THE EFFECT OF XENON PULSED-LIGHT TECHNOLOGY ON BIOFILM ADHERED TO STAINLESS STEEL SURFACES

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ABSTRACT

The Effect of Xenon Pulsed-Light Technology on Biofilm Adhered to Stainless Steel Surfaces

Stephanie Jacquez

In food processing, inadequate surface sanitation procedures lead to the formation of biofilms in which bacteria attach and aggregate in a hydrated polymeric matrix of their own synthesis. Formation of these sessile communities and their inherent resistance to existing sanitation procedures and agents are at the root of the risk of bacterial infections for consumers. Due to this existing problem, an effective method for reducing biofilm formation in dairy processing equipment is necessary for dairy products processing. Ultraviolet Pulsed light Technology has shown a positive effect in eliminating microorganism populations on food products. The objective of this work is to evaluate the effect of Pulsed light Technology on a biofilm of different dairy component matrices (e.g. Water (control); whey protein isolates (WPI), lactose, and sweet whey). This evaluation will be performed using the three strains of spore forming *Bacillus* species most common in commercial milk powder (*B. subtilis*, *B. coagulans*, and *B. licheniformis*). The matrix in which the evaluation was made consisted on allowing the attachment of endospores to on to a square 2.5cm x 2.5cm ASI 304 stainless steel coupon. Four Xenon light treatment levels (no treatment, 5 bursts, 10 seconds, 20 seconds and 30 seconds) were applied to the coupon surfaces using the Xenon model RC847 machine. The attachment of *Bacillus* to stainless steel in water as matrix was 1000 to 3000/ sq cm as measured in our laboratory. Results showed that there was a

significant difference in spore reduction depending on the matrix of the biofilm and with the intensity of the Xenon treatment. Reduction in spores ranged from 1 to 4.7 logarithmic reduction cycles depending on the material of the biofilm, the strain of spores and the intensity of treatment. We conclude that there is significant potential to use this technology in maintaining low spore counts in commercial dairy powders.

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CHAPTER 1: INTRODUCTION

One of the major hurdles in the food industry is foodborne illness. Although the U.S food supply is one of the safest in the world, the Center for Disease Control and Prevention (CDC) estimates that 48 million people become ill, 128,000 are hospitalized and 3,000 die every year due to a foodborne illness (CDC, 2016). Foodborne illness has become a significantly concerning topic around the world as the interest of importing and exporting food supply has increased over the years (Oliver et al,. 2005) . some of the most common pathogens found in raw milk are, *Campylobacter jejuni* , Shiga toxin-producing *Escherichia coli* ,*Listeria monocytogenes*, *Yersinia enterocolitica*, *Salmonella* isolates and *S. enterica* (Jayarao et al. 2006).

There are many stages in milk production where pathogens can be introduced to milk from the farm to the table: while milking due to manure or unsanitary milking environments, in bulk tank milk where pathogens and spores are free to multiply and finally in production of unpasteurized milk(Oliver et al,. 2005). Thanks to pasteurization, a procedure using a combination of time and temperature, and our increasing knowledge about the risk of consuming raw milk, FDA now considers pasteurized milk a safe food (FDA, 2007). Milk borne outbreaks have significantly decreased over the years due to strict food safety regulations and a plummet in raw milk consumption (Newkirk et al.2011).Pasteurization used in milk production successfully targets vegetative pathogenic bacteria but may miss, or induce the germination of spore- forming bacteria.

Spore-forming bacteria in milk are not commonly pathogenic but once germinated have an effect on end product shelf life and quality. More recently,

producers have set strict specifications on the bacterial spore forming concentrations of the dairy ingredients that they receive, especially those who manufacture infant formula. Some of these specifications are as follows: aerobic mesophilic and thermophilic spore counts <500 to <1,000 CFU/g for skim milk powder (SMP), non-fat dry milk (NDM) and whole milk powder (WMP) destined for infant formula; and <500 to <2,000 CFU /g for aerobic thermophilic spores in SMP and WMP destined for recombined or UHT products (Watterson et al. 2014). Biofilm composed of dairy components and spore forming bacteria can compromise the processing surfaces in the dairy industry and have an impact on the quality of the end product.

1.1 Purpose of Study:

The objective of this study was to evaluate the effect of Xenon Pulsed Light Technology (PLT) on biofilm composed of 3 different *Bacillus spp.* (*Bacillus subtilis, Bacillus licheniformis,* and *Bacillus coagulans*) and 3 types of dairy based media (5% whey protein concentrate, 5% lactose, 5% sweet whey and sterile deionized water as the control) adhered to stainless steel coupon surfaces. Also, a preliminary study of the effect of pretreated stainless steel coupons to compare the attachment of spores was conducted

CHAPTER 2: LITERATURE REVIEW

Various studies have been conducted that demonstrate the survival of various spore forming bacteria after pasteurization. In a study conducted by M.J Watterson and others, Evidence showed dairy powders, a product subjected to very high temperatures after pasteurization, thermophilic and some mesophilic spore formers were present. These spore formers included *B. licheniformis, B. subtilis*, *B. cereus* and *B. pumilus*, *A. flavithermus,* and *Geobacillu*s spp (Watterson et al.2014).

Although the majority of spore formers are not pathogenic, their growth and germination post pasteurization is one of the main determinants for product shelf life. The higher the spore counts in a product, the shorter its shelf life. Some of the effects of spores on dairy products are structural defects known as sweet curdling and bitty cream (Sheldemen et al. 2006). Although research has helped in detecting the culprit to curdled milk, the spore has a unique physiology that makes it difficult to destroy.

The characteristic that makes spore-forming bacteria so unique is their ability to withstand a harsh environment due to their unique core and cortex properties and various mechanisms that give them the ability to exit dormancy and return to vegetative growth in a re-emerging favorable environment (Moir and Smith 1990). When these microorganisms are starved for nutrients and exposed to a harsh environment, vegetative cells differentiate into a dormant structure known as an endospore. Within this state, endospores show no signs of metabolism preparing them for higher resistance against wet and dry heat, high hydrostatic pressure, desiccation, UV and γ-radiation, as well as antimicrobials (Sleiman et al., 2009).

2.1 Stages of Sporulation

A cell undergoes 8 stages sporulate starting from stage zero where the cell has upregulated levels of specific genes that cause it to begin sporulation. Sporulation was presumed to be irreversible. It was not until 2005 when a study conducted by Dworkin and Losick revealed that sporulation may be reversible if many *spo* genes are activated, and becomes irreversible only if certain sigma factor genes are active in the forespore and the mother cell (Tan and Ramaurthi 2014).

2.1.1 Stage 1 of Sporulation

This stage includes a condensed serpent-like chromosomal structure attached to the cell poles so that both the mother cell and the fore spore get one chromosome known as the Axial filament. Axial filament attachment is possible due to the RacA protein link on the chromosome ends in the GC rich regions and its interaction with DivIVA, the cell pole localizer, to ensure the chromosomal attachment to the cell poles (Lenarcic et al., 2009; Ramamurthi and Losick, 2009, Tan and Ramaurthi 2014).

2.1.2. Stage 2 of Sporulation

The most significant feature that differentiates replication of a cell and a spore is stage 2, the location of septation. An ordinary cell involves medial septation, meaning that the cell divides in the middle at cell division, while a spore undergoes asymmetric septation creating a mother cell and a forespore (Carniol et al., 2005; Tan and Ramaurthi 2014). In this septation step, only 30% of the forespore's chromosome is inside the actual forespore, the rest is still in the mother cell (Ptacin et al., 2008; Fiche et al., 2013; Tan and Ramaurthi 2014).

2.1.3. Stage 3 of Sporulation

This stage involves the engulfment of the forespore by the mother cell. The engulfment process involves the curving of the polar septum around the forespore in such a way that seems as though the mother cell has swallowed it. As a result, the forespore now has a double membrane and is free floating within the cytosol of the mother cell. the peptidoglycan wall is degraded for membrane fission and pinching off of the forespore from the mother cell (Abanes-De Mello et al., 2002; Tan and Ramaurthi 2014). Although it seems the peptidoglycan layer is fully removed as a step in stage 3 of sporulation, Tocheva et al., 2013, revealed that not only was some peptidoglycan present in the forespore, but it seemed to act as subsequent peptidoglycan remodeling events during the engulfment process as well as cortex assembly.

2.1.4. Stages 4-5 of Sporulation

Stages 4-5 are dedicated to the assembly of the coat and cortex of the spore both in which protect the spore from harsh environments. The outer shell of a forespore is called the coat and is composed of approximately 70 different proteins (Henriques and Moran, 2007; Mckenny et al., 2013; Tan and Ramaurthi 2014). The coat itself has four layers which include: the basement layer, inner coat, outer coat and crust. Each layer has a specific protein to help distinguish one layer from another (Mckenny et.al, 2010).

Stage 5 is the assembly of the cortex where its composition consists of a unique type of peptidoglycan to protect the spore from Heat and desiccation. This specialized peptidoglycan layer is found between the two membranes, inner membrane (IM) and outer membrane (OM) that surround the forespore. The cortex has two layers, the inner germ cell wall that is a thin layer between the outer forespore membrane, and an outer

cortex. The inner germ cell wall is a thin layer very similar to a vegetative cell but the cortex is differs from its vegetative counterpart due to the presence of muramic lactam (Tan and Ramaurthi 2014). There are many theories as to what exactly the function of the cortex's peptidoglycan layer is. One theory proposed by Ou and Marquis in 1970 was that the low level of cross linking found in this layer gave the spore the ability to expand and contract without having to germinate. Another study by (Lewis et al., 1960) and (Gould and Dring 1975; Popham et al., 1999) stated that the low degree of crosslinking of the peptidoglycan permits the spore to expand or contract to attain dehydration (Tan and Ramamurthi 2014).

2.1.5. Stage 6 of Sporulation

The stage of sporulation is considered the maturation of the spore. As illustrated in Figure 1, a mature spore is characterized by the tightly condensed chromosome in the forespore (Tan and Ramamurthi 2014). During sporulation the Mur proteins, proteins that produce peptidoglycan precursors, get upregulated. Once this upregulation has commenced, the peptidoglycan precursors move to the outer forespore membrane. The final stage, stage 7 of sporulation is the release of the resistant, dormant forespore into the environment by a lysed mother cell.

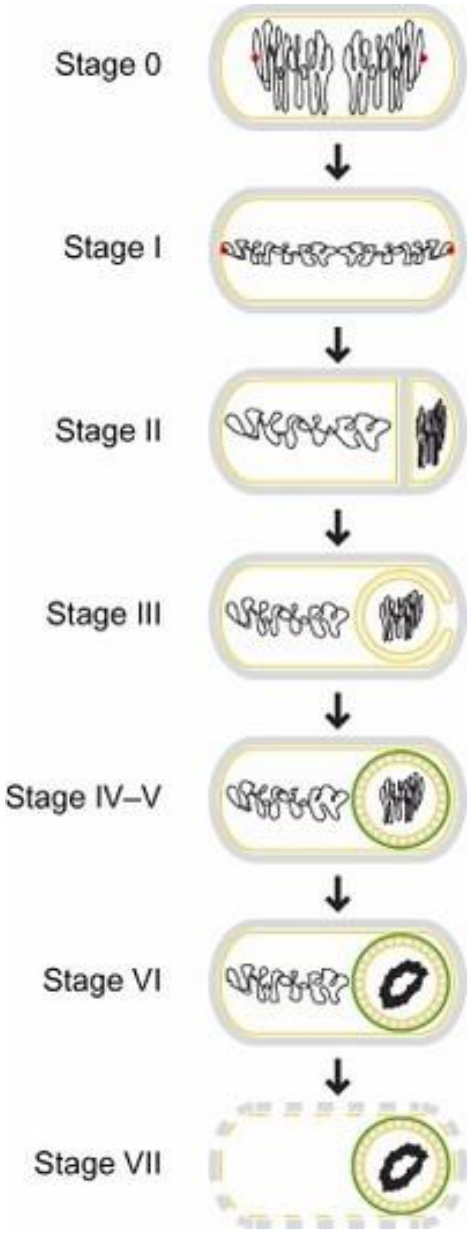

Figure 1. Schematic representation of sporulation stages in *Bacillus subtilis* provided by Tan and Ramaurthi (2014)

2.2. Spore Resistance

A bacterial endospore is resistant to chemicals as well as high heat. Its multiple layers make it extra difficult to control causing them to be problematic for food production as well as dairy production. For a period of time, researchers believed that a spore's ability to withstand a wet heat environment was due to the spore core containing a low water content dehydrating the core and enabling the stabilization of its proteins against thermal denaturation. It was not until later when evidence was found where the dehydration of the spore core could occur due to the immobilization of proteins and phenotypic heterogeneity for wet heat resistance (Eijlander et al., 2011). Heat resistance could also vary depending on the environmental conditions at sporulation (Scheldeman et al., 2006; S.A. Burguess 2010).

2.2.1. Sporulation Conditions

Spore resistance is affected by the sporulation conditions. In a study done by Nguyen et al., 2011, a wild type *B. subtilis* spore strain was cultivated in five different media conditions that included modified: aeration, pH, temperature, water activity, and calcium concentration. After cultivation, the spores were introduced to a high pressure treatment of 350MPa for 1 hour and heat treatments of 97°C for 30 minutes, It was found that the pH, temperature and water activity and calcium concentration at the time of cultivation had an effect on the spores' heat resistance as well as their germination (Hue Nguyn et al., 2011). If the vegetative state of the cell is exposed to a low aeration as well as a temperature of 45 °C, its spore state will have a more rigid cell wall and will be more heat resistant when compared to a vegetative cell exposed to the conditions of low water activity or 19°C where the spore contained a more fluid membrane and had a

higher sensitivity to heat (Hue Nguyn et al., 2011). Another factor that is found in milk and influences spore resistance is the presence of calcium ions.

2.2.2 Role of DPA in Spore Resistance

The absence of calcium ions is believed to inhibit the creation of Ca2+ dipicolinic acid and makes the spores more sensitive to heat (Hue Nguyn et al., 2011). Originated in the mother cell and transported to the forespore by a mechanism that is yet to be unfolded, Pyradine 2,6 dipicolinic acid (DPA) is one of the major ingredients in a dormant spore core and makes up 10% of the total spore dry weight (Murrell W.G 1967; Murrell W.G and A.D Warth 1965; Sleiman et al.,2001). It has been recognized by the help of a *B. subtilis* mutation excluding *dpaAB*, the gene responsible for the production of DPA, that the presence of DPA makes endospores more sensitive to wet heat but does not seem to impact dry heat resistance (Paidhungat et al., 2000; Sleiman et al.,2001).

