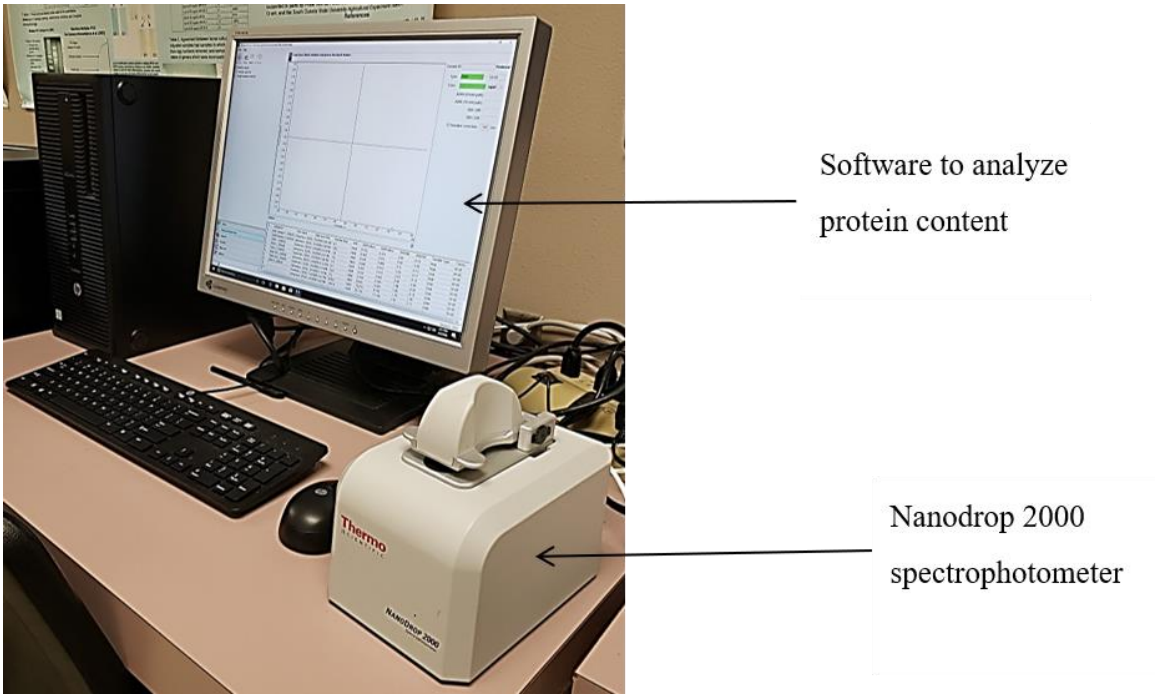


Figure 5. 4 Millipore membrane filtration unit used for the sterilization of the cheese whey

