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Influence of Sporulation and Germination Behavior of Bacillus Licheniformis on Microbial Quality of Skim Milk Powder

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INFLUENCE OF SPORULATION AND GERMINATION BEHAVIOR OF *BACILLUS LICHENIFORMIS* ON MICROBIAL QUALITY OF SKIM MILK POWDER

By

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

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Nancy Awasti

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
a.u.	Arbitrary Unit
BHI	Brain Heart Infusion
CFU	Colony Forming Unit
CIP	Cleaning in Place
FDA	Food & Drug Administration
HRS	Highly Heat Resistant
HTST	High Temperature Short Time
Log	log_{10}
mL	mili Liter
nm	nanometer
nM	nanoMolar
PBS	Phosphate Buffer solution
PMO	Pasteurized Milk Ordinance
RAPD	Random Amplification Polymorphic DNA
RSM	Response Surface Methodology
SAS	Statistical Analysis Software
UHT	Ultra High Temperature

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ABSTRACT

INFLUENCE OF SPORULATION AND GERMINATION BEHAVIOR OF *BACILLUS LICHENIFORMIS* ON MICROBIAL QUALITY OF SKIM MILK POWDER

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This dissertation includes modeling of sporulation and germination behavior of *Bacillus licheniformis* strains during raw milk holding, tracking the survival of sporeformers and spores of high sporulating *Bacillus* strain during skim milk powder manufacturing. In addition, preliminary studies were done to standardize a rapid spore detection technique by using ratiometric fluorescence. To understand the role of strain variability during raw milk holding conditions, population dynamics of two strains of *Bacillus licheniformis,* ATCC 6634 and 14580, were modeled as a function of temperature (4.0 -12.0˚ C) and duration (0 - 72 h), using regression analysis. Based on initial spiking of vegetative cells (approx. 4.0 log cfu/mL) and spores (approx. 2.0 log cfu/mL), regression equations elucidating *B. licheniformis* growth behavior during raw milk holding at low temperatures were obtained. Contour plots were developed to determine the time-temperature combinations, keeping the population changes to less than 1.0 log. Results suggested that for vegetative cell spiking study of *B. licheniformis* ATCC 6634 (S1), cell population changes remained below 1.0 log up to 72 h at 8˚ C. For *B. licheniformis* ATCC 14580 (S2), 1.0 log shift was observed only after 80 h at 8˚ C, indicating greater multiplication potential of S1 as compared to S2. As S2 was a readily sporulating strain, the vegetative spiking study showed spore formation at different

storage temperatures. In the presence of equivalent numbers of both types of sporulating strains in raw milk, despite strain variability, holding the milk at 8˚ C for not more than 72 h would keep any cell population changes below 1.0 log. In addition, under these storage conditions, the population would remain as vegetative cells that could easily be inactivated by pasteurization.

As BL ATCC 14580 was the high sporulating strain, therefore, further studies were conducted using this strain, wherein spiked milk samples were held prior to manufacturing of milk powder. A pilot-scale skim milk powder trial was conducted to evaluate the influence of holding conditions on final spore and sporeformer counts. After spiking, raw milk silos were maintained at two different holding temperatures i.e. at PMO based conditions (10˚ C for 4 h, followed by 7˚ C for 72 h, treatment 1) and at optimum holding condition based on contour plots (4˚ C for 24 h, treatment 2). Powders manufactured under these conditions were assessed for vegetative and spore population at different stages of processing. The overall final spore and vegetative cell counts in the powders manufactured under optimum holding condition (4˚ C for 24 h) were found to be significantly lower (0.58 ± 0.04 and 1.82 ± 0.05 log cfu/g) as compared to the conditions likely to practice by dairy plants (2.74 ± 0.03 and 1.03 ± 0.06 log cfu/g). This shows that milk powders with reduced vegetative and spore counts can be prepared by optimizing the raw milk holding conditions.

For standardization of a novel spore detection method, CaDPA content of spores was quantified using a ratiometric fluorescence technique. This method is based on the detection of CaDPA that enhances the luminescence of lanthanide ion when complexed with a semiconducting polymer. The intensity was recorded after chelating

semiconducting fluorescent polyfluorene (PFO) dots with terbium ions, sensitized by different volumes of CaDPA (0.1μM). The standard curve showed a linear relationship (R^2 = 0.98) in the experimental concentration range of 2.5 nM to 25 nM of CaDPA, with corresponding intensity (a.u.) of 545 to 2130 nm. In HPLC grade water, the minimum log spores detected were 1.36 ± 0.09 log cfu/mL with corresponding mean CaDPA content of <2 nM. On the other hand, for raw skim samples, the minimum log spores detected were 5.21 ± 0.07 cfu/mL with mean CaDPA content of approx. 2.0. For raw milk spiked samples, reduced fluorescence detection was observed and was approx. five times lower as compared to the spiked samples of HPLC grade water. The reduced fluorescence ability in raw milk can be due to the turbidity of the solution or interference of proteins, amino acids and other ions of milk. This study provides a proof of concept for a potential application of this technique to rapidly detect bacterial endospores in the dairy and food industry. Further studies are required to remove the inference of ionic components in milk to improve the efficiency of the protocol. Based on these studies we were able to establish the holding time-temperature values for raw milk holding that would result in the least change in the population of sporeformers and spores. Using these combinations, we were able to manufacture skim milk powder with lower counts of spores and sporeformers as compared to the PMO recommended holding conditions. The proof of concept generated through the spore detection protocol has the potential to be established as a rapid detection technique for spore counts in milk and related products.