2.3. Spore Germination

A dormant spore can survive for many years, but it only takes the right type of nutrients or ions and monovalent cations, available in the environment for the spore to germinate (Foerster and Foster 1966; Moir et al., 2002) .The process of spore germination is initiated by a series of signals given off by chemicals to specific receptors in a way that is still a mystery to scientists. The spore becomes vulnerable due to its loss of resistance and is reversed to a vegetative state (Scott and Ellar 1978; Setlow, 2003; Trolano et al., 2015). For germination to occur, nutrients must surpass the coat, cortex and germ cell wall to reach the inner cell membrane. The inner cell membrane contains nutrient germinant receptors (GRs) (Hudson et al., 2001; Setlow P. 2003;

Trolano et al., 2015.). It was believed that activity relating to germination was not initiated unless the spore cortex was hydrolyzed. It wasn't until later when a study done by Popham et al., 1996 proved that initiation is not dependent on spore cortex hydrolysis but is dependent on the activity of lytic enzymes found in the cortex. Cortex lysis, although not essential in germination initiation is still very important to germination completion. In dormant spores, 70 % of the lipids in the inner membrane (IM) are inactive and immobile but during germination, the activity of these lipids resemble a vegetative cell decreasing to 25% lipid immobility and the IM is shown to expand by 1.5 fold (Cowen et al., 2004; Torlano et al., 2015). Another factor that has been shown to have an effect on a spore and cause it to germinate and become more vulnerable is radiation.

2.4. Spores and Ultraviolet Radiation

When exposed to ultraviolet radiation, DNA undergoes a variety of lesions that include strand breaks, apurinic/apyrimidinic (AP) sites; UV induced pyrimidine dimers (PD) of many types as well as chemically altered bases (Nicholson et al., 2000, Pedraza-Reyes et al., 2012, Setlow P, 2007, Ramirez-Guadiana 2012). Endospores have shown to be 1 to 2 orders of magnitude more resistant to Ultraviolet radiation at 254 nm than its vegetative state. This resistance is due to a complex set of molecular interactions during the spore's developmental stages of sporulation, dormancy, and germination. When exposed to UV, two major repair mechanisms and a newly observed mechanism encoded by *ywj*D have been identified that contribute to an endospores increased resistance to ultraviolet radiation (Ramírez-Guadiana et al., 2012). One mechanism is the interaction of spores DNA with acid soluble spore proteins (SASPs),

and the ability to process the accumulation of spore photoproducts (SP) by repair systems that recognizes (SP) and splits them back into thymine residues (ii) and incising and excising by the nucleotide excision repair (NER) pathway (Nicholson et al., 2001, Ramírez-Guadiana et al., 2012).

2.4.1. Acid Soluble Spore Proteins

Acid soluble spore proteins (SASP) are spore coat components. These proteins are made late into spore synthesis and broken down during early germination (Setlow, 2007). SASP exists as two different conformations α/β all encoded by the *ssp* genes. It was reviewed by Setlow, 2007 that α/β SASP was the core component that provided spore DNA the most protection as it is found in high concentrations (5-10% of the total core) in *Bacillus* and *Clostridium* species (Setlow, 2006, Nicholas et al., 2005, Setlow 2004, Setlow, 2005, Driks, 2002, Setlow 2007). Binding between SASP and DNA was shown to be crucial for protection against UV radiation after the elimination of them caused spore UV sensitivity and increased protection upon their cross linking to DNA (Raju et al, 2006 and in press, Setlow 2007).

2.4.2. Spore Photoproducts

When a dormant endospore is exposed to ultraviolet radiation, the photochemistry found near its DNA develops spore photoproducts in which its most abundant is thymine dimer 5-thyminyl-5, 6-dihydrothymine informally known as (SP) (Donellan and Setlow ,1965, Varghese, 1970, Rebeil and Nicholson, 2001). One repair mechanism used by the spore is the reversal of (SP) to two thymines without excision from the DNA by SP lyase. SP lyase utilizes a 342-aa protein referred to as SpIB that has been confirmed by Rebeil and Nicholson to be part of the "radical SAM" protein

superfamily (Sofia et al., 2001, Rebeil and Nicholson, 2001).Given this new evidence as well as evidence proposed by earlier studies, Rebeil and Nicholson have proposed the role of SpIB in SP cleavage to two thymines *in situ* by SP lyase (Rebeil and Nicholson, 2001). As show in Figure 2, (i) When reduced centers of [4E-4S] are formed (ii) two of the subunits that make up SpIB dimerize to create SP lyase, Methionine and 5'-dAdo radicals are generated by the splitting of S-AdoMet after the [4E-4S] center has donated electrons (ii) The generated radical either directly or indirectly utilizes a proton from the C6' of SP (Rebeil and Nicholson, 2001). It is supported by Mehl and Begley (Mehel and Begley, 1999) that this cascade of SP lyase action leads to the β Scission of the bond between the two thymines and completes the reaction by back transferring of protons (Mehel and Begley, 1999, Rebeil and Nicholson,2001).

2.4.3. Nucleotide Excision Repair

The Nucleotide excision repair (NER) is a major repair mechanism found to alleviate an endospore from UV damage. The (NER) repair system functions by removing DNA damage and filling the open space by a DNA polymerase (Jaciuk et al., 2011). NER uses three identified proteins, UvrA, UvrB, and UvrC, to repair a wide range of DNA damages that include single base modifications, bulky adducts, backbone modifications and inter- or intrastrand cross-links (Howard-Flanders et al.,1966; Truglio et al., 2006; Jaciuk et al., 2011). Jaciuk et al., 2011 proposed that UvrA is the first protein to come in contact with the damaged DNA by sensing the bending, unwinding and any other irregularity of the normal DNA conformation. UvrA alone is not able to distinguish which DNA strand is the one that is damaged so it is suggested that UvrB is used to verify the damage and then UvrC cleaves the DNA at the fourth or fifth

phosphate 3' of the damaged area and at the eighth phosphate 5' of the lesion. UvrD cleans out the area by removing the unwanted oligonucleotides while polymerase I fills the gaps. It is not until DNA ligase I seals the nicks that the repair is complete (Sincar and Rupp, 1983; Jacuik et al., 2011). Avoiding the use of these repair mechanisms by microorganisms is always ideal. Protection from harsh external environments can be achieved by forming sessile communities more commonly known as biofilm.

2.5 Biofilm Formation

Feed streams in milk processing, due to milks rich composition of nutrients, provide an ideal environment for biofilm formation. Biofilm is described as a complex assembly of microorganisms coalesced within an extracellular matrix. It has been suggested by many that biofilm development is a five step process that includes: (1) The reversible attachment of the microorganism a solid surface, (2) the switching of reversible attachment to irreversible attachment when extracellular polymers (EPS) are produced by the adhered microbes (3) commencement of biofilm architecture, (4) the establishment of microcolonies deeming the biofilm as mature, (5) and finally the dispersal of portions of biofilm into the environment (costerton et al., 1995; Costerton, 1999; Sutherland, 2001;Myszka and Czaczyk,2011). Figure 3 shows each of the stages.

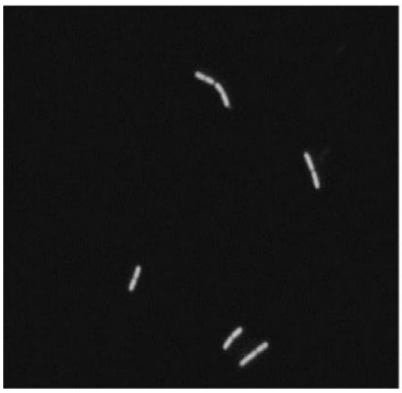
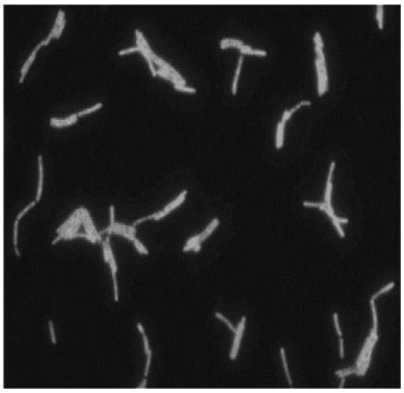
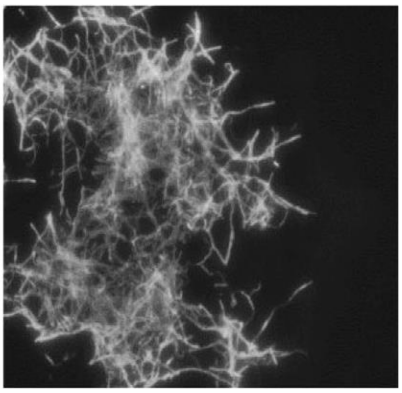

Figure 2. Biofilm stages Myszka and Cazaczyk, 2011

2.5.1. Reversible Attachment

There are many factors that affect the initial reversible attachment of endospores to stainless steel such as media type on the processing surface, surface charge, the hydrophobicity of the bacteria and the physical properties of the stainless steel. Stainless steel surfaces mainly contain a negative surface charge as well as most bacteria creating repulsion due to electrostatic interactions but it has been shown that because of an endospores hairy-like cell surface and their hydrophobicity, they are more likely to attach to surfaces than their vegetative counterparts (Ronner et al., 1990; Husmark and Ronner 1992; Kumar and Anand 1998; Anad et al., 2014). Surface conditioning media is also thought to change surface charge (Melo 1992; Carpentier and Cerf 1993; Subramani et al., 2009).Conditioning media containing milk proteins and lipids begins to form as soon as the liquid phase comes in contact with a hard surface changing the surface charge of stainless steel attracting spores with by different forces (Speers and Gilmour 1985; Mcguire and Swartzel 1989; Shi and Zhu 2009). Reversible bacterial adhesion begins through van der waals forces as well as electrostatic forces when the spore is approximately 2- 50 nm away from the processing surface. Depending on the conditions listed above and the hydrophobicity of the surface spore attachment is achieved (Miron et al., 2001, Myszka and Czaczyk, 2011). Hydrophobicity of a metal surface has so far been determined by measurements of its contact angle (Liu et al., 2004) .According to a study done by Bos et al [2004], microbial attachment was shown to be possible in the hydrophilic portion of the

hydrophilic/hydrophobic regions of stainless steel. Surface roughness is described as a measurement between peaks and valleys found on the metal surface and were found by Howard and Behrands (2006) to be correlated with cell attachment. Also, surface defects were found to affect attachment in that: (i)a rough surface has a larger surface area (ii) depressions in the material are ideal for microorganisms to colonize since the roughened material will provide protection from the shear forces that go along with production (Mitik- Deneva et al., 2008,2009; Myszka and Cazaczyk, 2011). Biofilm at this reversible stage is quite easy to remove with just rinsing; it is when the conversion to irreversible attachment is achieved that more effort is necessary to eliminate attached cells. Based on this evidence, it is clear that the surface of processing material has an impact on bacterial attachment and a means to work with these charges in order to prevent biofilm formation is necessary.

2.5.2. Production of Extracellular Polymers

Once reversible attachment is achieved by spore forming bacteria, like other studied microorganisms, produce extracellular polymers (EPS) deeming their attachment irreversible. Dipole- dipole interactions, hydrophobic, ion-dipole, ion-ion, covalent bonds, and hydrogen interactions hold the spores on the surface and have shown to upregulate specific adhesion genes that produce enzymes responsible for (EPS) production as fast as a few minutes after their initial attachment (Marshall et al., 1971; Zottola, 1994; Dunne, 2002; Myszka and Cazaczyk, 2011). EPS is described as the main component in biofilm formation since it is responsible for cell to cell, and substrate adhesion (Valle et al., 2006; Jun et al., 2009; Anand et al., 2014).The key component extracellular polysaccharide in EPS is composed mainly of carbohydrates,

(Kennedy and Sutherland, 1996) proteins, some nucleic acids and various humic substances (Nielsen et al., 1996; Liu and Fang 2002; Vu et al., 2009; Lee et al., 2010). EPS acts as a shield for spores and other microorganisms to the external environment and according to Sutherland 2001, many microorganisms EPS, including spore forming bacteria, contain 1,3-or 1,4-β-linked hexose residues making EPS more rigid. In dairy processing, biofilms were found to be composed of EPS and milk components (Flint et al., 1997; Mittelman 1998; Flemming et al., 2000). After the conversion of the biofilm to irreversible attachment, then biofilm maturation phase begins.

2.5.3. Commencement of Biofilm Architecture

The microorganisms within the biofilm grow and divide depending on how much nutrients are available in the initial conditioning media as well as the surrounding environment. As the bacteria grow within this matrix, microcolonies begin to form a layer of cells on the surface which also excrete EPS and so creating layers as more bacteria attach and produce more EPS providing additional protection to earlier cells (Kumar & Anand, 1998; Jefferson, 2004). The microbial distribution within these colonies is not uniform and some have been reported to be so complex that they contain water channels (Costerton et al., 1994; Kumar and Anand 1998; Avadhanula 2011) During this phase, if the adequate nutrients are available, biofilm grows a few millimeters in thickness in a matter of days creating three dimensional structures that resemble the shape of a mushroom (Dunne, 2002). It is at this stage were cell to cell signaling referred as quorum sensing (QS) is used within the biofilm.

2.5.4. Quorum Sensing

Quorum sensing is a term used when cells use chemical communication to check the species proportion and cellular population within the biofilm by producing, releasing, detecting, and responding to autoinducers (AI), hormone-like signal molecules. AI concentrations increase as the microbial population increases therefore QS is considered part of biofilm maturation due to its role in monitoring the environment for biofilm survival and development (Miller and Bassler 2001; Paresek and Greenberg 2005; Waters and Bassler 2005; Kociolek 2009). When a single spore forming bacteria activates QS it is not effective, it is when multiple spore forming organisms are in a sessile community that it is most useful. LuxI and LuxR are the two main regulatory proteins that work to recognize and synthesize the autoinducer. In gram positive bacteria, autoinducing peptides (AID) as well as autoinducer 2 (AI-2) are the cell signalling systems used (Bai and Rai 2011). Turovskiy and others 2007, have stated that QS helps biofilm grow by facilitating its response to the environment. QS has a significant importance in dairy processing as well as any other food production due to its regulation of deterioration of food caused by proteolytic, lipolytic, chitinolytic, and pectinolytic activities (Bai and Rai, 2011). Finally, when the biofilm has matured and there is enough spores accumulated, the flow of production causes the biofilm to slough off and be distributed to the final product and or attach to another surface and begin biofilm formation once again (Applegate and Bryers, 1991). However, biofilm formation varies depending on the environment, some biofilm such as ones found in dairy processing equipment do not reach the final stage of biofilm formation.