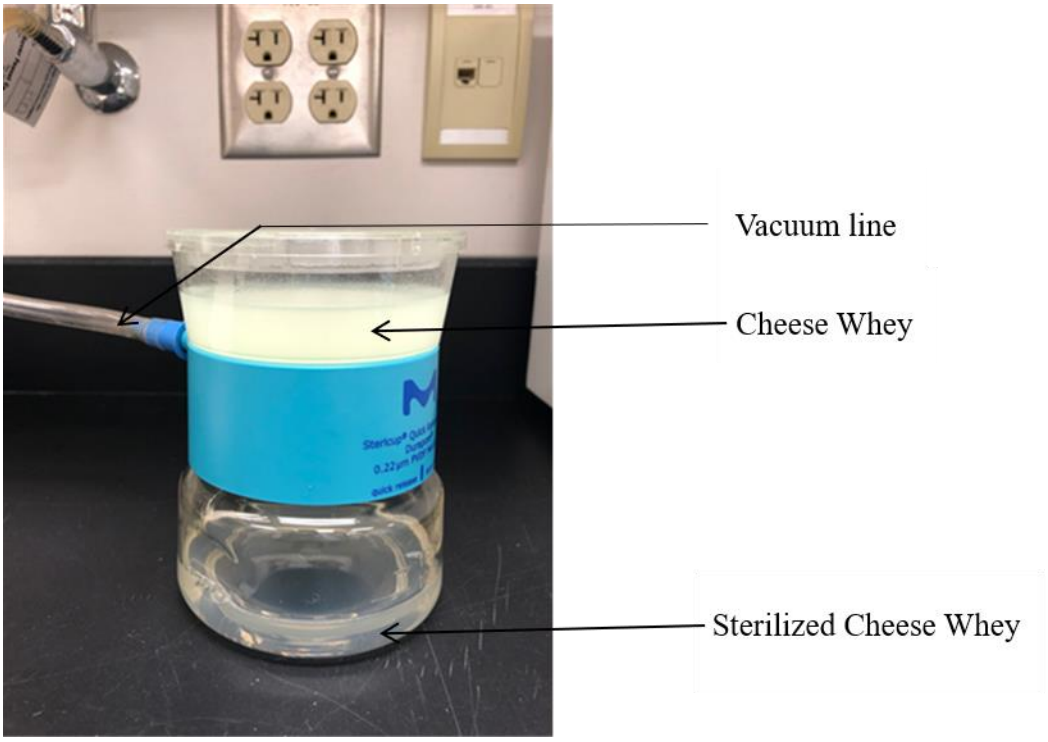


Figure 5. 5 Membrane sterilization steps before biofilm formation experiment

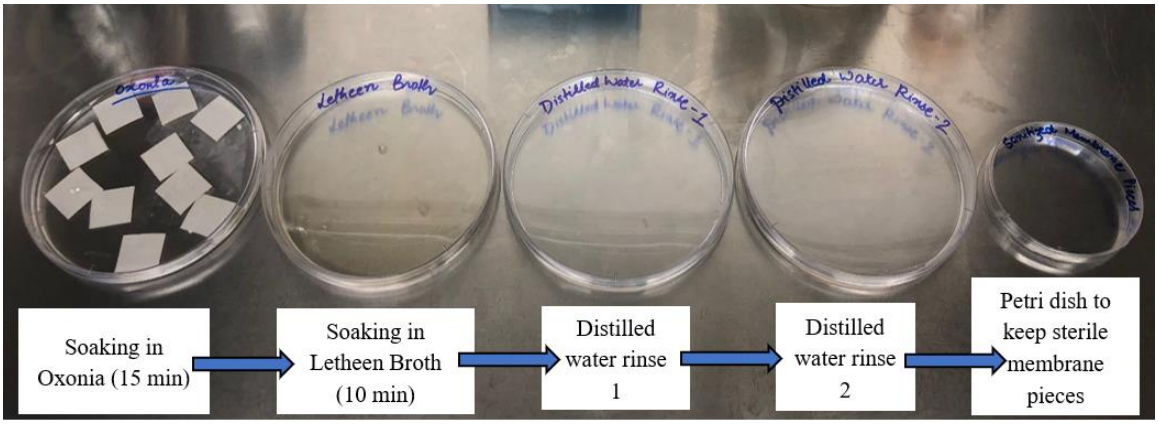


Figure 5. 6 Membrane pieces (1×1 inch2) arrangement inside the Petri dish containing whey inoculated with 7 log CFU/ mL of Bacillus subtilis in a shaking incubator.