INTRODUCTION

Bacillus species are major contaminants in food and dairy industry and have been responsible for milk and dairy products spoilage (Seale et al., 2015). Various quality and shelf stability issues caused by *Bacillus* species are sweet curdling, bitty cream, flat sour, off flavor, and ropiness (Berkeley, 2002, Heyndrickx and Scheldeman, 2008, Burgess et al., 2010). Previous studies stated the incidence of thermoduric psychrotrophs in raw milk and determined that a significant proportion of shelf life issues in milk and dairy products are linked to *Bacillus* species (Francis et al., 1998, Sharma and Anand, 2002). According to the Grade 'A' Pasteurized Milk Ordinance (PMO; FDA, 2017), after completion of the first milking cycle, raw milk is recommended to be cooled to 10˚ C (50˚ F) or less in four hours or less, and to 7˚ C (45˚ F) or less, within two hours after the completion of milking. After completion of each milking, many a time, due to economic and organizational reasons, raw milk is stored at low temperatures in the farm for long hours, and several times the supplied milk is not processed immediately. *B. licheniformi*s has frequently been encountered in milk powders, and pasteurized, as well as, UHT milk products (Barbano et al., 2006, Leitner et al., 2008, Buehner et al., 2015, Miller et al., 2015) it was also identified as the most predominant species in Midwest dairy farms (Buehner et al., 2014). These psychrotolerant spore-forming *Bacillus* species are regarded as a major challenge, as many of these organisms can withstand pasteurization and can germinate and outgrow in final product (Ivy et al., 2012, Masiello et al., 2014). A previous study (Kumari and Sarkar, 2014) reported the ability of *Bacillus* species to produce biofilms even at 4˚ C, which was isolated from chilling tank.

Skim milk powder manufacturing is a complex process which involves microbial as well as functional changes during all stages of processing. Among spoilage organisms, thermoduric *Bacillus* species are frequently present during all manufacturing steps. Spoilage is mostly caused by the heat-stable lipolytic and proteolytic enzymes or post-process contamination of the products during handling (Coorevits et al., 2008). These organisms, essentially in the form of spores, can survive pasteurization, evaporation as well as spray drying temperatures, and are an important source of milk powder contamination (Kalogridou-Vassiliadou, 1992, Scheldeman et al., 2006). *Bacillus* spores are capable of surviving pasteurization treatments, which can reduce 99.9% of the vegetative population but, provides a suitable environment for the subsequent germination and growth of spores which tend to form biofilms on the surfaces of processing equipment (Palmer et al., 2010). The endospores are formed as a defense mechanism to unfavorable environmental conditions which would hinder its growth/existence in the vegetative state and are resistant to most agents that would normally kill the vegetative cells they are formed from. Bacterial build-up during storage of raw milk may lead to the maintenance of ‘persistence cycles' of thermodurics that may lead to microbiological quality issues such as ‘bacterial or spore-spikes' in the final products such as milk powder (Hayes et al., 2001). Scott et al. (2007) reported evaporators and plate heat exchanger as the predominant sporulation sites during whole milk powder manufacturing. The problem of bacterial outgrowth could be addressed early on during milk processing by optimizing raw milk holding conditions. Numerous studies have evidenced the growth of vegetative cells at refrigeration temperatures during milk processing (Kent et al., 2016) but none of the studies explored the sporulation

behavior in raw milk held at refrigeration storage and during powder processing run at pilot scale. Regardless of all the above aspects, the influence of the length of storage, temperature and powder manufacturing conditions on germination, sporulation or multiplication behavior of *Bacillus* sporeformers, and their ability to form spores at farm and pilot scale is not understood clearly. Therefore, it is important to control spores and sporeformers at initial stages of milk powder processing and thus understanding the influence of time-temperature combinations that favor minimum multiplication or sporulation of sporeformers by using process optimization strategies, could help control bacterial load in raw milk during holding as well as during powder manufacturing.

Taking an example of *B. licheniformis*, we hypothesized that different strain may behave differently in terms of germination, multiplication, and sporulation in during raw milk holding before processing and powder processing run especially during evaporation. If the sporulation and germinations sites are known during the overall milk processing into powder, then those specific conditions can be optimized to reduce sporeformers and spores in the final product. To understand the influence of processing conditions, current dissertation conducted studies that studied the influence of refrigeration holding and strain variability on raw milk holding at refrigeration temperatures, the effect of lab scale evaporator namely, rotatory evaporator and a pilot scale, Niro drier on population dynamics of common sporeformers and spores. These studies will help us understand the influence of individual treatment such as holding, evaporation and spray drying on population dynamics of *Bacillus* species. Understanding the influence of powder processing conditions that favors either sporulation or germination of spores can help understand the behavior of *Bacillus* species and help identify the steps responsible for the

outgrowth of microbes. Such information can be helpful to control spores and sporeformers by using process optimization strategies.

As the current spore detection methods rely on cultural techniques and have limitations of time, efficiency, and sensitivity, attempts were made to standardize a ratiometric fluorescence technique to rapidly detect bacterial endospores. Based on a previous study (Li et al., 2013), the current study investigated CaDPA concentration in *Bacillus* spores using ratiometric fluorescence technique, an important biomarker and major component of bacterial spores. Development of a rapid spore the detection kit can be helpful in detection of spores in milk and milk products within about 30 min.

The overall objectives of this study were to 1. understand the influence of refrigerated storage, evaporation and drying temperatures on sporulation and germination behavior of *B. licheniformis* strains during skim milk powder manufacturing, and 2. To standardize a rapid spore detection technique using ratiometric fluorescence spectrophotometry.

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CHAPTER 1

Literature Review

Bacillus species are major contaminants in food and dairy industry and have been responsible for milk and dairy products spoilage (Seale et al., 2015). Predominant spore-forming bacteria of concern to the dairy industries belong to the genus *Bacillus* and vegetative and spore forms of this species may contaminate foods including dairy products through different sources (De Jonghe et al., 2010). The vegetative cells range from 0.5 x 1.2 to 2.5 x 10 µm in diameter. Their optimal growth temperatures range from 25 to 37˚ C, although, thermophilic and psychrotrophic species are capable of growing at temperatures as high as 75˚ C or as low as 3˚ C, respectively. Overall, these bacilli are responsible for many organoleptic and physical defects of different foods including milk and dairy products, thereby, causing a great economic loss.