2.5.5. Process Biofilm

Biofilm composed of thermophilic bacilli, the type regularly seen in dairy production, is referred to as process biofilm (Flint, 2007). Although process biofilms are still poorly understood, it is believed that due to the varying pressures such as pH, product composition, high temperatures, and water activity found throughout dairy processing, process biofilms are limited to a single bacterial species (Burgess et al.,2010). Studies done at a laboratory scale have suggested that process biofilm in areas like the surface of a plate heat exchanger that are regularly cleaned, have high shear rates as well as no dead zones, only form a monolayer and never reach the multilayer stage of the traditional biofilm formation. It is in the areas where milk flow is inconsistent for example, underneath distribution plates of the evaporators, spores may get trapped in milk foulant allowing for a multilayered structure (Burgess et al., 2010; Flint et al., 2001;).Although our understanding of this biofilm is limited, there are many studies on methods to eliminate or prevent the attachment of bacteria.

2.6. Current Methods to Eliminate and Prevent Existing Biofilm Formation

A list reported by Myszka and Cazaczyk, 2011, are shown in Table 1., lists various physical methods that have shown effect on existing biofilm which include: scraping and ultrasonic technique, the use of water at very high temperatures, low electrical currents and antibiotics. There are also chemical methods listed such as: chlorine, peroxygen and the use of quaternary ammonium compounds (QAC). Besides chemical and physical means of eliminating an already existing biofilm, enzyme based cleaners and surfactants work to prevent microbial adhesion (Whittaker et al.,1984).

The most practiced method in any food production facility for removal of debris and bacteria is the manual scraping of surfaces. As studied by Qian et al., 1999 on a

biofilm composed of *E.coli* , the manual scraping in combination with a sonication of 100-15 KHz was able to remove a thick layer of biofilm. In another study by Wirtanen & Matilla--Sandholm, 1993; Burfoot et al., 2009, where water at a temperature as high as 125°C for 30 min seemed to control the formation of biofilm composed of *P. aeruginosa*; however, this method proved ineffective against three day old biofilm of the same species and results varied depending on the type of bacteria.

Cubet et al., 2004, investigated the use of low electrical currents in combination with antibiotics on biofilm composed of E. coli. The transfer of biocide ions through the biofilm showed to transfer antibiotics rapidly at lethal concentrations. Although the study was found successful, the limiting factor again was the stage and age of the biofilm (Cubet et al., 2004).

There are a variety of chemicals that are currently being used in industry to eliminate biofilms that include the use of: chlorine, peroxides, acids and quaternary ammonium compounds (QAC). Due to the oxidizing and disinfecting values, chlorine has been commonly used throughout the food industry (De Beer et al., 1994). Chlorine has shown to be most effective after 5 to 30 min of contact time to reduce the load of *Pseudamonas* spp and proved to be effective at removing EPS material produced by *Salmonella* spp. on stainless steel surfaces (Gelians et al., 1984; Ronner and Wong,1993). Even more effective than chlorine are chloramines because they are better able to penetrate biofilm but require a longer contact time (Samrakandi et al.,1997).

Peroxygen sanitizers are bactericidal against endospores (McDonell & Russel, 1999). Harkonen et al., 1999 showed that peroxygen sanitizers were more effective on some biofilms than chlorine but required more contact time.

Quaternary ammonium compound cleaners are foam, cationic, surfactants cleaning activity (McEldowney and Fletcher, 1987).They are used in industry often to clean surfaces such as floors, containers and any other surface that does not need to be rinsed or can allow a longer contact time during production since QAC is more effective with increased time (Chmielewski & Frank, 2003).Even Though all of these physical and chemical methods of removing biofilm have proven to be successful, age of biofilm, as well as existing debri and soils are still factors that play into their effectiveness and their ability to eliminate endospores on surfaces.

Another type of cleaner that is used is an enzymatic based cleaner commonly referred to as QuatroZyme. QuatroZyme is a combination of many enzymes:lipase, protease, cellulose, and amylase, enzymes found by Tang et al., 2010 to work better than any other enzymes tested in the past. Enzymatic cleaners function in a unique fashion in that chemicals and detergents neutralize charged particles and resuspend them where as enzyme based cleaners hydrolyze the exopolymers where microbes are embedded (Whittaker et al., 1984). Although enzymes have these capabilities, because of their large molecular weight they are incapable of degrading biofilm so it is recommended to mix them with an anionic biodegradable detergent to enhance their performance (Coolbear et al., 1992). Enzymes have also been utilized to prevent surface fouling. Leroy et al., (2007) demonstrated in their study that the application of the enzyme savinase prevented the attachment of marine biofilms.

Surfactants are made up of surface-active compounds that provide an additional uniform wetting effect to surfaces giving them additional cleaning (Cloete and others 1992;Lutey1995). The mechanism behind surfactants is through the binding of ionic sites in proteins with electrostatic and hydrophobic interactions. A group of four distinct surfactants are classified based on their hydrophilic groups: anionic, cationic, nonionic, and zwitterionic (Madaeni and others 2010, Anand et al., 2014). Anionic surfactants are in particular interest in the dairy industry since their hydrophilic groups interact with whey proteins to decrease the surface tension of molecules that are in contact with each other (Anand et al., 2014). A major disadvantage associated with use of surfactants is not only their high concentrations in waste water streams created during food production but also their detection in rivers and lakes (sakai et al 1998; Lavorante et al., 2007Latif et al., 2012; Hampel et al 2012; Bazel et al., 2014).Aside from the mentioned surface treatments, the use of Ultraviolet radiation as an alternative to chlorine has been found to be quite effective; however, conventional UV is ineffective toward spores. Based on the above evidence on methods to prevent or eliminate spore containing biofilm, it is clear that a more powerful, potent, and rapid treatment is needed in order to reduce the endospore population within a biofilm or to prevent their initial adhesion.

2.6.1. Xenon Pulsed Light Technology

Pulsed light (PL) technology is the use of short light bursts to decontaminate surfaces by inactivating bacteria with a broad range of ultraviolet radiation. Xenon lamps are a rapid, high energy treatment that consists of wavelengths ranging from (200-1100 nm) including far UV (200-300 nm), near UV (300-380 nm) and infrared (780-1100 nm)

(Ogihara et al., 2013). The mechanism of action by Xenon (PL) is reviewed by Gomez-Lopez et al., (2007) where power is magnified by storing electricity in a capacitor over fractions of a second and releasing it in a short time (millionths or thousandths of a second). The light flash has a high peak power and consists of wavelengths from 200 to 1100 nm (Dunn, Bushnell, Ott and Clark, 1997; Dunn, Ott and Clark, 1995; Gomez-Lopez et al., 2007). Not only does this technique produce flashes with high peak power, but also create a greater relative production of light with shorter bactericidal wavelengths (MacGregor et al., 1998;Gomez-Lopez et al., 2007).

PL has many advantages over continuous wave UV light (CW-UV), a food preservation technique using UV-C, and has been an approved method. In the food industry where disinfection is preferred at a faster pace, PL is the preferred method (wang et al.,2005). Rice and Ewell, (2001) proved that the same amount of fluency was reached in 40 seconds using PL than 3h using CW-UV. CW-UV lamps are made of mercury while Xenon PL lamps do not making PL systems more environmentally friendly. Xenon lamps do not leave any residues after their use in comparison with much of the cleaners deeming PL the "Greener" option and there is not the concern that these chemical residues will end up in the final product or cause ecological concerns (Gomez-Lopez et al., 2007). PL has been used effectively to remove both non-food and food related bacteria on various surfaces such as: agar (Ogihara et al.,2013), plastic films Fernandez et al. (2009),glass, various liquids including buffers, juices and water (Sauer and Moraru, 2009; Palgan et al., 2011), even on the actual food stuff surfaces (Ozer&Demirci,2006; Can et al., 2014). PL has also been used successfully on dairy products. Dunn et al., 1991 used cottage cheese that contained *Pseudomonas* spp. and

treated it with pulsed UV light at 16 J/cm2 with pulse duration of 0.5 ms, and obtained a 1.5-log reduction. Krishnamurthy et al.,2007 used xenon PL to investigate the effect of sample distance from the UV strobe, number of passes, and flow rate of the milk inoculated with *Staphylococcus aureus* and found that with treatments combinations of 11 cm, two passes, and 20 ml/min resulted in reductions up to 7.26 log CFU/mL. The effect of xenon PL was also evaluated on the surface of hard cheeses.

Authors such as Can et al., 2014 used PL technology on the surfaces of packaged and unpackaged, hard cheeses. Cheese surfaces were spread with *P. roqueforti* as the spoilage microorganism, and *L. monocytogenes* as the pathogenic microorganism. It was found that PL reduced populations of both microbes in packaged and unpackaged cheese but worked more effectively on *L. monocytogenes* yielding a 2.9 and 2.8 log CFU/cm2 with the treatment combination of 8 cm for 30 seconds compared to *P. roqueforti* that resulted in a 1.1- log reduction at the same combination (Can et al., 2014). The difference in PL effect is thought to be since mold spores are more resistant than their vegetative counterparts and because *P. roqueforti* mold spore colonies are darker in color than *L. monocytogenes* colonies (Can et al., 2014). Other cheese quality parameters, cheese color and peroxidation, were measured after the application of mild (5 seconds at 13 cm), moderate (30 seconds at 8 cm), and extreme (40 seconds at 13 cm) xenon PL treatment conditions (Can et al., 2014). It was found that there was no significant difference after application of the moderate treatment in both color and peroxidation; however, changes started occurring after moderate and extreme conditions (Can et al., 2014). Based on the given literature, it is clear that spore forming bacteria, especially spores involved in a biofilm complex, need high treatment

levels of Xenon PL to be inactivated and that this technology would be best used on the processing surfaces and not on the actual food stuff surface in order to avoid any physical or sensory disturbances of the final dairy product.

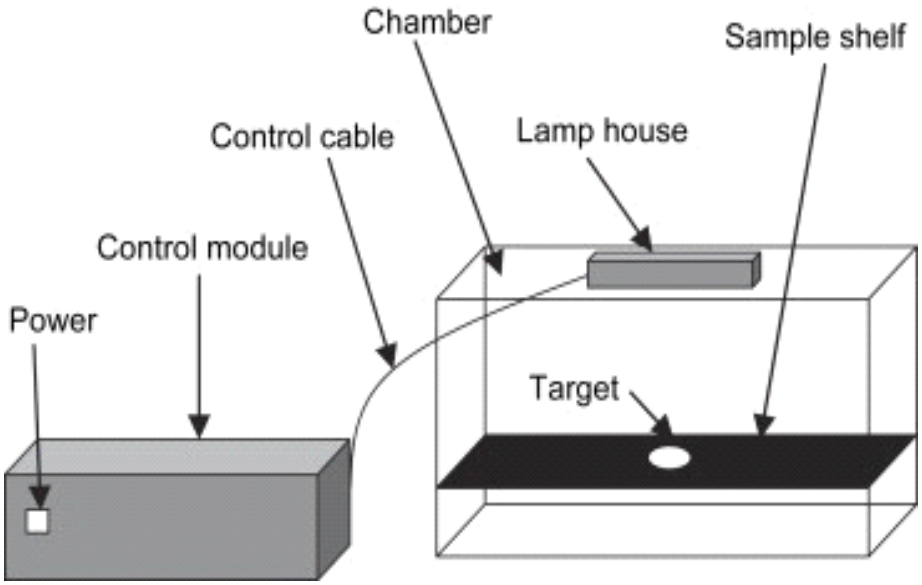

Figure 3. Diagram of Xenon Pulsed- Light apparatus provided by Gomez-Lopez et al., (2007).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Sweet whey was obtained from daily cheese manufacturing at the Cal Poly Creamery (San Luis Obispo, CA, USA). Whey protein Isolate (WPI) 9400 was provided by Hilmar (Hilmar, CA, USA), and L5-500 a-D- (+) - Lactose Monohydrate as well as the starch and agar used for growth media were obtained from Fisher Scientific (Fair Lawn, NJ,USA).Square coupons with dimensions, 2.5 cm x 2.5 cm x 0.1 cm of Stainless steel ASI 304 , typical metal used on dairy processing surfaces, was used to make the coupons for this experiment. Xenon Corp. device, model SteriPulse-XL 3000 (Xenon Corporation, USA) with an output of (3100V dc- 3800Vdc) was applied, as treatment, to the stainless steel surfaces.

3.2 Endospore Stock Solution Preperation

Endospores were grown according to a protocol developed by Dr. Rafael Jimenez-Flores' research group. A single loop from each spore stock solution was aseptically transferred to sterile tryptic soy broth (TSB). A water bath heat- shock step, initially developed by Whittaker and others 1984, of 80°C ±1 °C for 12 minutes was applied to each tube to activate spores followed by an ice bath for 10 minutes.

Cultures were streaked for isolation on tryptic soy agar (TSA) and 0.2% (w/v) soluble starch. After 24h of incubation at 55°C, a representative isolated colony was used to inoculate 9mL of TSB which was incubated overnight at 55°C until tubes appeared turbid.

The cultures were streaked for heavy grow on 0.2% starch TSA and incubated at 55°C for 7 days. Plates were placed inside of bags to avoid dehydration and after the 7

days phase contrast microscopy was used to evaluate sporulation. Once 90% sporulation was observed, plates were incubated again overnight to induce further sporulation.

Spores were collected from the plates by adding 5mL 0.1% peptone solution to each plate and scraping them from the agar surface using sterile hockey sticks into sterile 50 mL falcon tubes. Cells were then pelleted at 4000 rpm for 20 minutes at 4°C and the supernatant was discarded. The cell pellet was then resuspended with 25 mL of 0.1% peptone solution before being submitted into a heat treatment of 70°C for 30 min. After the heat treatment, the cells were centrifuged at the same conditions mentioned previously. The supernatant was discarded before 9 mL of filter sterilized lysozyme solution (0.3mg/mL lysozyme, 15mM $MgCl_2$, 0.067 M K-phosphate buffer; pH 7.0) was added in each falcon tube in order to digest the remaining vegetative cells. The tubes were vortexed and incubated for 1 hour at room temperature. After these procedures, the tubes were heat treated at 60°C for one minute to deactivate the lysozyme before being centrifuged.

Spores were once again pelleted at 4000 rpm for 20 minutes at 4°C and the supernatant was discarded to rinse the pellet. The rinsing procedure was repeated three times. 30 mL of sterile deionized water was added to the final spore pellet and the spore stock solution of each microorganism were maintained at 4°C for approximately 3 months. Concentrations of the spore stock solutions were determined using direct microscopic count and recorded on each tube.

3.3 Stainless Steel Surfaces

In order to create a biofilm by dairy media and spores, stainless steel coupons with dimensions of 2.5 cm x 2.5 cm x 0.1 cm and a hole in one corner (1mm diameter) were sanitized before each experimental run using the conditions adapted from Rosmaninho et al. (2007) and Palmer et al. (2010) that imitate CIP procedures. The coupons were immersed in a 1% NaOH solution (w/v) at 80°C for 15 minutes at 400 rpm. The stainless steel coupons were then rinsed with distilled water at room temperature for 5 minutes then immersed in a 50% (v/v) nitric acid solution at 70°C for 15 minutes at 400 rpm to passivate the surface. After the initial passivation, the nitric acid solution is lowered to 1% (v/v). Coupons are then washed with distilled water for 5 minutes at room temperature and then autoclaved at 121°C for 15 minutes.