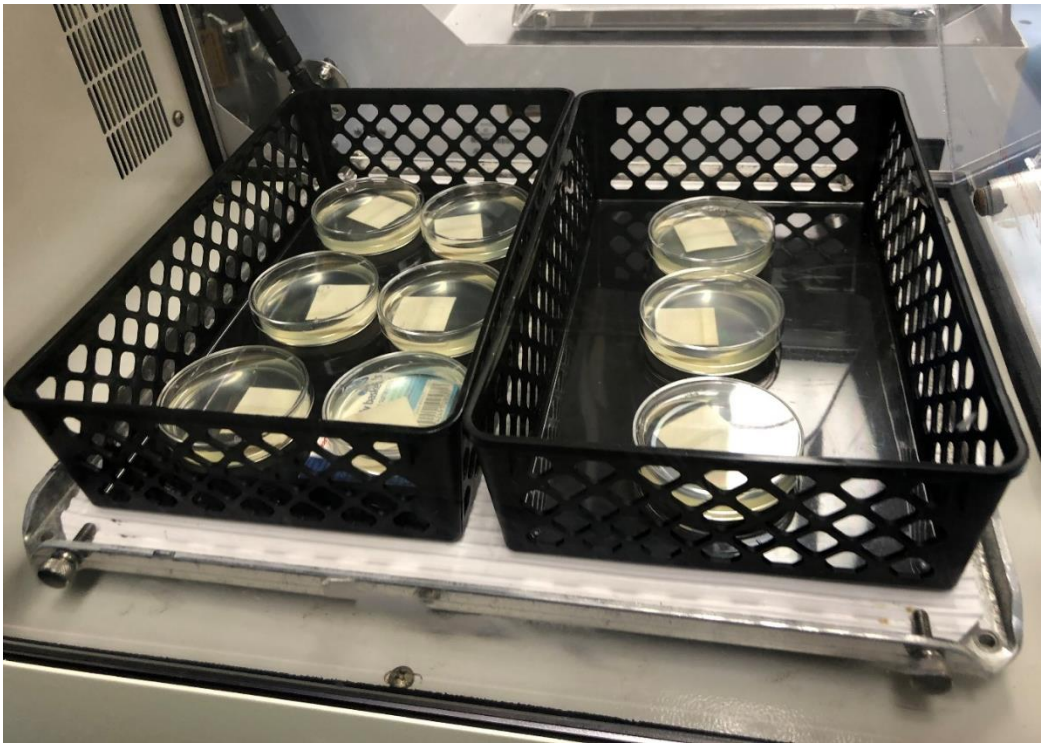


Figure 5. 7 Swabbing the retentate side of the membrane piece to retrieve biofilm embedded cells.

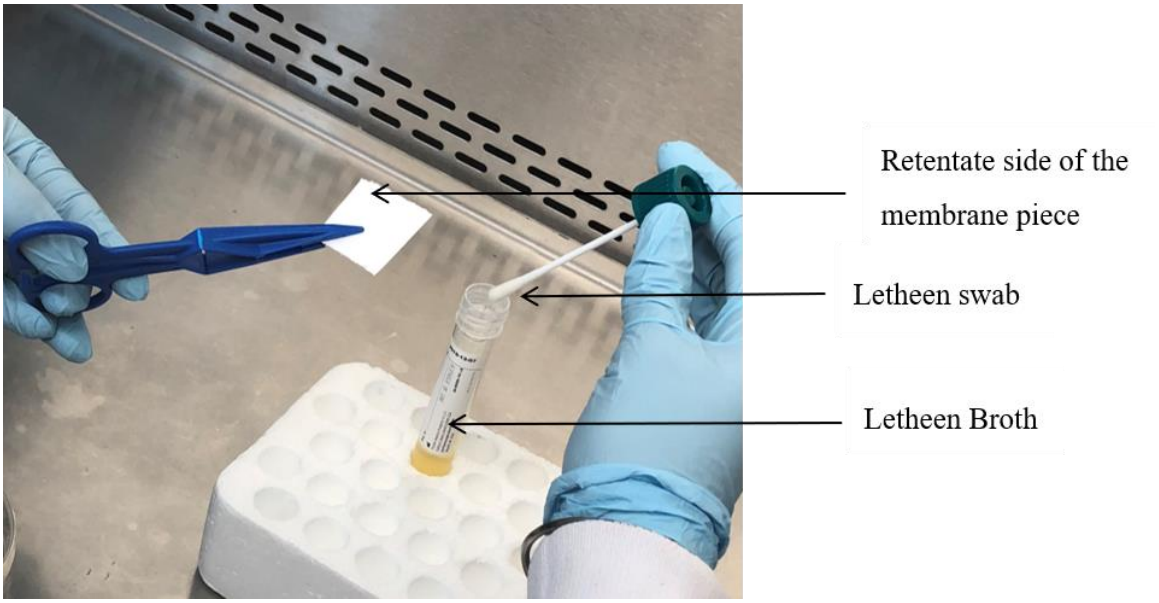
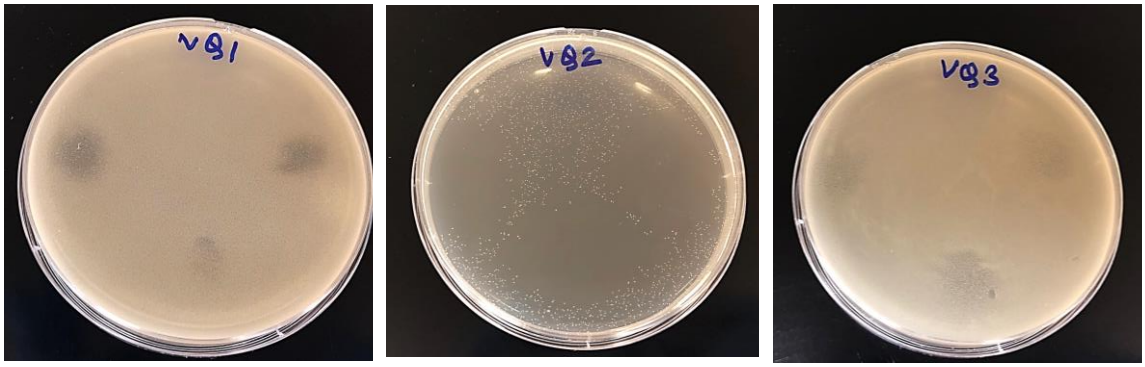