1.1 Prevalence of *Bacillus* species in raw milk and milk powder

Spoilage by *Bacillus* species has been reported not only in raw milk but also in pasteurized milk (Magnusson et al., 2006), in milk powders (Murphy et al., 1999), and even in the commercially sterilized and UHT treated milks (Scheldeman et al., 2005). Such spoilage is mostly caused by the heat stable lipolytic and proteolytic enzymes or post process contamination of the products during further handling (Coorevits et al., 2008). Raw milk is an appropriate medium for bacterial growth and hence the quality of milk is mostly dependent on the population of the microorganisms in it. Raw milk can be contaminated with the bacteria before, during, and after milking. Spore forming bacilli are frequently being described to be associated to the spoilage of raw and pasteurized milk or milk products. Presence of *Bacillus* species and their spores before pasteurization

play a significant role for such degradation (Slaghuis et al., 1997).The undesirable effects of *Bacillus* species are mainly described in terms of production of food spoilage enzymes that may lead to organoleptic and compositional changes in milk and milk products. Various quality and shelf stability issues caused by *Bacillus* species are bitty cream, sweet curdling, off flavor, flat sour, and ropiness (Berkeley, 2002, Heyndrickx and Scheldeman, 2008, Burgess et al., 2010).

After *Pseudomonas*, *Bacillus* is the second predominant species frequently isolated from refrigerated raw milk (Sørhaug and Stepaniak, 1997, Vithanage et al., 2016). The major bacilli that contaminate and spoil milk and dairy products are *B. cereus, B. subtilis, B. licheniformis, B. stearothermophilus, B. coagulans* (Robinson, 2002), *B. sporothermodurans* (Pettersson et al., 1996, De Jonghe et al., 2008), *Brevibacillus bortelensis, Paenibacillus* (Scheldeman et al., 2006, Huck et al., 2007), and *Anoxybacillus flavithermus* (Scott et al., 2007). Among these species, *B. licheniformi*s has frequently been encountered in dry milk powders, and pasteurized, as wells as, UHT milk products (Barbano et al., 2006, Leitner et al., 2008, Buehner et al., 2015, Miller et al., 2015) It was also identified as the most predominant species in Midwest dairy farms (Buehner et al., 2014). If adequate hygienic conditions are not practiced at farm level, then as high as 10^2 to 10^6 cfu/mL of spores may be encountered in raw milk (Te Giffel et al., 2002). These heat-stable *Bacillus* spores could survive milk processing steps, and at optimum conditions they germinate and cause spoilage (Becker et al., 1994, Rukure and Bester, 2001, Chen et al., 2004), due to the production of enzymes (proteases and lipases) and acid (Chen et al., 2004). McGuiggan et al. (2002) studied different factors that influence the population of the different spore-formers in raw milk and found the number

of mesophilic spore-formesr in raw milk ranging from $1.4x10^{1}$ to 2.4×10^{5} spores/mL in raw milk. They further described that such organisms were generally found to be positively associated with the mean environmental temperatures and somatic counts in raw milk.

Spore counts in raw milk are relatively lower and generally remain around 50 cfu per ml of milk. During milk powder processing, raw milk is concentrated by approximately 10 times, which itself makes the concentration of spores in milk powder to be approximately 500 cfu/gm even if no significant growth is considered during milk processing within the plant (McGuiggan et al., 2002, Rückert et al., 2004). The processing cycles and the clean in place (CIP) systems are designed to keep the number of bacterial counts within the specification. But several other factors such as bacterial quality of raw milk, plant hygiene, biofilms, and thermal operating conditions of the plant play an important role in increasing the number of bacteria in the final product (Rueckert et al., 2006).

1.2 Source of sporeformers

Bacilli are ever present in nature and can easily contaminate milk through different sources. They can grow well in a wide temperature range and hence could fall under any of the psychrotrophic, mesophilic or thermoduric group of an organism (Meer et al., 1991, Robinson, 2002). They produce enzymes that can degrade protein and fat present in milk. When the cells of bacilli are stressed due to any reason, they respond to stress by converting themselves into spores, which help them resist those stresses and remain viable for a longer period of time (Setlow et al., 2001, Henriques and Moran, 2007). Bacterial spores contaminate almost all sorts of dairy foods through several sources such

as milk from an unhealthy animal, biofilm, resistance and persistence of spore forming strains in processing lines, soil, transportation, personal hygiene, environmental sources, and others. Multiplication of cells following germination and outgrowth of spores can take place at wide ranges of intrinsic and extrinsic factors such as temperature, pH, and water activity (Carlin, 2011, Peter and Eric, 1997). The major sources of raw milk contamination on the farm are exterior of udder, surfaces of equipment coming in contact with milk, inadequate cleaning, and sanitizing procedures, and the miscellaneous environmental factors (Schmidt, 2009). Slaghuis et al. (1997) categorized such factors as pre-milking, during milking, and post-milking factors.

Among all sources, soil is one of the major habitat of spore-forming bacteria, and contaminated soil can serve as a source of contamination of milk via dirty teats (Novak et al., 2005, Hong et al., 2009, Carlin, 2011). Slaghuis et al. (1997) reported spore counts in soil up to 10^5 to 10^8 cfu/g. Fecal materials and manure can directly contaminate soil, water, and raw milk. Hong et al. (2009) carried out a study to compare the *Bacillus* spore counts in soil and feces, and their result revealed that soil contained 10 to 100 times more aerobic spore than the human fecal material, however, the average counts in feces were also significant (10^4 cfu/g). Feeds and bedding materials may also serve as sources for the spore contamination of milk (Magnusson et al., 2007, Slaghuis et al., 1997). However, feed concentrate, silage, fresh grass, and hay contain low spore population, which makes them less important factors for spore contamination (Novak et al., 2005). Magnusson et al. (2007) reported less than 50 *Bacillus* spores/g in fresh bedding material, whereas used bedding material had a considerably higher spore content. They further detected vast differences in the spore counts (900 to 62,000 spores/g) obtained from the samples of

bedding material in the surface layer, and there was a decrease in counts towards the bottom of the layer. Te Giffel et al. (2002) reported the level of spores in silage to be 10 to 10^5 per gram, and inferred silage to be a significant source of contamination of raw milk with spores. Hull et al. (1992) reported that *Bacillus* spores in raw milk normally remained at the level of $5x10^3$ spores per mL. The counts were reported to depend on housing of cattle contamination of teats, and water supply with soil. Spore counts per teat of cow ranged from 10^2-10^5.