3.4 Biofilm Creation

In order to compare the effect of pulsed light technology on spore forming bacteria, the coupons were subjected to 20 hours of fouling with four different dairy matrices (5% lactose (w/v), 5% WPI (w/v), sweet whey (unfiltered to imitate processing conditions) (v/v), and distilled water) at 200 rpm and 55°C After the incubation period, biofilm was created on the coupon surfaces using a 1% spore solution for an additional 20 hours.

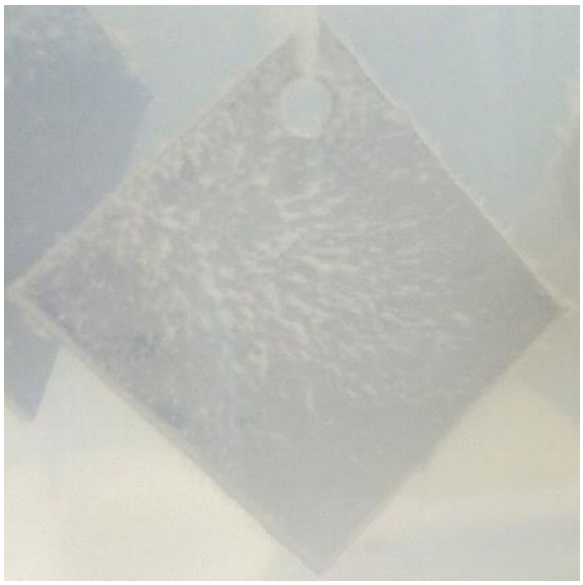

Figure 4. WPI fouled coupon with spores after 20 hours of incubation stainless steel coupons were incubated with a 5% (w/v) WPI solution at 55°C 200rpm for 20 hours. The solution was then discarded and replaced with a 1% (v/v) solution under the same incubation conditions. A mossy hair like film was visible after incubation with 1% spore solution.

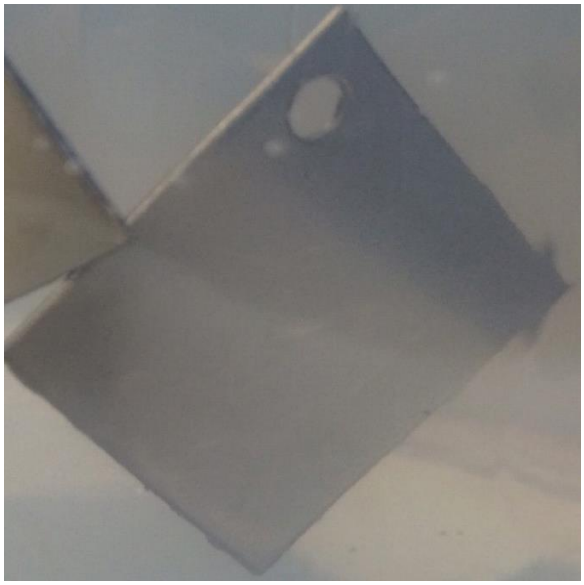

Figure 5. Sweet whey fouled coupon with spores after 20 hours of incubation. Stainless steel coupons were incubated with a 5% (v/v) sweet whey solution at 55°C 200rpm for 20 hours. The solution was then discarded and replaced with a 1% (v/v) solution under the same incubation conditions. Unlike coupons initially fouled with WPI, coupons incubated with sweet whey did not have a visible film on the coupons.

3.5 Experiment 1. Xenon Pulsed Light Treatment Application

After the coupons were subjected to the appropriate fouling conditions, each coupon was dipped three times in sterile deionized water to remove any loosely adhered spores. Coupon surfaces were treated with 5 UV treatment levels (No treatment, 5 bursts, 10 seconds, 20 seconds and 30 seconds) each placed at a distance of 10.5 cm away from the ultraviolet lamp. After treatment, the coupons were placed inside 50 mL falcon tubes and vortexed for two minutes with 0.1% peptone solution to detach spores from the coupon surfaces and obtain a heterogeneous microbial solution. After these procedures, samples were pour plated on 0.2% (w/v) starch TSA and incubated at 55°C for 48 hours.

3.6 Experimental Design

Four Coupons were treated with the same media in a single jar (MJar). Coupons that shared the same jar were treated with the same media at the same time. Coupons were then treated with the same strain in a single (SJar). Coupons that shared the same SJar were treated with the same strain and were treated earlier with the same MJar at the same time (trial). Lastly, Individual coupons were treated with a single pulse type, and were plated three times.

3.7 Experiment 2. Determining the Difference in Spore Germination Based on Fouling Media

To determine if there is a difference in spore germination depending on fouling media, four different solutions composed of 5 % solution of lactose (w/v), WPI (w/v), sweet whey (v/v) and sterile deionized water were incubated with 1% (v/v) spore solutions (*B. licheniformis* and *B*. *coagulans*) at 55°C and 200 rpm for approximately 20 hours. To compare the maximum amount of time of incubation from experiment 1 to the

initial state of the spore, a spore solution was incubated at time 0 hours and time 20 hours , 1 mL were pipetted into 1 mL centrifuge tubes and centrifuged at 14.1 thousand rpm for 20 minutes two times to obtain a visible pellet. 100 µL of sterile deionized water were then used to resuspend the pellet and 10 µL were used for endospore staining. Spore germination was determined by direct microscopic count. Both vegetative bacteria and spores were counted.

3.8. Experiment 3. Pretreatment of Stainless Steel Coupons to Compare Spore Adhesion on Surfaces

After the initial stainless steel coupon sanitation procedures, the coupon surfaces were treated with either: canola oil, 3 minutes of continuous PL treatments at a distance of 10.5 cm from the lamp and canola oil, 3 minutes of PL, and no treatment at all. After pretreatment procedures, the coupons were subjected to a 1 % spore solution and incubated at 55 °C and 200 rpm for approximately 20 hours. After the incubation period, the coupons were placed inside 50 mL falcon tubes and vortexed for two minutes with 0.1% peptone solution to detach spores from the coupon surfaces and obtain a heterogeneous microbial solution. After these procedures, samples were pour plated on 0.2% (w/v) starch TSA and incubated at 55°C for 48 hours.

3.9 Spore Enumeration

The number of CFU/ cm^2 was determined by the following equation (Bernardes 2012).

$$\frac{CFU}{cm^2} = \left(\frac{\frac{M}{D} x \frac{V_D}{V_A}}{A}\right)$$

Where $V_D$is the volume of peptone solution used for rinsing (mL); V_A is the volume used for the aliquot plated mL; M the colony number after incubation on the plate (CFU); D is the decimal dilution; A is the area of the test coupon (cm^2).

CHAPTER 4: RESULTS

Each bacillus strain behaved differently towards the PLT exposure depending on the fouling media but this difference did not deem to be statistically significant. Even Though *B. coagulans* experienced no less than an average of a one log reduction on all treatments with all fouling media, the highest average log reduction happened when sweet whey fouled coupons were subjected to a PLT treatment of 20 or 30 seconds and followed the expected trend of increasing average log reduction with increasing PLT time.

When an ANOVA test was conducted, table 5, the analysis showed that media type used was statistically significant having a P- value of <.0003. After conducting a Bonferroni adjustment it was determined that the media WPC produced a higher mean response than lactose, SW, and water. The mean response of lactose, SW, and water were statistically indistinguishable as they shared the same letter in the mean response in table6. We also identified that there was no statistical difference in strain type, table5. Also, there was no interaction between media type and strain shown at the 1% significance level but there were some differences noted with a graphical analysis, figures 7,8,10 and 11. Pulse level also had a p value of <.001at a 1% significance level table5. By using the Bonferroni adjustment it was suggested that Pulse level 0 produced a higher mean response than all other pulse levels. Pulse level 4 produced a mean response lower than all other pulse levels (0, 1, and 2) other than level 3.

Experiment1.

It is demonstrated in the control group fouled treated with water as media (Figure 10), how *B. coagulan* exposed to 5B of PLT obtained a 1.15 log reduction while at 10

seconds held a 2.25 log reduction. A 1.97 log reduction was achieved after 20 seconds of exposure to the treatment as well as 2.25 logs reduced after 30 seconds. *B. licheniformis* obtained a 1.89 log reduction at 5B and a 2.89 log reduction at 10 seconds. 20 seconds and 30 seconds both deemed an average log reduction of 3.25. 5B applied to *B.subtilis* resulted in 1.42 logs of reduction and 10 seconds of PLT exposure produced 1.59 logs of reduction. 1.5 logs of reduction were obtained after 20 seconds and a maximum reduction of 2.04 logs after 30 seconds was seen.

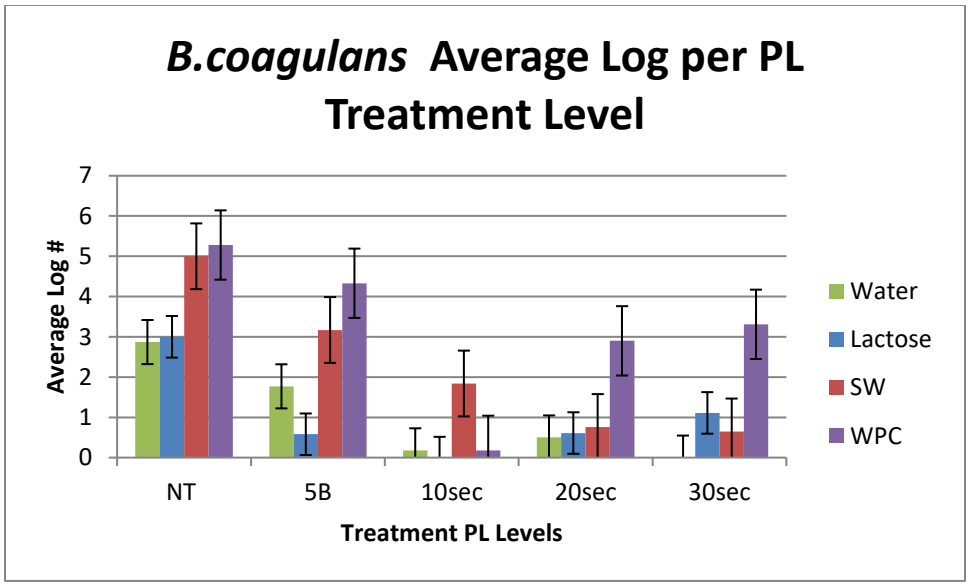

Figure6. The Average Log number of *B. coagulans* incubated in different media at treatment levels (NT, 5B, 10sec, 20sec and 30sec).

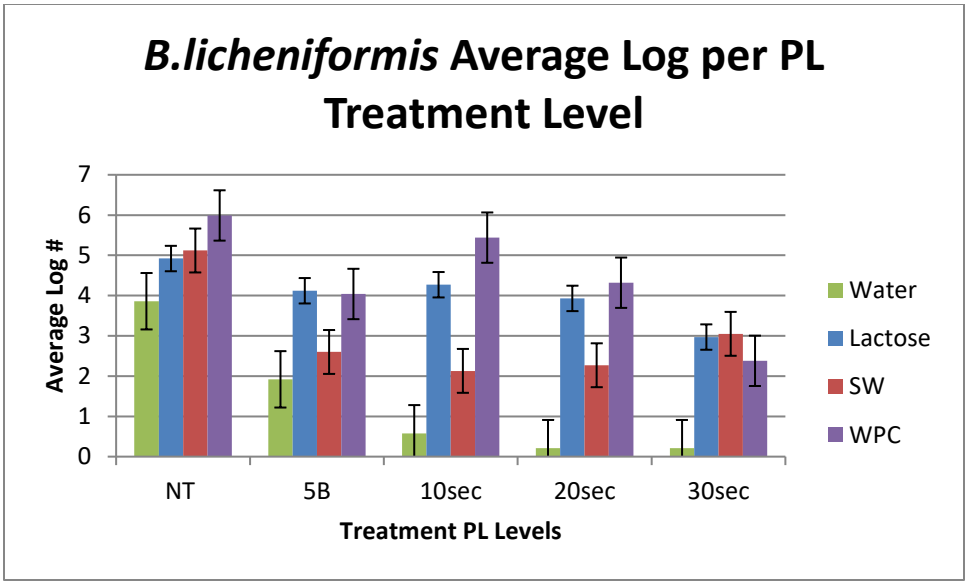

Figure7. The Average Log number of *B. licheniformis* incubated in different media at treatment levels (NT, 5B, 10sec, 20sec and 30sec).

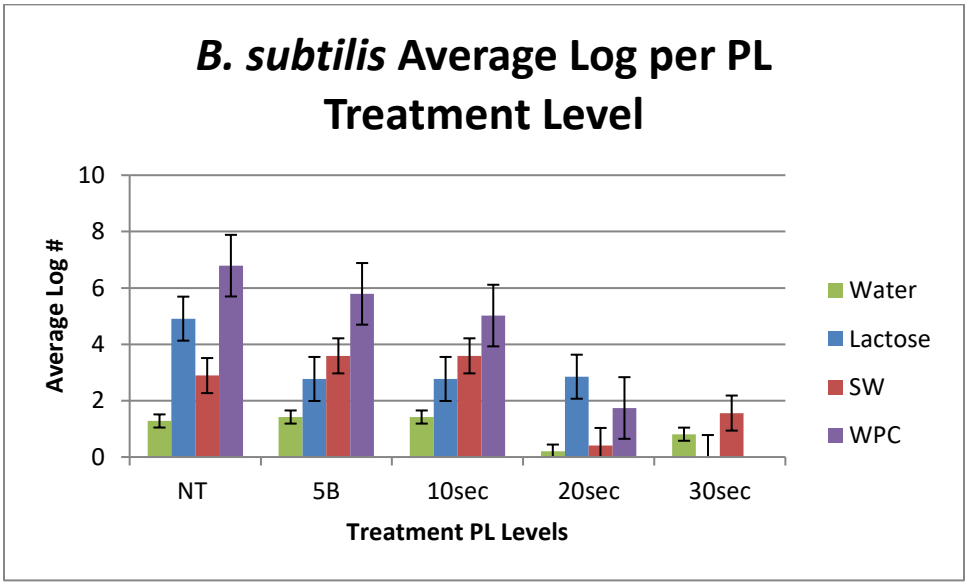

Figure8. The Average Log number of *B. subtilis* incubated in different media at treatment levels (NT, 5B, 10sec, 20sec and 30sec).