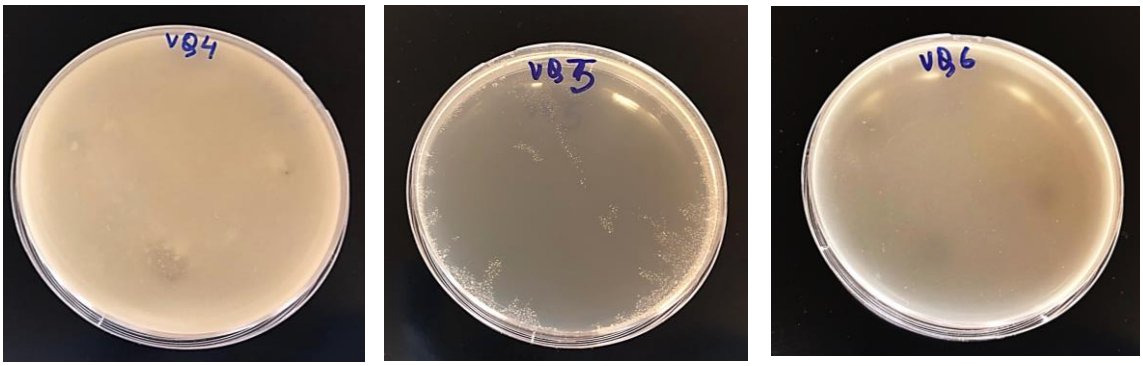
Figure 5. 8 Clear zones of inhibition obtained by dialyzed CFE of Bacillus subtilis against Micrococcus luteus.

Micrococcus luteus (control)

Figure 5. 9 Inhibition spectrum of dialyzed CFE of Bacillus subtilis against the constitutive microflora of an 18-month-old Ro membrane biofilm.

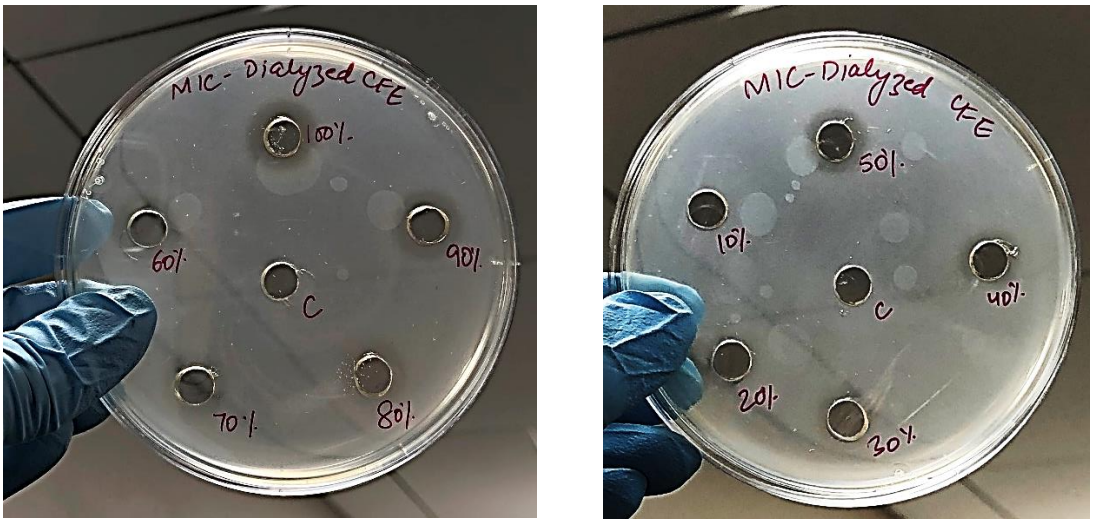


Bacillus subtilis (VQ1) *Exiguobacterium sp. (VQ2)* *Bacillus licheniformis (VQ3)*



Bacillus sp. (VQ4) *Exiguobacterium aurantiacum (VQ5)* *Acinetobacter radioresistens (VQ6)*

Figure 5. 10 The minimum inhibitory concentration of dialyzed CFE having 175.45 mg/ mL protein content (150 mL of CFE reduced to 1 mL using dialysis tubing in 7 days yielded 175.45 mg/ mL protein content).