There are many different sources on the farm environment that lead to the contamination of different types and forms of microorganisms in milk. Milking personnel, milking equipment and utensils, air, dust and aerosols, and water used for cleaning are the major external contamination sources of milk (Schmidt, 2009). Magnusson et al. (2007) analyzed the air and rinse water samples collected during milking. The spore content in air was very low with a maximum of 100 spores/m^3, whereas rinse water collected at the outlet of the milking system contained up to 250 spores per liter, even though 38 out of 63 samples analyzed did not show spores in detectable number.

1.3 Thermal resistance among *Bacillus* species

The organism producing higher heat resistant (HRS) spore was first isolated from the milk sample taken from the bypass of indirect heating UHT device in Belgium and was later identified as *Bacillus sporothermodurans.* The problems related to its occurrence in UHT milk samples were simultaneously reported in other European and Non-European countries (Scheldeman et al., 2005, Scheldeman et al., 2006). Spores of thermophilic bacteria are activated immediately after heat treatment, and their

germination and propagation into milk or milk products cause a number or food spoilage problems. Spores are not only resistant to the commonly applied thermal processes, but some of them also adhere to surfaces of the processing equipment resulting in biofilm formation (Scheldeman et al., 2006). On the basis of a wide range of favorable growth temperatures, the resistance of spores to industrial pasteurization, and their ability to grow at refrigeration storage; thermoduric, thermophilic, and psychrotrophic spore formers are considered of significance to the dairy industry (Hull et al., 1992).

Thermoduric bacteria can survive the pasteurization process, but may not necessarily be thermophilic (Hull et al., 1992). Once they survive the pasteurization process, they are carried over to the final product and later on result in defects and spoilage. Almost 100 % spores of *Bacillus* survive at 63˚ C for 30 minutes. They not only escape pasteurization but can also germinate and colonize into very large number during the manufacturing process, and cause several problems related to product quality and safety (Hileman, 1940, Silva and Gibbs, 2010).

The term thermophilic bacteria applies particularly to bacteria which grow at elevated temperatures normally between 40 - 65˚ C (Scott et al., 2007). Few organisms are referred as thermoduric such as *B. stearothermophilus, Geobacillus stearothermophilus, B. coagulans, B. licheniformis, B. subtilis,* and *B. sporothermodurans* and can grow up to 55˚ C, even though their optimum growth temperature may be at a mesophilic range. (Martin, 1981, Yoo et al., 2006, Burgess et al., 2010).

Psychrotrophic bacteria can be defined as the bacteria which, irrespective of their optimum growth temperature, can survive and grow at or below 7˚ C (Meer et al., 1991,

Shah, 1994). Evidence from the literature suggest that thermoduric psychrotrophs may be simply variants of mesophilic organisms that adapted to grow at lower temperatures (Collins, 1981, Meer et al., 1991). From the spoilage point of viewpoint, psychrotrophs are the most important organisms because they can grow and spoil milk and dairy products at even refrigeration temperature (Champagne et al., 1994, Granum and Lund, 1997).

In general, the psychrotrophs in milk are less than 10% of the total bacterial load in raw milk, even though they acquire higher attention as they grow rapidly and predominate other microflora during refrigeration (Shah, 1994). Psychrotolerant aerobic spore-forming bacteria, particularly *Bacillus* and *Paenibacillus* spp., which are thermoduric psychrotrophic in nature, are recognized as second most important organisms responsible for lowering the shelf life of HTST milk (Ranieri et al., 2009).

1.3.1 Heat resistance based on different thermal treatments. Yuan et al, (2012) characterized three strains of *Bacillus* on the basis of heat treatment and reported that spores of all the strains survived all time-temperature combinations of pasteurization treatments, whereas spores of only *Geobacillus* strain survived UHT treatment. Based on the thermal resistance of various *Bacillus strains,* the population levels in raw milk and powder were evaluated by Kent et al., 2016. They reported *Bacillus licheniformis* as a most prominent strain in raw milk and powder. Out of five different treatments, mesophilic spore counts (isolated using heat treatment temperature of 80˚ C for 12 mins) were found significantly higher in raw milk whereas, for powder samples, spores from three treatment namely, high heat resistant mesophilic and thermophilic spore and especially thermoresistance spore method showed no significant difference in numbers.

1.4 Influence of microbial quality of raw milk on end-product

The number of spore-forming psychrotrophs in raw milk may vary from 1 to 1600 spore per mL, whereas their presence in pasteurized milk has been quoted between <0.5 to 300 spores per mL. It has been suggested that about 25 % of the issues regarding shelf life of conventionally pasteurized milk in the U.S. may be related to the thermoduric psychrotrophs (Collins, 1981, Meer et al., 1991). Inadequate hygiene during milk processing can result in an increase of thermophilic numbers up to the level of 10^4 cfu/g in milk products like powders. The level and type of spores are based method used for detection (Kent et al., 2016). Even though raw milk is a major source of thermophilic bacilli in milk products, higher load in final products is not necessarily correlated with the initial loads in raw milk. As per previous studies, soil, feces, unhygienic conditions, bedding, and silage may result in aerobic spores ranging from 5 to 8 log cfu/mL but even then, the average number spores in raw milk is as low as 1-2 log spores/mL (Slaghuis et al., 1997, Pereira and Sant'Ana, 2018).