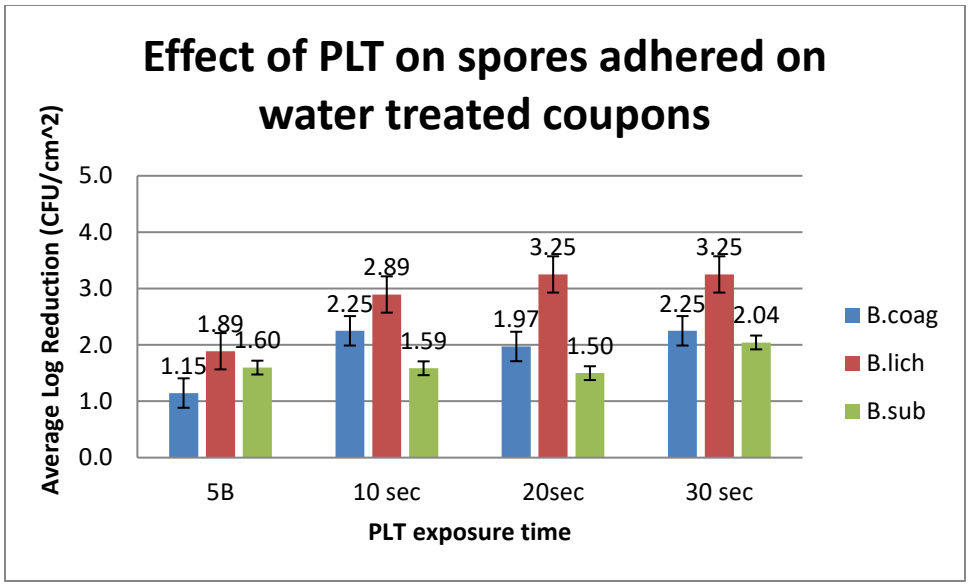

Figure9. *B.coagulans, B. licheniformis, and B.subtilis* adhered to coupons pretreated with water and exposed to increasing time intervals of pulsed ultraviolet radiation in the xenon machine.

Lactose fouled coupons containing both *B. licheniformis* and *B. subtilis* followed a trend of increasing average log reductions with increased exposure time in Figure 8. *B. licheniformis* reached 1.30 log reductions with as little as 5 consecutive pulsed light bursts (5B), at 10 seconds a log reduction of .87 was obtained; 20 seconds of continuous exposure produced an average of 1.22 log reduction and as much as 2.18 log reduction at 30 seconds. *B. subtilis* also had a .98 log reduction at 5B and escalated to 2.03 log reduction for 10 second and 20 seconds of exposure and 4.13 log reduction at 30 sec. having the maximum log reduction using lactose as the fouling media of all three endospores. *B. coagulans* reached a 2 log reduction at 5B and a 2.58 log reduction at 10 seconds; however, the log reduction was reduced to 2.19 at 20 seconds and 2.03 log reduction at 30 seconds. Figure 9 is a visual example of colony forming units on agar plates from lactose and WPC fouled coupons with treatments of No treatment, 5B, 10 sec and 20 seconds of PLT exposure. Not pictured are water treated

and sweet whey treated samples as well as coupons treated at 30 seconds of PLT exposure.

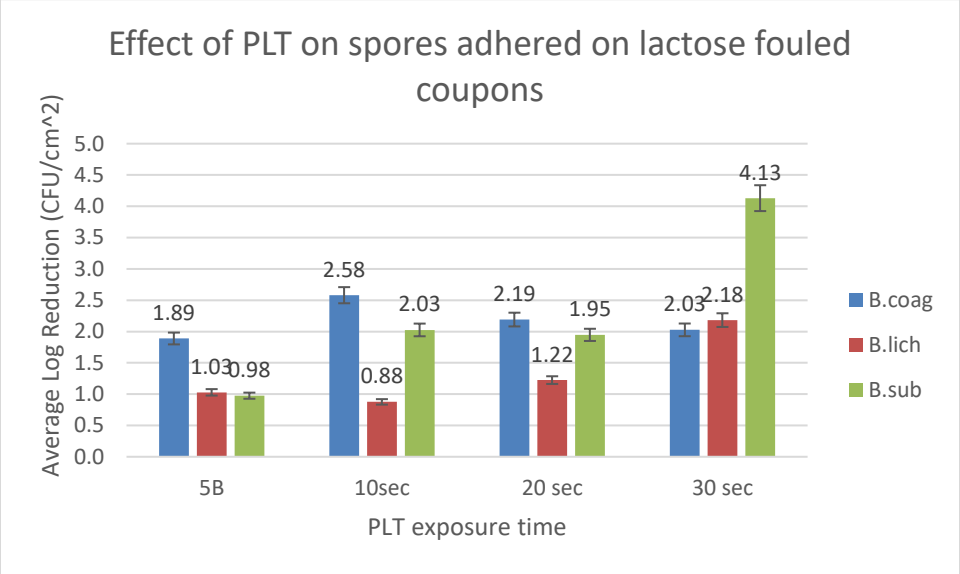

Figure10. *B. coagulans, B. licheniformis, and B. subtilis* adhered onto lactose fouled stainless steel coupons and exposed to increasing time intervals of pulsed ultraviolet radiation in the xenon machine.

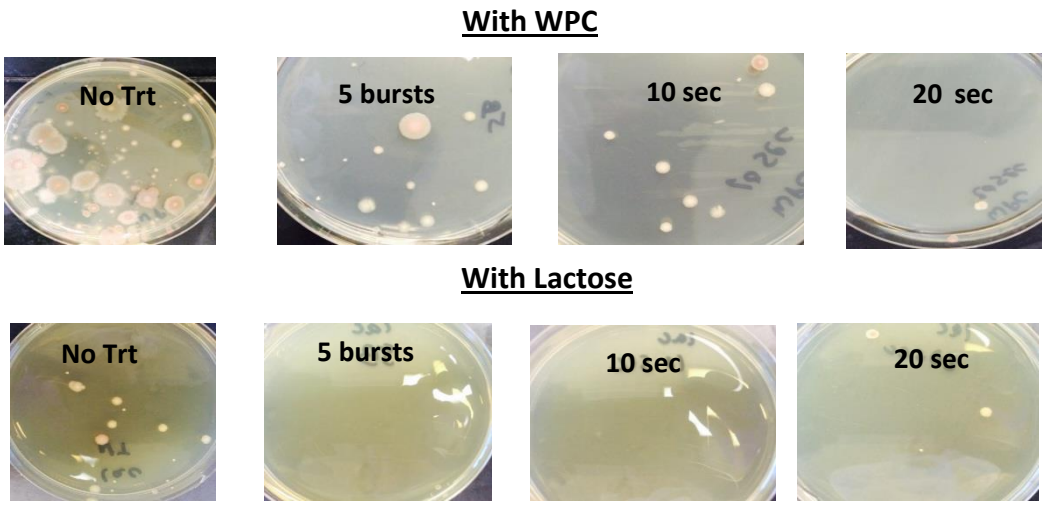

Figure11. Colony forming units on TSA agar plates from WPC and Lactose fouled coupons subjected to different UV treatments. (30 sec treatments not pictured)

SW fouled coupons containing *B. coagulans* initiated with a 2.1 log reduction with 5B of PLT followed by 3.20 log reductions at 10 seconds increasing to 4.17 log reductions at 20 seconds and concluding with a maximum of a 4.28 log reduction when exposed to 30 seconds of PLT in Figure 10. At 5B, *B. licheniformis* had a 2.64 log reduction and 3.11 log reduction at 10 seconds. 20 seconds of PLT exposure produced a 3.08 log reduction and a lower log reduction of 2.53 at 30 seconds. *B. subtilis* proved more resistant to treatment with SW as the fouling media with only .09 log reductions at 5B of treatment and .82 log reductions at 10 seconds. 20 seconds of treatment increased log reduction to 3.32 and 2.49 at 30 seconds.

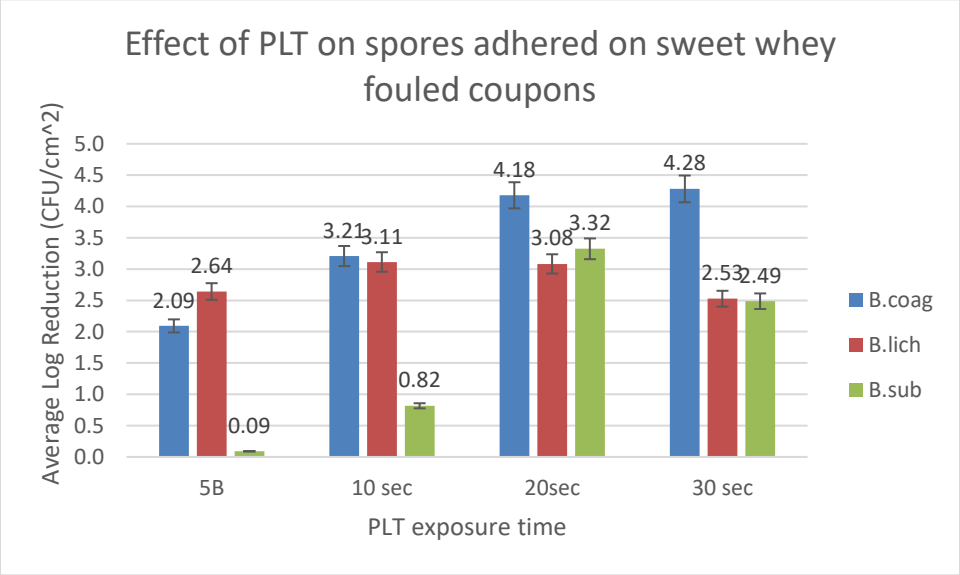

Figure12 .*B. coagulans, B. licheniformis, and B. subtilis* adhered onto sweet whey (SW) fouled stainless steel coupons and exposed to increasing time intervals of pulsed ultraviolet radiation in the xenon machine.

Coupons fouled with whey protein isolates (WPI) Figure 11, at 5B, *B. coagulans* showed a log reduction of 1.2 followed by a 2.68 log reduction at 10 seconds. At 20 seconds a log reduction of 2.31 was achieved and at 30 sec a log reduction of 1.78 was

observed. *B. licheniformis* showed a 1.96 log reduction but only a 0.89 log reduction at 10 seconds. An increased rate in reduction occurred at 20 seconds with a 1.79 log reduction followed by a 3.73 reduction at 30 sec. Log reductions at 5B and 10 seconds were 0.97 and 1.09 for *B. subtilis* and 1.75 logs for 20 seconds but had an increase in log reduction with 4.69 log reductions after 30 seconds of exposure. Figures 18-20 in the Appendix depict the response of each individual strain on the different media subjected to each level of treatment. Both *B. licheniformis* and *B. subtilis* yielded a higher average log reduction at 30 seconds of PLT with biofilm composed of WPI media. *B. coagulans* had a higher average log reduction at 30 seconds of PLT exposure as well but with biofilm composed of SW. Although there was no statistical difference between spore strain type, there was a significant difference on the reaction of PLT based on the media so a second experiment was conducted.

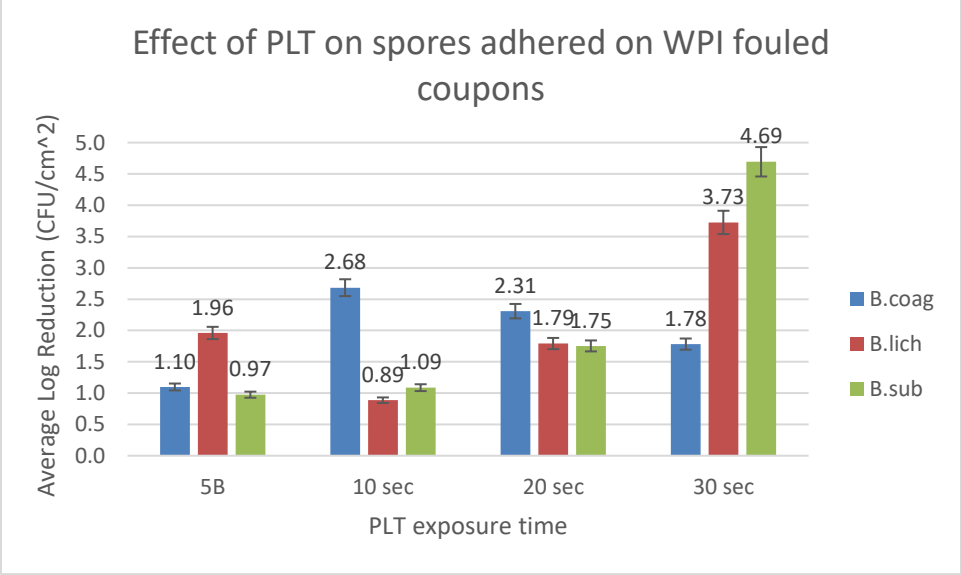

Figure13. ***B. coagulans, B. licheniformis, and B. subtilis*** **adhered onto whey protein isolate (WPI) fouled stainless steel coupons and exposed to increasing time intervals of pulsed ultraviolet radiation in the xenon machine.**

Experiment 2: Direct Microscopic Count to Compare Germination Rates of *B. licheniformis* and *B. coagulans*.

A simple direct microscopic count was conducted on *B. licheniformis* and *B. coagulans* incubated on each type of media at time 0hours and at 20hours to check the initial germination of each spore strain Figures12 and 13. This portion of the experiment was conducted with the hypothesis that if spores had a higher germination rate at 20 hours, then the spore would have a higher log reduction with as low as 5 bursts of pulsed UV. *B. coagulans* experienced an increase in vegetative cells on all medias after 20hours of incubation with the most dramatic increase with lactose and WPI. At 20 hours, B*. licheniformis* showed log DMC/mL values that followed the same trend as the average log reductions at 5B having the highest vegetative cell counts with sweet whey followed by WPI, water and lastly lactose (Figures 12 and 19). At 20 hours, *B. licheniformis* vegetative cell counts increased up to 3 logs with WPI from time 0 hours and increasing on all media except for lactose where vegetative cells counts were reduced below detection limits at 20hours.