Note: The notation ‘C’ represents the control (HPLC grade water)

SUMMARY AND CONCLUSIONS

Biofouling is one of the most critical problems faced in cleaning and sanitation of dairy separation membranes. As the feed stream containing background microflora encounters the membrane surface, the feed bacteria initiate attachment and colonization on the membrane surfaces. These are also known as biofilms, which lead to biofouling and reduce the flux rate. Even routine cleaning protocols prove to be ineffective. This results in frequent and premature membrane replacements, due to product quality and safety concerns, and a severe decline in the flux rate. The membrane replacement cost is very high and leads to significant economic loss to the dairy industry. The present research work offers new insights on the role of natural antimicrobials, released by an isolate of a membrane biofilm, to develop novel alternative strategies to combat biofilm formation on membrane surfaces.

For the first research study, an 18-month-old RO-membrane from a cheese whey plant was used to isolate the biofilm microflora using cultural techniques. Distinct colonies were identified based on colony morphology, gram staining, and MALDI-TOF. Six isolates were identified as *Bacillus licheniformis, Exiguobacterium aurantiacum, Acinetobacter radioresistens*, *Bacillus subtilis* ('rpoB' sequencing), and one unidentified species each of *Exiguobacterium* and *Bacillus*. As selective isolates within the biofilm microflora acquire resistance and emerge predominant within the biofilm microflora. The emergence of predominance was studied using a co-culture combination technique and the results were further validated using natural selection in a mixed-species growth environment. *Bacillus subtilis* emerged as the predominant organisms among the biofilm constitutive microflora.

The colony and cell morphology were also studied to develop a better understanding of the common type of cell structures responsible for forming resilient biofilms. The cell morphology revealed the presence of rod-shaped cells with varied rod lengths, with 50% isolates depicted sporulating behavior. Several studies have shown that bacterial spores are much more resilient than vegetative cells. Therefore, spore formers might be responsible for forming resilient biofilms that cannot be effectively cleaned with the help of existing CIP protocols.

The predominant strain, *B. subtilis* was the only isolate that was found positive for the string test among the isolated biofilm microflora. Also, it was noteworthy that hyper-mucoviscosity was observed with a string length of > 5mm. The presence of mucoid colonies has also been associated with the predominance of certain strains over others that do not produce such mucoid colonies. Therefore, the presence of mucoid could also be related to *B. subtilis* emerging predominant with the biofilm constitutive microflora.

In the second study, a similar approach was adopted as that of study 1. In which, two UF membranes were procured from two different locations. One was a 15-month-old UF membrane procured from a whey processing commercial plant located in the mid-west region used to concentrate whey. Another UF membrane was procured from the southwest region from a milk processing plant. The UF membrane was 6-8-month-old used for skim milk processing. Culturing techniques were used to isolate the biofilm constitutive microflora. Distinct colonies from the 15-month-old UF-membrane were identified as *Enterococcus faecalis*, *Rahnella aquatilis*, and two unidentified species of *Exiguobacterium* and 6-8-month-old UF membrane revealed the presence of *Klebsiella oxytoca*, *Citrobacter freundii*, *Bacillus licheniformis*, and one unidentified species of

Enterococcus using colony morphology, gram staining, and MALDI-TOF identification. Therefore, these isolates can be further studied to establish any emergence of predominance within biofilm microflora and help to create a targeted cleaning procedure to remove resilient biofilms from the surface of separation membranes.

Whereas, studies have reported the predominance of spore-forming *Bacillus* spp. in the RO membranes which might be responsible for generating more resilient biofilms. Also, our previous study on the RO-membrane revealed the presence of three spore-forming *Bacillus* spp. including *Bacillus subtilis* and *B. licheniformis* as a part of the biofilm microflora from an 18-month-old RO-membrane. It is noteworthy that the presence of gram-positive as well as gram-negative bacteria was observed in both UF and RO membranes which could be the result of post-pasteurization cross-contamination. It is important to note here that this is only a preliminary study to compare the biofilm microflora of the UF membrane with RO, and further studies are required to draw any conclusions.