A study done by Walstra et al, (2006) reported that microbial quality of raw milk may not necessarily contribute in spore and spore-forming bacterial contamination of final product, but the major contribution of contamination is from production practices and post-processing contamination. Processing conditions and proper sanitization practices in dairy plant plays a crucial role in controlling the spore and sporeformer populations. Biofilms within processing lines, loaded with sporeformers and spores may serve as a continuous source of contamination of product (Ronimus et al., 2003, Pereira and Sant'Ana, 2018). Despite the low number in raw milk, improper CIP and poor sanitization of the dairy plants allow the retention and extensive multiplication of these

bacteria within the processing system, especially in the longer processing cycles (Burgess et al., 2010). Therefore, from the above discussion, it's clear that several factors play an important role in increasing the number of bacteria in the final product such as an initial microbial load of raw milk, processing conditions, plant hygiene, biofilms, and thermal operating conditions of the plant.

1.5 Influence of low-temperature storage during raw milk holding on population dynamics of *Bacillus* species

According to the Grade 'A' Pasteurized Milk Ordinance (PMO; FDA, 2017), after completion of the first milking cycle, raw milk is recommended to be cooled to 10˚ C (50˚ F) or less in four hours or less, and to 7˚ C (45˚ F) or less, within two hours after the completion of milking. After completion of each milking, many a time, due to economic and organizational reasons, raw milk is stored at low temperatures in the farm for long hours, and several times the supplied milk is not processed immediately. Since after milking, raw milk is cooled to 8˚ C or less, however, abused temperature (>8˚ C or more) conditions during milking intervals and transportation can significantly impact the microbiological attributes of raw milk leading to the outgrowth of the microbial population (Lafarge et al., 2004, Salustiano et al., 2009). Holding raw milk for up to 96 h at temperatures higher than 6˚C (O'Connell et al., 2016) can impair its chemical, physical as well as microbiological quality and thus, reduces its suitability for processing various dairy products (Barbano et al., 2006, Leitner et al., 2008). After *Pseudomonas*, *Bacillus* is the second predominant species frequently isolated from refrigerated raw milk (Sørhaug and Stepaniak, 1997, Vithanage et al., 2016). These psychrotolerant spore-forming *Bacillus* species are regarded as a major challenge, as many of these organisms can

withstand pasteurization and can germinate and outgrow in the final product (Ivy et al., 2012, Masiello et al., 2014).

A previous study (Kumari and Sarkar, 2014) reported the ability of *Bacillus* species to produce biofilms even at 4˚ C, which was isolated from chilling tank. Based on the strain specificity, these *Bacillus* species can multiply, germinate or may form spores under stressed conditions such as temperatures below and above their optimum range. These heat-stable *Bacillus* spores could survive milk processing steps, and at optimum conditions they germinate and cause spoilage (Becker et al., 1994, Rukure and Bester, 2001, Chen et al., 2004), due to the production of enzymes (proteases and lipases) and acid (Chen et al., 2004). Bacterial build-up during storage of raw milk may lead to the maintenance of ‘persistence cycles' of thermoduric that may lead to microbiological quality issues such as ‘bacterial or spore-spikes' in the final products (Hayes et al., 2001). Numerous studies have evidenced the growth of vegetative cells at refrigeration temperatures during milk processing (Kent et al., 2016) but none of the studies explored the sporulation behavior in raw milk, stored at low temperatures. Regardless of all the above aspects, the influence of the length of storage and temperature on germination and sporulation behavior of different sporeformers, and their ability to form spores during raw milk holding at the farm and plant silos is not understood clearly. Different strains of *B. licheniformis* could behave differently in terms of germination, multiplication, and sporulation in raw milk held at refrigeration temperatures for longer durations. Therefore, understanding the influence of time-temperature combinations that favor minimum multiplication or sporulation of sporeformers by during raw milk holding can be helpful

in increasing the efficiency of thermal treatments and reducing the bacterial load in end-product.

1.6 Influence of powder processing steps on final counts in the product

During milk powder manufacture, thermophilic bacteria are able to grow within the section of processing plant where the temperature for thermophile growth is ideal. Regeneration sections of heat exchangers and evaporators are the sections where the operating temperature remains between 45 to 75˚ C, which favors the growth of thermophiles resulting up to 10^6 cfu/g in the milk powder (Scott et al., 2007). The growth and sporulation ability of such *Bacillus* strains during evaporation and other thermal treatments can result into survival of these bacteria during hostile conditions thus resulting into persistence and constant contamination of end-product for long-term. Three species of bacilli viz. *G. stearothermophilus, B. licheniformis and Anoxybacillus flavithermus* constituted 91.9 % of the total bacterial population in milk powder collected from 18 different countries and screened by a RAPD based survey (Rückert et al., 2004). In other studies, Rueckert et al. (2005), and Rueckert et al. (2006) reported seven strains of *Bacillus* that commonly occurred in milk powder. These were identified to be *G. stearothermophilus* (strain A), *A. flavothermus* (Strain B, C, D), *B. licheniformis* (Strain F, G) and *B. subtilis*.

Murphy et al. (1999) determined thermophilic counts of milk at the intermediate stages of milk powder processing. He reported that preheat treatment of milk used in the manufacture of low heat powder, resulted in the growth of thermophilic counts up to a level of log 5 cfu/ ml. Bypassing the preheater was found to reduce the growth of bacteria at evaporation stage, and ultimately reduced their numbers in milk powder. Limiting the

production cycle to 12 h substantially reduced the number of thermophiles in milk powder. In another milk powder processing plant, no spores were detected in the milk in feed balance tank, whereas the number in the evaporator pass sample reached up to log 4.1 cfu/mL (Scott et al., 2007).