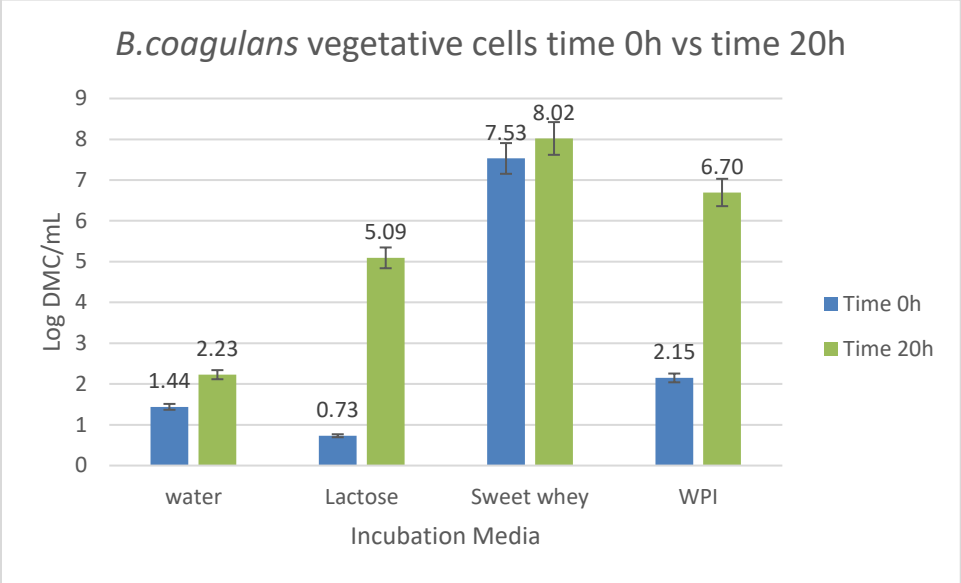

Figure14. Direct Microscopic Counts (DMC) of *B. coagulans* vegetative cells at time 0h and 20h.

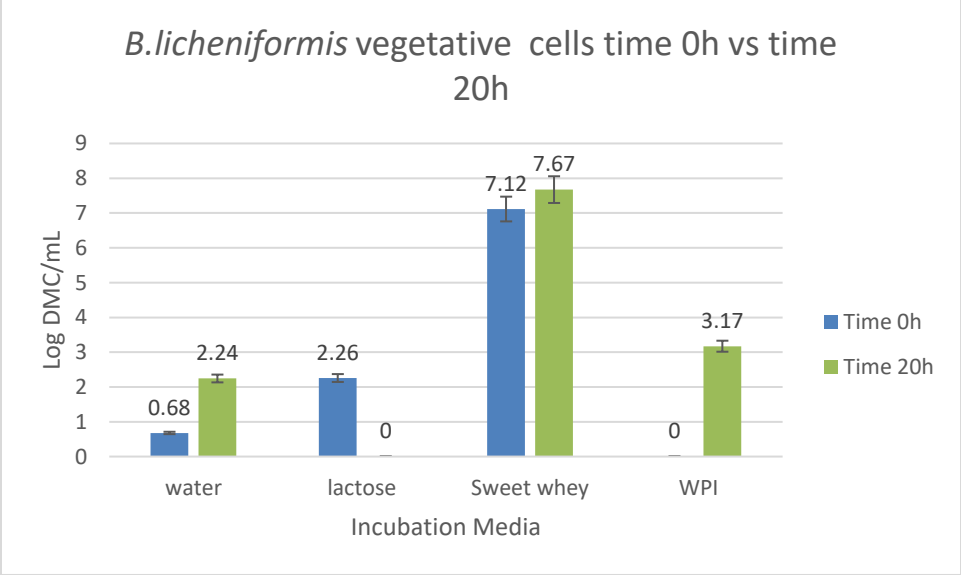

Figure15. Direct Microscopic Counts (DMC) of *B. licheniformis* vegetative cells at time 0h and 20h

Experiment 3: Pretreatment of Stainless Steel Surfaces for Spore Attachment Evaluation.

Sweet whey is higher in fat, since fat had an impact on the attachment to the coupon, a preliminary experiment was done were stainless steel coupon surfaces were pretreated and spore attachment was evaluated. A surface pretreatment of either no pretreatment (control) , canola no pulsed light (C no PL) (Figure16) , three minutes of Xenon UV PL (PL no C)and canola with 3 minutes of UV PL (PL&C) (Figure 17), were applied to stainless steel coupons before being subjected to a 20h incubation of a 1% spore solution at 55°C 200rpm. Canola oil was chosen for this experiment since dairy cow diets sometimes contain canola to increase milk fat content. The triacylglycerols (TAGS) and esterified long chain fatty acids found in this diet based milk are part of the surface fouling, conditioning media regularly found in dairy processing equipment. A statistical analysis was not conducted for this portion of the experiment, however, there was not a graphical difference between spore attachment on pretreatment groups NP, PL no C and PL&CA. A series of coupons were sprayed with canola oil then subjected to 3 minutes of continuous pulsed light radiation. This set of coupons was labeled as pulsed light and canola (PL&C). After the exposure the coupons were allowed to cool before they were incubated with 1% (v/v) spore solution for 20 hours at 55°C and 200rpm. NP, PL&C and PL no C showed very similar attachment with C no PL having the lowest attachment Figure19.

Figure16. Stainless steel coupon pretreated with canola oil.

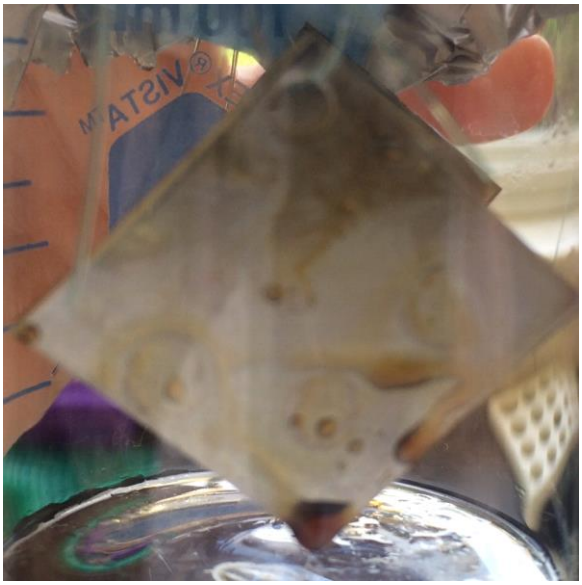

Figure17. Stainless steel coupon pretreated with canola and 3 minutes of continuous pulsed light radiation from the Xenon machine.

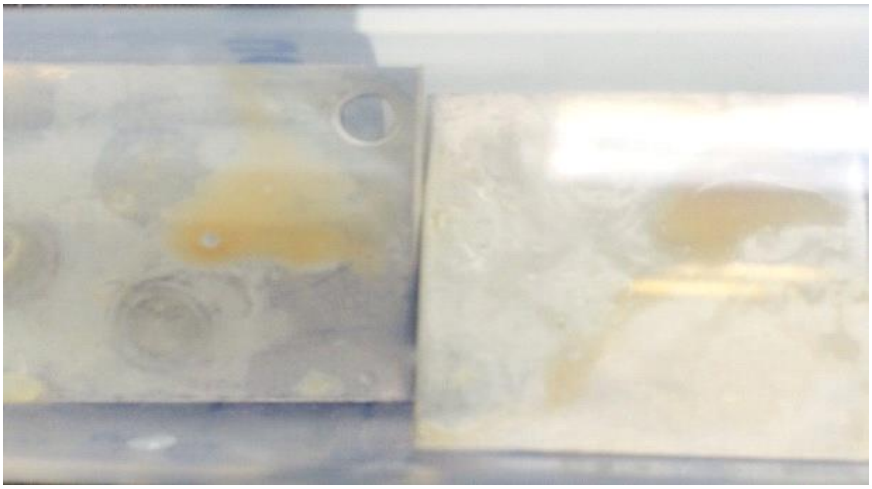

Figure18. Stainless steel coupon pretreated with canola and 3 minutes of continuous pulsed light radiation after 20 hour of incubation with 1% B. licheniformis spore solution.

.

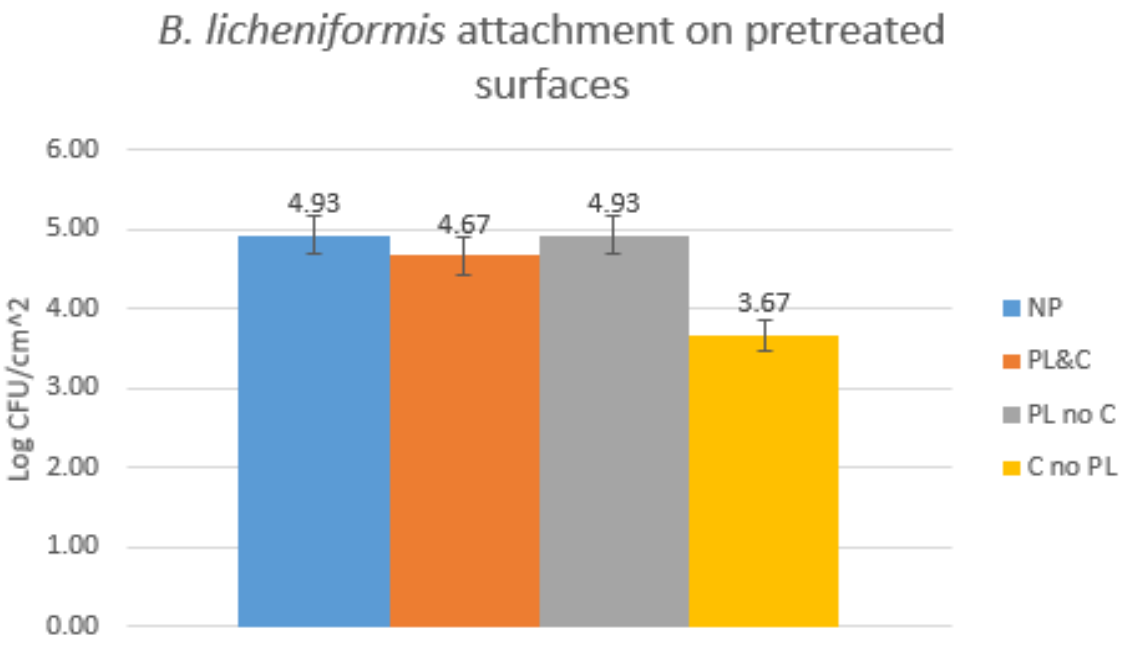

Figure19. Spore attachment on pretreated stainless steel surfaces

CHAPTER 5: STATISTICS

The statistical model for experiment 1 was estimated using the REML (restricted maximum likelihood) method with a mixed-model ANOVA in JMP version 11.1.The statistical model for this analysis has 7 things that must be tested (in the following order):the Media*Strain*PLT interaction ;the Media*Strain, Media*PLT, and Strain*PLT interactions; and the Media, Strain, and PLT main effects. Each test was done using an ANOVA F-test. Using the Bonferroni adjustment, all 7 tests were done with a 1% individual significance level. For Experiment 2, the same type of analysis was conducted at the 5% significance level. There was no statistical analysis conducted for experiment 3.

CHAPTER 6: DISCUSSION

Each bacillus strain behaved differently towards the PLT exposure depending on the fouling media but this difference did not deem to be statistically significant. Even Though *B. coagulans* experienced no less than an average of a one log reduction on all treatments with all fouling media, the highest average log reduction happened when sweet whey fouled coupons were subjected to a PLT treatment of 20 or 30 seconds and followed the expected trend of increasing average log reduction with increasing PLT time.

Deionized water is a transparent substance that is stripped of ions and minerals found in regular water found on tap. In Figure 7, Stainless steel coupons that were treated with water and *B. licheniformis* spores had a steady increase as xenon PLT exposure time increased. Even though there were log reductions with *B. coagulans* and *B. subtilis*, the trend seemed to fluctuate slightly with *B. coagulans* and only slightly changed until 30 seconds of exposure with *B. coagulans*. An interesting observation was that water treated coupons did not yield the highest log reductions compared to coupons fouled with thicker more opaque dairy solutions. In a study conducted by Artiguez et al., 2011, the impact of liquid thickness and flow rate on L. innocua inactivation in a flow- through unit was observed with the conclusion that inactivation was greater in thinner media (Artíguez et al., 2011). This difference suggests that spore inactivation is more complex than the transparency or thickness of contaminated liquids. Also there is a possibility of shadowing effects occurring due to aggregated spores on the coupon surface as well as some spores embedding in microscopic grooves or other imperfections of the stainless steel.

B. coagulans was the only spore strain that followed the expected trend of increasing average log reduction as PLT time increased both *B. licheniformis* and *B. subtilis* had a bell curve trend Figure 10, with *B. subtilis* having the more dramatic shape. Since the sweet whey that was used was not filtered, It is possible that lower level treatments of 5B and 10 seconds have eliminated the already germinated spores or lactic acid bacteria and caused other bacteria present to sporulate with longer treatment levels. Since 30 seconds of xenon pulsed light creates a significant amount of heat, it is possible that sweet whey creates a crust that protects embedded *B. licheniformis* and *B. subtilis* spores making it harder for UV to reach it. Also this encrusting of the media makes it difficult to obtain all of the adhered spores for enumeration which could also play a part in the results obtained.

As noted by Gould 1969, Even though spores are dormant in unfavorable environments, if the spore detects the presence of germinants, it will return to a vegetative state deeming it vulnerable to such things as UV radiation. Nutrient germinants include amino acids, sugars and purine molecules, each very specie and strain specific (Gould 1969; Setlow 2013). As noted in the figures above, the PLT treatment of 20 and 30 seconds varied significantly within spore strain depending on the fouling media confirming Gould's discovery that each media may contain a nutrient germinant favored by one specie over the other that trigger germination. It is important to note that actual metabolism of these nutrient germinants does not occur since a dormant spore has no metabolic activity rather they bind to their indicated proteins termed germinant receptors (GR) (Setlow 2013).

These results were expected since in the previous experiment at 5B, the log reduction was the lowest with lactose suggesting that spores were still dormant and that lactose may have caused vegetative cells found at time 0h to sporulate rather than to germinate. Surprisingly, there was an increase in vegetative cells in water treated *B .licheniformis* spores. It is unexpected since deionized water is striped of most of its ions and minerals that could cause spores to germinate. Nonetheless the possibility of the very small amounts of ions and minerals left behind with the combination of the incubation temperature of 55°C if not the temperature alone could cause some germination.

A similar phenomenon occurred with *B. coagulans* having an increase in vegetative cell counts on all media. *B. coagulans* had the expected average log reductions with sweet whey and lactose but had unexpected results with WPI. Even though spores incubated in WPI had a high Log DMC, the average log reduction at 5B was as much as water treated spores but seems to increase log reduction at 10 seconds and made a big jump at 30 seconds. Results with WPI fouled coupons showed a large leap in log reduction at 30 seconds of PLT exposure. It has been proven in previous studies that WPI has the ability to encapsulate bacteria shielding it from external factors. Mainly, this process is meant for probiotics or culture bacteria but could also have the same effect with spore forming bacteria (Khem et al., 2016). It has been noted that above 65°C whey proteins begin to unfold and its hydrophobic structure is exposed but this phenomenon has been documented to also happen at 55°C during the spray drying process and with certain moisture content (Haque et al., 2013) . A recent study done with the gram positive bacterium *L. plantarium* commonly used for

fermentation, concluded that when WPI was heated, it was unfolded and a hydrophobic core, mainly β-lactoglobulin the component making up 50% of the protein fraction, made an interaction with the hydrophobic bacterium surface encapsulating it for added protection (Khem et al., 2016). Since spores are also gram positive bacteria and gram positive bacteria have an overall hydrophobic cell surface they could have been encapsulated by WPI enabling the UV in the Xenon machine to reach the spore at shorter exposure time. This could explain why log reductions demonstrated in Figure 11, were low at shorter PLT exposures. But with the combination of 30 seconds of PL and the heat generated on the stainless steel coupon from the treatment, the average log reductions of all three spore strains made a significant leap.