In the third study, the factors influencing the predominance of *B. subtilis*, within the constitutive microflora were investigated. This study included the antimicrobial activity of *Bacillus subtilis* isolate (within the membrane biofilm microflora) as a potential cause of its predominance. The emergence of predominance could be associated with several factors including competition for the nutrients, faster-growing rate of one microorganism over the others, production of metabolites by the cells, production of bacteriocins, secretion of broad and narrow spectrum toxins with coupled privatized antitoxin, etc. Freeze-dried CFE from *B. subtilis* was prepared and the antimicrobial activity was screening using indicator strain, *M. luteus*. On depicting zones of inhibition, 12 h freeze-dried CFE was tested against the constitutive microflora

and some common foodborne pathogen. Revealing its broad spectrum of inhibition against gram-positive and gram-negative bacteria, and some common pathogens such as *Listeria monocytogenes*, *Bacillus cereus*, and *Salmonella enteritidis*.

On characterization, the antimicrobial substance was found to be proteinaceous in nature and amino acid profiling revealed the total protein content of 51% (wt/wt) having its major constituent to be glutamic acid (comprising 11.30% (wt/wt) of the total protein content). Studies have stated the importance of glutamic acid in exhibiting antimicrobial activity and have associated its presence with more effective bactericidal activity. Also, the freeze-dried CFE was thermally stable on exposure to the common temperature used for sanitizer applications (23.8°C for 5 and 10 min) and over a pH range of 3.0-6.3. The minimum inhibitory concentration was observed at a 10% concentration of the original. To our knowledge, antimicrobial activity against membrane biofilm constitutive microflora, by the antimicrobial substance produced by a *B. subtilis* isolate of the membrane biofilm origin itself, has not been reported before. In conclusion, this study revealed that the spore former *B. subtilis* of a membrane biofilm origin is a promising source of an antimicrobial substance that could be associated with its predominance within the biofilm constitutive microflora.

The fourth study was conducted as a proof of concept to evaluate the effectiveness of the antimicrobial substance from *B. subtilis* for cleaning membrane biofilms, under in vitro conditions. For this, dialyzed CFE was prepared at a protein concentration of 97.01 mg/ mL. The antimicrobial activity was tested against the indicator strain, *M. luteus*, and the constitutive microflora. The dialyzed CFE, even with lower protein content than that of freeze-dried CFE (175.95 mg/ mL), inhibited all isolates of the constitutive microflora, including the producing strain itself. Therefore, for the evaluation trial, a 72 h *B. subtilis*

in-vitro membrane-biofilm was generated and was exposed to a modified CIP protocol by replacing the sanitizer step with the dialyzed CFE preparation. The in-vitro biofilms were developed (6.68 ± 0.12 CFU/ inch^2) using the isolated *B. subtilis*. These biofilms were exposed to a commercial cleaning and sanitation protocol. After 4 steps of the process, the residual biofilm counts were reduced to 2.18 ± 0.54 logs CFU/ inch^2. These counts got further reduced to 1.20 ± 0.09 logs CFU/ inch^2 on using the *B. subtilis* antimicrobial substance in place of the commercial sanitizer during the cleaning process. The study thus provides a proof of concept for the higher efficacy of the natural antimicrobial substance released by *B. subtilis* in cleaning resilient biofilms and shows a promise for the future development of a biosanitizer.

Based on the above findings, future studies are necessary to improve the efficacy of the natural antimicrobial to control biofilm formation on membrane surfaces. This natural antimicrobial compound needs to be further studied, purified, and concentrated to achieve maximum reductions. More studies need to be carried out to understand the active sites responsible for inhibition, the effect of protein folding on the inhibition spectrum using CFE in freeze-dried and dialyzed form, and the formulating chemistry. Also, pilot-scale studies are required to entirely determine the exact industrial applicability of the dialyzed antimicrobial preparation.

APPENDIX

Abstract 1 for ADSA Graduate Student Dairy Foods Poster Competition: 2019 American Dairy Science Association Annual Meeting, June 23-26, 2019, Cincinnati, Ohio, Journal of Dairy Science, Volume 102, Supplement 1.