Apart from thermophilic strains, other species of *Bacillus* such as *Bacillus licheniformis, B. subtilis and B. coagulans* were also present in milk powders (Gopal et al., 2015). During drying, the inlet temperature of dryer usually ranges from 180 – 230˚ C (Moejes & Boxted, 2017), whereas in most cases the temperature that product experiences is about 60-100˚ C and is much lower to kill most of the spores of various species. As different strains and species of *Bacillus* respond to heat stress differently, therefore it is important to understand the influence of duration, temperature, water activity, initial load, strain variability and processing conditions during powder manufacturing on survivability and growth of vegetative as well as spore population. The evidence suggests that the number of spores in milk powder increases basically due to two major reasons; increase in spore concentration along with the concentration of milk, and favorable growth conditions within specific segments of processing line that supported the growth of thermophiles during longer manufacturing cycles.

1.7 Rapid spore detection methods

Spores are very resistant microbial structures that survive adverse conditions and can germinate when conditions are favorable. Detection of bacterial spores and determining their numbers are very useful in many applications. Detecting bacterial spores in dairy and food processing environments, water, dairy and food matrix can help reduce spoilage and shelf life issues in final product. Aerobic sporeforming *Bacillus* species are known as

a major contaminant in food and dairy industry (Seale et al., 2015). *Bacillus*, being a second pre-dominant species, is frequently isolated from raw milk and responsible for many shelf life issues of milk and milk products (Sharma and Anand, 2002). The *Bacillus* sporeformers are of importance to the food and dairy industry as these generally produce heat- resistant spores that may survive industrial sterilization and ultimately become incorporated into final products. Detection and enumeration of bacterial endospore concentrations are time consuming tasks. Therefore, timely detection of *Bacillus* species before milk processing is vital, to identify the source of contamination and strategies to reduce or control *Bacillus* build up in the later stages of the process.

Number of methods has been developed during past decades including plating techniques, biological methods such as polymerase chain reaction (PCR's) and immunoassays and optical methods. The most frequently used methods of quantification of spores are microscopy and plate culture counting methods, which are slow and tedious. Plating methods for spore enumeration may take up to 72 hours for the results to be available (Sharma and Prasad, 1992, Rosen et al., 1997). Whereas, biological methods usually require costly reagents and requires sample processing time before analysis. Thus, there remains a need for simple and cost-effective method that can help rapid identification of aerobic *Bacillus* spores in food and dairy matrix.

From past two decades, optical method for detection and enumeration of spores based on dipicolonic acid (DPA) have occupied a great deal of attention. Most of the modern methods work using dipicolonic acid (DPA) as a spore marker (Rosen et al., 1997, Bell et al., 2005). Rosen et al. (1997) have described a method based on terbium dipicolinate photoluminescence. In this method terbium chloride ($TbCl_3$) is added to an aqueous

suspension containing bacterial endospores. Another method based on delayed gate fluorescence detection of dipicolinic acid (DPA) chelated with lanthanide metals has been described. The optimized system had a detection limit of 2 nM DPA, which also allowed a real-time monitoring of the germination of bacterial spores by continuously quantifying exuded DPA. A minimum detection limit of 10^4 *B. subtilis* spores per ml could be attained (Hindle and Hall, 1999). Yet another rapid spore enumeration assay, Rapid SSEA, has been reported. Rapid SSEA is based on imaging and counting individual bacterial spores in a microscopic field, where a magnified contrast is generated by a highly luminescent complex of (Tb3+) and dipicolinic acid (DPA) which is released from spores during germination (Yung et al., 2006). A method using lysozyme and performic acid as germination inducer and staining of the germinated spore with acridine orange has been described (Sharma and Prasad, 1992). Surface-enhanced Raman spectroscopy (SERS) based assay of spore DPA to enumerate *B. anthrax* spores has been described by Bell et al. (2005).

As a follow-up to a previous study (Li et al., 2013), further investigation should be done to evaluate CaDPA concentration in *Bacillus* spores using ratiometric fluorescence technique. This technique can be useful rapidly enumerate total spore counts in raw milk and is based on the detection of calcium dipicolinate (CaDPA), an important biomarker and major component of bacterial spores. Lanthanide ions (Ln3+) have a high affinity for CaDPA, and thus the binding could enable a very sensitive assay with bright luminescence. Most of the lanthanide-based sensors only use the change of fluorescence intensity to estimate the concentration of CaDPA. While, the ratiometric fluorescent detection method, being validated in this study, could measure the relative changes of

fluorescence intensities at two different wavelengths. Development of these rapid spore detection kits can be helpful in detection of spores in milk and milk products within farm facilities itself within a duration of approx. 20-25 min.

1.8 Optimization strategies, based on regression models, to control spore population

Currently, the dairy and food industry are more concerned in attaining strategies whereby spore-former and spore counts can be controlled or reduced to safe levels. Thus, Response Surface Methodology is a powerful mathematical and statistical technique widely used in developing and optimizing response variable in the industrial world (Lenth, 2009). RSM was initially described by box and Wilson, allows the estimation of various process parameters and their interactions on the dependent variables. Understanding the influence of time-temperature combinations that favor minimum multiplication or sporulation of sporeformers by using process optimization strategies, could help control bacterial load in raw milk. As the conditions like temperature and duration of holding raw milk can vary continuously in bulk silos, and throughout the powder processing run therefore application of optimization strategies can be helpful in knowing the optimum time temperature conditions.

In a recent study Buehler et al., (2018) used predictive models to predict spoilage patterns in HTST pasteurized milk which can be helpful in analyzing various approaches in reducing milk spoilage. Therefore, in current study, RSM can be used to analyze different parameters namely temperature and duration to optimize the outcome and to analyze the response variable, low degree model can be applied. The contour plots can be applied to generate time-temperature combinations where least or no sporulation ability of strains occur. Regression equations can be applied to predict the individual strain

population numbers for specific temperature and duration of raw milk holding. This increases the ability to create predicted values for vegetative cell population growth at a wide time-temperature range, which can be easily generated using regression equations. Based on germination, multiplication and sporulation patterns of individual strain, such population changes can be predicted using regression equations that can be applied at production farms and dairy industry. Such optimized milk holding conditions would prove useful for the dairy industry, and thus in future can be used as a strategy for shelf life determination of raw milk. The contour plots can readily be used even over an extended time-temperature range of storage to predict the behavior of sporeformers during raw milk holding in bulk silos at production farms and dairy plants.