Different fouling media could also create varying surface charge on the coupons affecting initial spore attachment. Since it was mentioned above that WPI has the possibility of unfolding and exposing a hydrophobic center, it is possible that this conditioning media is creating an attraction for spore adherence onto the stainless steel surface (Khem et al., 2016). Surface tension is also believed to have a great impact on the adhesion of microorganisms. The attachment of microorganisms onto the surface before the application of UV is a very important consideration since surfaces with an even layer of conditioning media will harbor more cells and will have a higher chance to develop sessile communities (Bernardez et al., 2012).Surface tension is defined as the force acting on the surface of a liquid, tending to minimize the surface area (Atkins, 1994). Meaning that a liquid with high surface tension has more capillary action and slides off of some surfaces while low surface tension liquids will spread and stick to a surface increasing microbial adhesion as well as increasing the difficulty of cleaning.

The surface tension of whole milk is approximately 44 mN/m while the surface tension of water is 72 mN/M (Chandan, 1997). Lactose and other salts are believed to increase surface tension. This theory was proven to be true in this experiment since the initial bacterial attachment at 20h was greater with coupons conditioned with whey protein isolate than with lactose or water treated coupons (data not shown). An interesting phenomenon occurred with sweet whey conditioned coupons as they had as much initial attachment as lactose treated coupons. Sweet whey is higher in fat, since fat had an impact on the attachment to the coupon, an experiment was done were stainless steel coupon surfaces were pretreated and spore attachment was evaluated.

A surface pretreatment of either no pretreatment (control), canola no pulsed light (C no PL), three minutes of Xenon UV PL (PL no C) and canola with three minutes of applied to stainless steel coupons before being subjected to a 20h incubation of a 1% spore solution at 55°C UV PL (PL &C) were applied to stainless steel coupons before being subjected to a 20 hour incubation of a 1% spore solution at 55°C 200rpm. Canola oil was chosen for this experiment since dairy cow diets sometimes contain canola to increase milk fat content. The triacyglycerols (TAGS) and esterified long chain fatty acids found in this diet based milk area part of the surface fouling, conditioning media regularly found in dairy processing equipment. Graphical results demonstrated an unexpected result since canola subjected to 3 minutes of Pulsed light ultraviolet radiation becomes oxidized and more than likely changes the surface charge of the coupon providing more or less attachment than the control group. Although oxidized canola made no difference in attachment from the control group, canola alone without being subjected to pulsed UV had 1.26 Log $\frac{CFU}{cm^2}$ less attachment than coupons with

oxidized canola. Milk contains X amount of cholesterol……canola oil contains phytosterols that are very similar in molecular structure; major sterols are listed below in table 1. (Canola Council of Canada). Pryzybylsi and Esking, 1991, found that the storage of foods fried with oil containing phytosterols had oxidation products that mimicked the oxidative products of cholesterol suggesting that canola oil sterols could, in fact, undergo the same oxidative reactions as cholesterol. One such oxidative reaction is the One- electron reduction where reductants and lipoprotein cholesterol can reduce to oxyl radicals and trigger peroxidative damage(Korytowski et al., 1999). In a study done by Girotti 2001, leukemia cells were exposed to increasing light fluency and accumulated O_2- derived sterol epoxides deeming a lethal effect on the cells. Although this may be true for the Leukemia cell, there is not an existing study to suggest that these findings are true for spore forming bacteria. Also, the data provided in this study does not support Girotti's study and demonstrates a higher cell attachment in coupons pretreated with canola oil and Pulsed light than with coupons pretreated with canola oil alone . This phenomena can be explained by the order of the treatment as these lethal reactions could have already subsided by the time the spore solution was introduced into the experiment.

Major Sterols Found In Canola Oil	
Sterol	**%**
Brassicasterol	13.8
Capesterol	27.6
β-sitoserol	52.3
Total sterols	6900.0 mg/kg
Total esterified sterols	423.5 mg.kg

Table1. List of Major Sterols Found in Canola Oil

Another observation that was noted was that after 20 hours of incubation the oil from coupons, pretreated with canola and 3 minutes of Xenon PL, remained on the stainless steel surface instead of dispersing in the water turning opaque (Figure18) . Processed canola oil has pigments that include chlorophyll, xanthophyll and carotenes. These pigments have been suggested by Przybylski, 1993 to inhibit catalysts used for hydrogenation when oxidation is promoted in the presence of light. Hydrogenation could produce soft but solid appearing fats (Stayer et al., 2006). The Hydrogenation of canola oil can then increase saponification values making the sanitation of processing equipment much more rigorous (Przybylski, 1993).

Saponification is the process of converting fat into soap by treating it with an alkali, a generally used process in the food industry as part of the equipment sanitation regime. Saponification value is the weight of alkali needed to saponify 1 gm of fat. The Saponification value of canola oil is 168-181mg but once hydrogenated, this value increased to 188-192 (Przybylski, 1993).

CHAPTER 7: LIMITATIONS

Even though the use of Xenon pulsed light showed to have a great impact on spore survival, we still cannot say that we can use this technique over existing sanitation methods because no other sanitation technique was tested for comparison purposes. It would be recommended that the xenon machine be applied as part of a sanitation regime instead of a standalone process. Since only a mild three step rinse process of the conditioned coupons was performed before being subjected to the Xenon, there was an incrustation of the media adhered on the coupon and it was difficult to remove all of it during the vortexing process. This observation suggests that during an actual processing environment, if there is a biofilm on the surface, treatment with UV may make it more difficult to clean. It would therefore be recommended to apply hurdle technology and include manual or mechanical scrubbing of the equipment before applying Pulsed light to avoid leftover residue that will act as conditioning media for more microorganisms. Safety should always be considered when applying new equipment in the process. Personnel working with the equipment should be properly trained as well as use the appropriate personal protective equipment (PPE) recommended by OSHA. Applying pulsed light to the actual product has been shown to create off flavors. So it is recommended on limiting this technology to processing surfaces. Even though pulsed light was applied only to stainless steel surfaces in this experiment, there is a possibility that sloughing of treated biofilm may end up in the finished product which may raise concern of consuming since this is a process that involves some sort of radiation.

CHAPTER 8: INDUSTRY APPLICATIONS

Xenon pulsed light Technology has not been incorporated into the dairy industry due to past research suggesting a negative impact in its use directly onto the product but has been used in other sterilization processes. However, if it were to be incorporated in dairy products industry for sanitation applications on processing surfaces, it would be best utilized in enclosed systems such as vats, milk holding tanks, milk trucks and Dewhey belts that are made of stainless steel material. Also, it is recommended that this technology be used as the last step in a sanitation regime to ensure that food residue is not heated onto the surface making equipment more difficult to clean. In order be incorporated into food production, there still needs to be some research to conduct.

CHAPTER 9: FUTURE RESEARCH

There is still much research that needs to be conducted before this technology is used in dairy products processing facilities. According to the FDA (2015), there are still many topics that should be further researched before this technology is implemented into any sanitation regime. It would be in the industries best interest to identify the critical process factors and their effect on the Xenons' microbial inactivation.Since not everything in the dairy industry is liquid and clear, more validation of the suitability of the pulsed light technology for solid foods and non-clear liquids where penetration depth is critical is necessary. Since Xenon pulsed light uses white light, it is important to consider the potential formation of unpalatable and toxic by-products and how the fat content of dairy changes with or absorbs white light. Microorganisms have the tendency to adapt to harsh environments over time so there may be potential for common pathogens or surrogate organisms to develop resistance towards pulsed light treatments. Since there was some evidence that pretreated coupons with regular canola had a lower Log $\frac{CFU}{cm^2}$ attachment than coupons exposed to Xenon PL, more studies should be done to determine if canola itself can cause spore germination and aid in the effect of xenon PL on the Log reduction. Also the effect of photooxidation that canola undergoes under light should be studied on its effect of these spores.

CHAPTER 10. CONCLUSION

In conclusion, there are many factors that affect the creation of biofilm on stainless steel surfaces in dairy processing. Such factors include the flow of product stream, the varying temperatures throughout the process, the effectiveness of a CIP system, and even the sanitary design of equipment. Even though Xenon pulsed light technology did yield some promising results; there are many factors to take into consideration. In an industry such as the dairy industry, where milk normally travels quite a distance from the milk holding tanks all the way to the cheese vats and beyond, there are many places in which a biofilm could be developed. It has been suggested by the results of previous studies that the composition of the feed stream contributes to spore attachment to processing stainless steel surfaces. This study, however, suggests that the components of this feed stream may also contribute to the effectiveness of Xenon white light (PLT) on spores. The components of the feed stream may also contribute to the protection or germination of spores. Depending on components reaction to radiation and incubation temperature, the components may actually encapsulate the spore adding yet another layer of protection or provide the spore with the adequate germinants to produce germination. Also there is the possibility that with the extra heat produced by the application of PLT may make it more difficult to clean equipment to it is suggested to use this technology at the end of a sanitation regime for best results. According to this study, the type of media and the time of exposure of the Xenon PLT on stainless steel surfaces is statistically

significant. Although there were no significant interactions, graphically, it was observed that some spore strains, even though not statistically significant, showed a difference on each PLT treatment depending on the media. On some media the spores reacted as expected and showed a higher log reduction on prolonged exposure but with other media some spore strains showed a sudden drop in log reduction on a more extended exposure time.

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APPENDIX

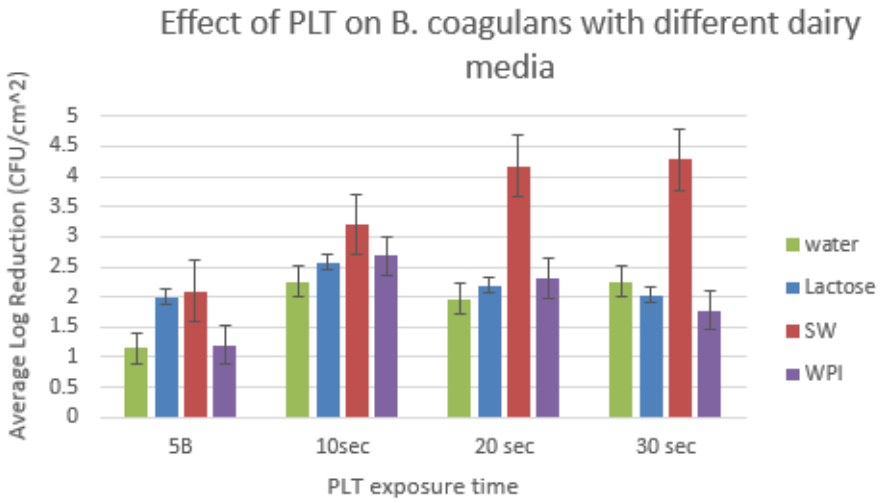


Figure 20. The Effect of Pulsed Light Technology on *B. coagulans* with different dairy media was examined with the determination that the largest Average Log reduction and the expected trend occurred with SW at 30 seconds. Results with other media demonstrated more of a bell curve with this spore strain.

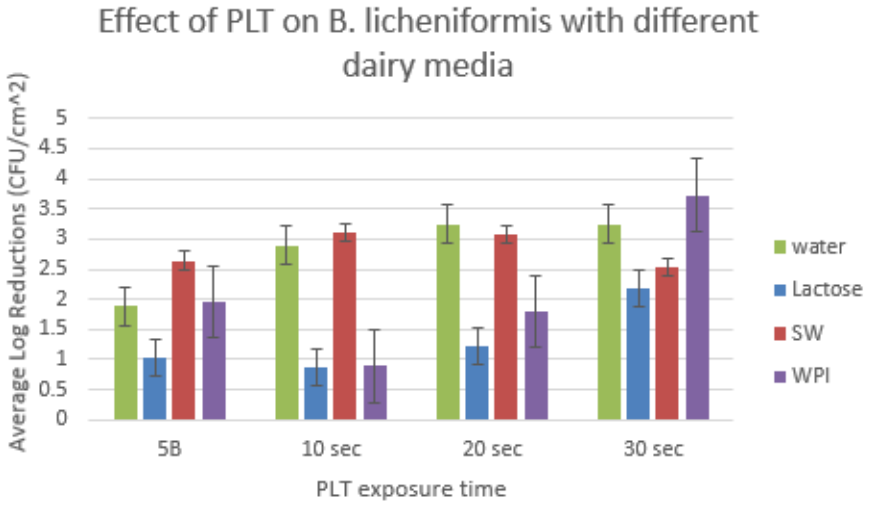


Figure 21. The Effect of Pulsed Light Technology on *B. licheniformis* with different dairy media was examined with the determination that the largest average Log reduction occurred with WPI at 30 seconds. Trends were as expected, an increase of average log reductions with an increase in treatment exposure were observed on all medias.

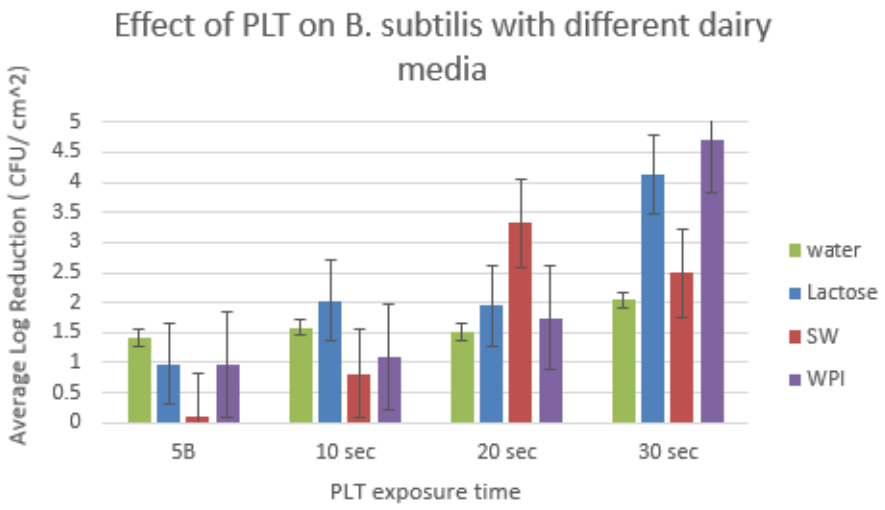


Figure 22. The Effect of Pulsed Light Technology on *B. subtilis* with different dairy media was examined with the determination that the largest average Log reduction occurred with WPI at 30 seconds followed by Lactose. All media demonstrated an expected trend of increased average log reduction except for coupons fouled with SW where a bell curve was more prominent with this spore strain.