A natural antimicrobial from Bacillus subtilis, a predominant constituent of membrane biofilms.

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Current cleaning and sanitation protocols may be ineffective in cleaning separation membranes and result in the formation of resilient multispecies biofilms. These old biofilms may show bacterial predominance on prolonged use of the membranes. In our previous study, we isolated organisms such as *Bacillus subtilis, Bacillus licheniformis*, *Exiguobacterium aurantiacum,* and *Acinetobacter radioresistens* from an 18-mo old reverse osmosis membrane. Competitive exclusion studies revealed the predominance of B. subtilis within the membrane biofilm microflora. This study investigates the antimicrobial activity of B. subtilis as a cause of its predominance. The culture was incubated in tryptic soy broth (TSB) at 37°C, and microfiltered to prepare cell-free extracts (CFEs) at 8-, 10-, 12-, 14-, 16- and 18-h intervals. The CFEs were freeze-dried and re-suspended in minimum quantities of HPLC grade water to create concentrated solutions. The antimicrobial activities of CFEs were tested using agar-well assay against the biofilm constitutive microflora and some common food pathogens. The experiments were conducted in triplicates and means were compared for significant differences using a general linear mixed model procedure of SAS. The results indicated the highest

antimicrobial activity of 12 h CFE of B. subtilis against other *Exiguobacterium* sp., *E. auranticum*, *A. radioresistens*, *Listeria monocytogenes*, and *Bacillus cereus*, with average inhibition zone sizes of 16.5, 16.25, 20.6, 18.0, and 13.8 mm, respectively. On treating with proteinase K, the CFE completely lost its antimicrobial activity, establishing it to be a proteinaceous compound. The amino acid profiling revealed the total crude protein in CFE to be 51% (wt/wt) having its major constituent to be glutamic acid (11.30% wt/wt). The CFE was thermally stable on exposure to the common temperature used for sanitizer applications (23.8°C for 5 and 10 min). Based on this study, the proteinaceous antimicrobial compound produced by B. subtilis may result in its predominance.

KEYWORDS: biofilms, antimicrobial activity, predominance

Abstract 2 for ADSA Graduate Student Poster Presentation-Dairy Foods: Microbiology 2: 2019 American Dairy Science Association Annual Meeting, June 23-26, 2019, Cincinnati, Ohio, Journal of Dairy Science, Volume 102, Supplement 1.

Competitive exclusion study demonstrates Bacillus subtilis as a predominant constitutive microorganism of RO membrane biofilms

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Microbial attachment and colonization on separation membranes lead to biofilm formation. The constitutive microflora might compete and result in certain species emerging as predominant, especially within older biofilms. To understand the microbial interactions within biofilms, the emergence of predominance was studied in the current investigation. An 18-month-old reverse osmosis membrane was procured from a whey processing plant. The membrane pieces (1 x 1 inch2) were neutralized by dipping in Latheen broth. Their resuscitation was done in Tryptic Soy Broth (TSB) at 37°C, followed by plating on Tryptic Soy Agar (TSA) to recover the constitutive microflora. Distinct colonies of isolates were further identified using MALDI-TOF as *Bacillus subtilis*, *Bacillus licheniformis*, *Exiguobacterium aurantiacum*, *Acinetobacter radioresistens*, and two unidentified species of *Exiguobacterium* and *Bacillus*. Further, the competitive exclusion study helped to establish the predominance using a co-culturing technique. Fifteen combinations of two isolates each were prepared. For which, isolates were spiked in the ratio of 1:1 in TSB, incubated at 37°C for 24 h, followed by plating on TSA. The isolates on plates were distinguished based on colony morphology, gram staining, and MALDI-TOF. For establishing the predominance, plate counts were compared using analysis of variance. In

all the co-culture combinations, *B. subtilis* emerged as predominant with a mean log counts of 6.73±0.23 CFU/ mL. It was interesting to note that another isolate, *B. licheniformis*, competed equally with *B. subtilis*, while not with others. The predominance of *B. subtilis* was further validated using the process of natural selection, where the broth with overnight-incubated membrane piece (with mixed species biofilm) was inoculated in fresh TSB and incubated for another cycle. Five such sequential transfers resulted in demonstrating the predominance of *B. subtilis* based on its population density.

KEYWORDS: RO membrane, constitutive microflora, competitive exclusion