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Chapter 2

Influence on Population Dynamics of Low Sporulating Strain During Raw Milk Holding at Different Refrigeration Temperatures – A Case Study

ABSTRACT

Bacillus licheniformis is a widely reported spore-former in raw milk and milk powders. The organism, being thermotolerant, is considered a challenge during milk processing. It would be of interest to understand the growth dynamics of *Bacillus* species during raw milk storage at low temperature in farm and plant silos. The current study was conducted to observe the changes in a population of vegetative cells and spores of *B. licheniformis* ATCC 6634, spiked in raw milk samples at about 4.0 and 2.0 log cfu/mL respectively. The vegetative cell spiked milk samples were stored at 4 and 8˚ C, for durations of 0 and 72 h whereas, spore spiked milk samples were incubated at 4, 6, 8, 10 and 12˚ C for up to 72 h and enumeration was performed after every 24h interval. Standard protocols were followed for microbial analysis. Spore enumeration was done by heating the spiked milk samples at 80˚ C for 12 min prior to plating on Brain Heart Infusion agar. Three trials, in replicates of three, were conducted, and the data were analyzed using two sample t-test, analysis of variance, and first order regression model. For vegetative cell spiking, log vegetative counts increased to 4.09 log cfu/mL after 72 h at 4˚ C whereas at 8˚ C the counts increased to 4.42 logs cfu/mL. A significant difference ($p < 0.02$) was thus observed in the mean counts after 72 h of holding for 4 and 8˚ C. On the other hand, the corresponding spore population mainly remained unchanged during 72 h at different storage temperatures. The results thus suggest that *B. licheniformis* may multiply to a greater extent when milk is held at temperature of 8˚ C or above. Whereas, no lack of fit

($p = 0.294$) was observed for spore values, and the entire regression surface was not significant. The regression equations were applied to generate contour plots for estimating the population shift across the holding temperature and duration. The contour plots were also used to determine the time-temperature combinations, where 1.0 log increase in population was observed. In the vegetative cell spiking study of *B. licheniformis* ATCC 6634, cell population remained mainly unchanged for 72 h up to 8˚ C, with more than 1.0 log change observed only at 10˚ C and above, therefore suggesting expeditious multiplication of low sporulating strain. The experimental log sporeformer and spore numbers were validated with that of contour plot generated values, which were originally applied using regression equations. Overall, if raw milk is predominated by population of low sporulating strain then, contour plots suggested using time-temperature combination of 8˚ C or below up to 72 h for holding milk. Current results exhibited that population dynamics of a low sporulating *Bacillus* species is influenced due to temperature and duration of holding raw milk. Importantly, the current study provides regression equation that can be used at farm and dairy industry to predict microbial outgrowth during raw milk holding and thus in future can be used as a strategy for shelf life extension of the final product.

2.1 INTRODUCTION

Bacillus species are known as a major contaminant in food and dairy industry responsible for food-borne diseases and milk and milk product spoilage (Granum et al., 1993, Seale et al., 2015). Previous studies reported the presence of thermoduric psychrotrophs in raw milk and determined that 25% of shelf life problems in milk and

milk products are associated in the United States is linked to *Bacillus* species (Francis et al., 1998, Sharma and Anand, 2002). As per Pasteurized Milk Ordinance (PMO) requirements, the total microbial count in Grade A milk leaving the farm is <100,000 cfu/mL and should be less than <300,000 cfu/mL after pooling milk in processing plant (Barbano et al., 2006). According to the Grade ‘‘A’’ Pasteurized Milk Ordinance (PMO, 2017) guidelines, after completion of the first milking cycle, raw milk should be cooled to 10˚ C (50˚ F) in less than four hours, and to 7˚ C (45˚ F) or less, within two hours after the completion of milking. A previous study (Wehr and Frank, 2004) examined standard methods for dairy products and analyzed that, general quality parameters exist in the industry that limits thermophilic spore-formers to a maximum of 2,000 cfu/g.

After *Pseudomonas, Bacillus* is the second predominant species frequently isolated from refrigerated raw milk (Sørhaug and Stepaniak, 1997, Vithanage et al., 2016). Among *Bacillus* species, *Bacillus licheniformi*s is considered as a potential source of spoilage of milk and milk products including dry milk powders, pasteurized as wells as UHT milk products (Barbano et al., 2006, Leitner et., 2008, Buehner et al., 2015, Miller et al., 2015). Due to economic and organizational reasons, it becomes impossible to deliver milk after completion of each milking, to dairy plant and industry. Therefore, milk is stored at low temperatures in the farm for long hours and several times, supplied milk is not processed immediately. Holding raw milk for long hours impairs its chemical, physical as well as microbiological quality and thus, reduces its suitability for processing various dairy products by intensifying milk impairment (Barbano et al., 2006, Leitner et al., 2008). Thermoduric psychrotrophs *Bacillus* can multiply even in hygienically produced raw milk and is dependent on the duration and temperature of storage (Griffiths

et al., 1987, Griffiths et al., 1988). In the dairy industry storing raw milk at refrigerated conditions is a prerequisite process and many a times raw milk is held for three to five days in bulk tanks before delivering to processing plants. However, abused temperature conditions during milking intervals and transportation can significantly impact the microbiological attributes of raw milk leading to the outgrowth of psychrotrophs (Lafarge et al., 2004). Therefore, our study analyzed the effect of different refrigeration storage temperatures and durations on population dynamics of raw milk spiked with *B. licheniformis* ATCC 6634 spiked in raw milk.