Bacillus strain	Fouling Media	PLT treatment	Average Log reduction
B.licheniformis	Water	5B	1.15
B. coagulans	Water	5B	1.89
B.subtilis	Water	5B	1.6
B.licheniformis	Lactose	5B	1.03
B. coagulans	Lactose	5B	1.89
B.subtilis	Lactose	5B	0.98
B.licheniformis	SW	5B	2.09
B. coagulans	SW	5B	2.64
B.subtilis	SW	5B	0.09
B.licheniformis	WPI	5B	1.1
B. coagulans	WPI	5B	1.96
B.subtilis	WPI	5B	0.97

Table 2. Average Log Reduction of *B. licheniformis, B. coagulans,* and *B. subtilis* in different fouling media (water, lactose, SW or WPI) at 5B treatment.

Bacillus strain	Fouling Media	PLT treatment	Average Log reduction
B.licheniformis	water	10 sec	2.89
B. coagulans	water	10 sec	2.25
B.subtilis	water	10 sec	1.59
B.licheniformis	Lactose	10 sec	0.88
B. coagulans	Lactose	10 sec	2.58
B.subtilis	Lactose	10 sec	2.03
B.licheniformis	SW	10 sec	3.11
B. coagulans	SW	10 sec	3.21
B.subtilis	SW	10 sec	0.82
B.licheniformis	WPI	10 sec	0.89
B. coagulans	WPI	10 sec	2.68
B.subtilis	WPI	10 sec	1.09

Table 3. Average Log Reduction of *B. licheniformis, B. coagulans,* and *B. subtilis* in different fouling media (water, lactose, SW or WPI) at 10 sec treatment.

Bacillus strain	Fouling Media	PLT treatment	Average Log reduction
B.licheniformis	water	20 sec	3.25
B. coagulans	water	20 sec	1.97
B.subtilis	water	20 sec	1.5
B.licheniformis	Lactose	20 sec	1.22
B. coagulans	Lactose	20 sec	2.19
B.subtilis	Lactose	20 sec	1.95
B.licheniformis	SW	20 sec	3.08
B. coagulans	SW	20 sec	4.18
B.subtilis	SW	20 sec	3.32
B.licheniformis	WPI	20 sec	1.79
B. coagulans	WPI	20 sec	2.31
B.subtilis	WPI	20 sec	1.75

Table 4. Average Log Reduction of *B. licheniformis, B. coagulans,* and *B. subtilis* in different fouling media (water, lactose, SW or WPI) at 20 sec treatment.

Bacillus strain	Fouling Media	PLT treatment	Average Log reduction
B.licheniformis	water	30 sec	3.25
B. coagulans	water	30 sec	2.25
B.subtilis	water	30 sec	2.04
B.licheniformis	Lactose	30 sec	2.18
B. coagulans	Lactose	30 sec	2.03
B.subtilis	Lactose	30 sec	4.13
B.licheniformis	SW	30 sec	2.53
B. coagulans	SW	30 sec	4.28
B.subtilis	SW	30 sec	2.49
B.licheniformis	WPI	30 sec	3.73
B. coagulans	WPI	30 sec	1.78
B.subtilis	WPI	30 sec	4.69

Table 5. Average Log Reduction of *B. licheniformis, B. coagulans,* and *B. subtilis* in different fouling media (water, lactose, SW or WPI) at 30 sec treatment.

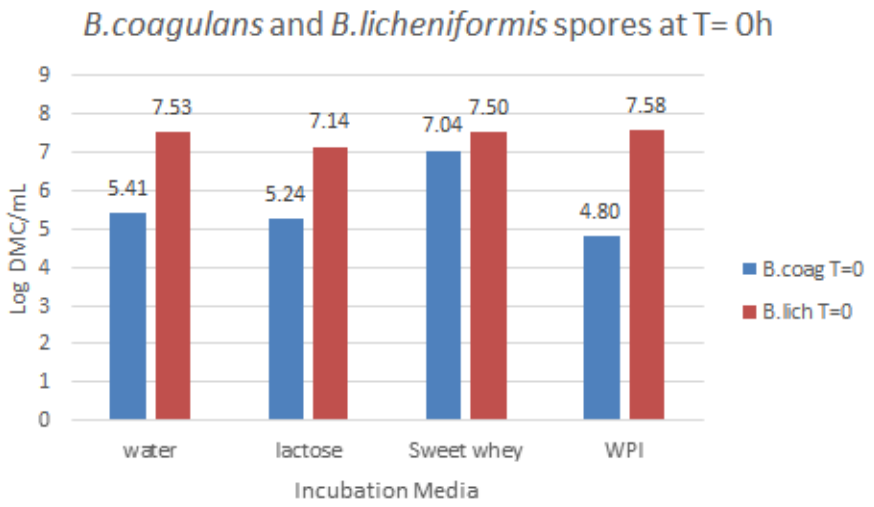


Figure23. *B. coagulans* and *B. licheniformis* spores were plotted at Time 0 (T=0) with *B. licheniformis* having a higher Log DMC/mL across all media types

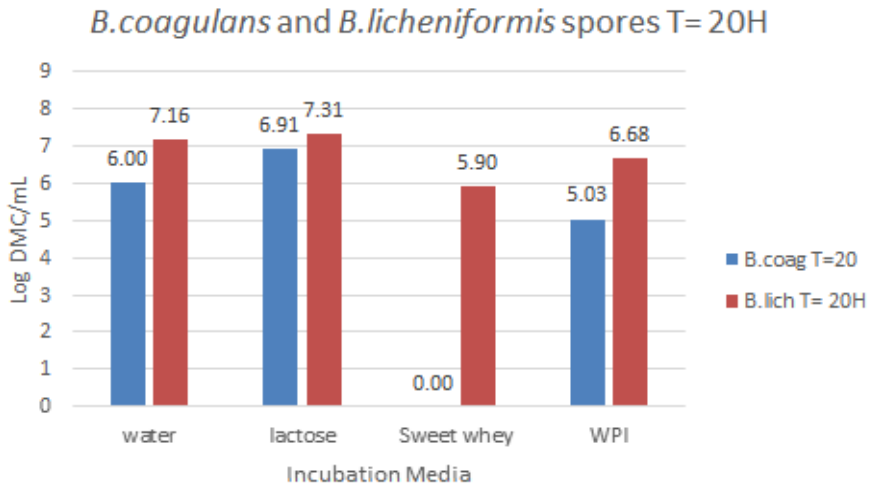
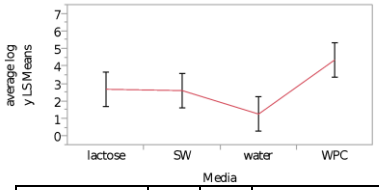


Figure24. *B. coagulans* and *B. licheniformis* spores were plotted at Time 20 (T=20) with *B. licheniformis* having a higher Log DMC/mL across all media types.

Source	NumDF	DenDF	F Ratio	Prob > F
Media	**3**	**6**	**37.9645**	**0.0003**
Strain	2	16	5.7383	0.0132
Media*Strain	6	16	1.4913	0.2433
PLT	**4**	**96**	**31.2910**	**<.0001**
Media*PLT	12	96	1.0953	0.3730
Strain*PLT	8	96	2.5816	0.0135
Media*Strain*PLT	24	96	1.7190	0.0342

Table5. One- way analysis of Variance Table.
*The terms in bold are significant at the 1% significance level (7% overall significance level) and will be analyzed further. Terms with p-values between 0.01 and 0.05 do not have strong evidence of differences in the average response in this experiment. However, future experiments could show that they are important.

Media type



Media			Mean Response
WPC	A		4.370
lactose		B	2.697
SW		B	2.624
water		B	1.293

Table6. The mean response for the different levels of the media factor. The table and plot above shows the mean response for the different levels of the media factor. Levels that share the same letter are not significantly different at the 1% individual significance level (7% overall significance level).

Level1	- Level2	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
WPC	**Water**	**3.076741**	**0.2892663**	**1.63814**	**4.515339**	**0.0002**
WPC	**SW**	**1.745630**	**0.2892663**	**0.30703**	**3.184228**	**0.0038**
WPC	**lactose**	**1.672962**	**0.2892663**	**0.23436**	**3.111561**	**0.0047**
lactose	Water	1.403778	0.2892663	-0.03482	2.842377	0.0113
SW	Water	1.331111	0.2892663	-0.10749	2.769709	0.0145
lactose	SW	0.072667	0.2892663	-1.36593	1.511266	0.9938

Table 7. The mean response for the different levels of the Media Factor
*Each row shows the difference between two levels (Level1 minus Level2). P-values below 0.01 in the table indicate significant differences. (Similarly, these differences will have lower and upper confidence limits than do not contain zero.) Significant differences are bold.

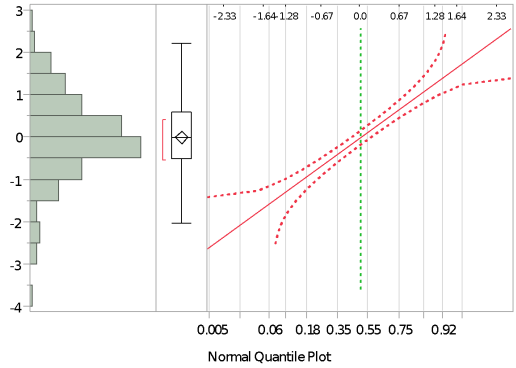
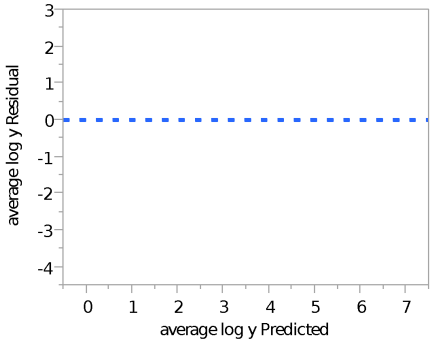
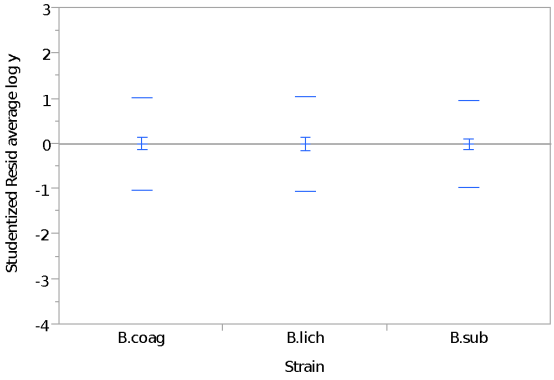


Figure 25. Normal Quantile plot.
Residual plots show that there are no serious violations of model assumptions. The residuals are reasonably Normal and have similar variability across levels of the different factors.This histogram and normal probability plot of residuals show approximate Normality.

This plot of residuals vs. predicted values shows a moderate fan-shape, but no serious violation of equal variance. (The diagonal line of points at the bottom left is due to how the data were measured.)



The plots below show residuals vs. each treatment factor (



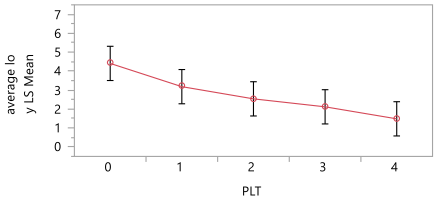
Means and Std Deviations

Level	Number	Std Dev
B.coag	60	1.01681
B.lich	60	1.05245
B.sub	60	0.95357

Table 8. Standard deviation of residuals for each level of the three factors (media, strain, and pulse).

*****The tables show the standard deviation of residuals for each level of the three factors. None of the factor levels have a residual standard deviation that differs substantially.

Pulse Type



Pulse					Mean Response
0	A				4.409
1		B			3.182
2		B	C		2.538
3			C	D	2.118
4				D	1.484

Table 9. Mean response for the different levels of the pulse factor.

*Levels that share the same letter are not significantly different at the 1% individual significance level (7% overall significance level)

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
0	**4**	**2.925186**	**0.2822731**	**1.97999**	**3.870384**	**<.0001**
0	**3**	**2.291297**	**0.2822731**	**1.34610**	**3.236496**	**<.0001**
0	**2**	**1.870741**	**0.2822731**	**0.92554**	**2.815940**	**<.0001**
1	**4**	**1.697869**	**0.2822731**	**0.75267**	**2.643068**	**<.0001**
0	**1**	**1.227316**	**0.2822731**	**0.28212**	**2.172515**	**0.0003**
1	**3**	**1.063981**	**0.2822731**	**0.11878**	**2.009180**	**0.0026**
2	**4**	**1.054445**	**0.2822731**	**0.10925**	**1.999644**	**0.0029**
1	2	0.643425	0.2822731	-0.30177	1.588624	0.1606
3	4	0.633888	0.2822731	-0.31131	1.579087	0.1721
2	3	0.420556	0.2822731	-0.52464	1.365755	0.5715

Table 10. Difference between two levels (Level 1 minus Level 2).

*P-values below 0.01 in the table indicate significant differences. (Similarly, these differences will have lower and upper confidence limits than do not contain zero.) Significant differences are bold.

Diagnostics

Residual plots show that there are no serious violations of model assumptions. The residuals are reasonably Normal and have similar variability across levels of the different factors.

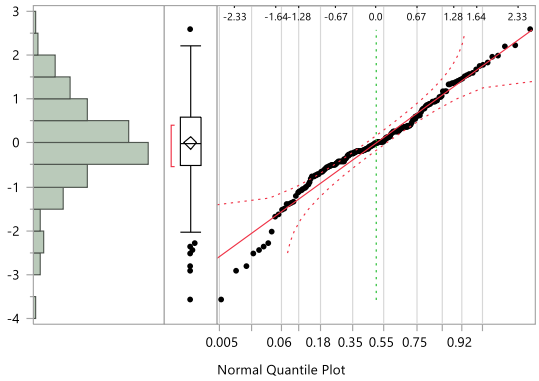


Figure 26. Hstogram and normal probability plot of residuals.

These show approximate normality.

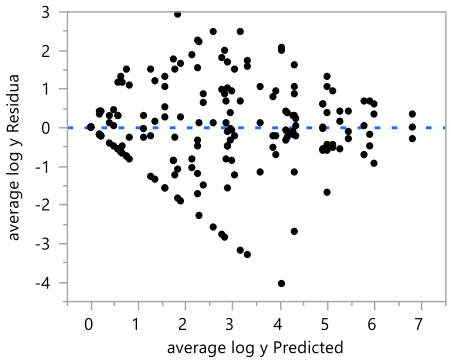


Figure 27. Plot of residuals vs. predicted values.

This plot shows a moderate fan-shape, but no serious violation of equal variance. (The diagonal line of points at the bottom left is due to how the data were