Regardless of all the above aspects, only a few studies and research has undertaken the microbial behavior in raw milk quality between its production and processing. In this study, the microbial attribute of spiked raw milk is accessed to observe the behavior of spores and vegetative form of *B. licheniformis* ATCC 6634 at low temperatures, when held for several hours. The growth pattern of *B. licheniformis* was observed after spiking raw milk with 4.0 log of vegetative cells and 2.0 log of spores in relation to its different storage temperatures and duration of holding. Therefore, our study hypothesizes that raw milk holding at low temperatures, may involve germination and sporulation of strain that may provide a chance for microbial build-up in the raw milk, leading to microbiological quality issues such as ‘spore-formers or spore-spikes’ in the final products.

Currently, the dairy and food industry are more concerned in attaining strategies whereby spore-former and spore counts can be controlled or reduced to safe levels. Thus, Response Surface Methodology is a powerful mathematical and statistical technique

widely used in developing and optimizing response variable in the industrial world (Lenth, 2009). RSM was initially described by box and Wilson, allows the estimation of various process parameters and their interactions on the dependent variables. As the conditions like temperature and duration of holding raw milk can vary continuously in bulk silos, therefore, this study involves different combinations of treatments x_1 and x_2 to accurately estimate the response or dependent variable i.e. the enumeration of spore-formers and spores. Thus, RSM is employed using different parameters namely temperature and duration to optimize the outcome and to analyze our response variable, low degree model was applied. RSM with first and second order model is applied to our data to optimize the time-temperature conditions where least or no significant shift in log cfu/mL of spore-formers and spores are observed. Generated second order regression equations were used to apply contour plots ts are used, to help predict the optimum time-temperature combinations. Percent dissolved oxygen (% DO) and pH are investigated as covariates during spiked raw milk storage study.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial Strain Collection and Growth Conditions

This study includes a pure culture of *B. licheniformis* ATCC 6634, purchased from the American Type Culture Collection of Microorganisms (ATCC). *B. licheniformis* was grown in Brain Heart Infusion (BHI; BD Difco™) by incubation at 32˚ C. The culture was preserved for future use by using protocol suggested by (Perry, 1995). The pellets of actively grown culture were prepared at mid-exponential phase by centrifuging at 4500 × g for 30 min. Phosphate buffer saline (PBS) with pH 7.4 was used to suspend

pellet, and the culture was preserved in 1.8 mL cryogenic vials (CRYOBANK^{TM} – Copan diagnostics Inc., CA, USA). For further use, the cryovials were stored at -80 ˚C in a deep freezer (NuAire ultralow freezer, NuAire Inc. MN, USA).

2.2.2 Growth Curve and Dose Standardization

Growth curve of Bacillus licheniformis ATCC 6634 was analyzed in fresh BHI broth and culture was plated after every 2 h of inoculation using BHI agar and absorbance was also recorded at 600 nm. Growth rate constant (k) and mean generation time (T_d) was calculated as described by (Powell, 1956). For dose standardization, activated cultures of *B. licheniformis* was centrifuged and later was suspended in 1.0 mL of phosphate buffered saline (PBS Different concentrations of the culture were obtained by adjusting the OD (approx. 0.3) at 600 nm (Spectronic 200, Fisher Scientific, Passaic, NJ, USA) and pour plating was done to validate the viable counts. Plates were incubated at 32˚ C for up to 24–48 h, and plates with 25–250 colonies were enumerated for calculating total counts (Wehr and Frank, 2004).

2.2.3 Sample Collection, and Inoculation

Challenge studies were conducted using aseptically collected (500 mL each) raw milk from South Dakota State University (SDSU) dairy farm. The raw milk samples were transported in refrigerated conditions (7° C or less) and stored at 4 ± 0.03° C before use. Raw milk samples (500 mL) were spiked separately with pre-standardized dose vegetative cells (approx. 4.0 log cfu/mL) and spores (approx. 2.0 log cfu/mL) of *B. licheniformis* ATCC 6634. Spiked raw milk samples were incubated in a refrigerated

incubator (FSC-97-990E, Fisher Scientific, Passaic, NJ, USA) at the respective temperatures and durations. Plates were incubated at 32˚ C for 24–48 h, and plates with 25–250 colonies were enumerated for calculating total counts (Wehr and Frank, 2004). Endospores were prepared using method as recommended by Khanal et al. (2014). Approximately 1.0 mL of actively growing broth culture of each of the spore-formers was spread-plated on the BHI agar plates. The plates were incubated at the appropriate incubation temperatures for up to 15 d. To monitor the level of sporulation, spore staining was performed occasionally throughout the incubation period and after achieving 90% of sporulation, spores were harvested using a similar technique described by Wang et al. (2009).

2.2.4 Experimental Design

Two factors were studied to understand the population dynamics of *B. licheniformis* namely: temperature and duration of holding. Parameters such as pH (Fischer AE150, Fischer Scientific, Passaic, NJ, USA) and dissolved oxygen (Dissolved Oxygen Meter Pen, Fischer Scientific, Passaic, NJ, USA) were used as covariates and were recorded for all time-temperature combinations during our study.

2.2.4.1 Vegetative spiking. Developing First Order Regression Model

At the beginning of this study, for vegetative spiked raw milk, a rotatable design (9 experiments) with three levels (-1, 0 and +1) of two independent variables (temperature and duration) were used initially to obtain first order regression model and was potent in searching the direction of optimum domain (Table 2.1). A two-factor rotatable (9 experiments) design, with axial points (experiments N1 to N4, Table 2.1),

and central points in five repetitions (experiments N5 to N9) was used to assess the influence of two factors (temperature and duration of holding) on sporulation, germination and population dynamic behavior of *B. licheniformis* for raw milk spiked with vegetative cells. Nine experiments were augmented with five replications in the center points (6˚ C for 36 h) to check the bias within several sets of experiments and to estimate the pure error. The aim of repetition of the central points was to supply a measure of the pure error of the regression analysis (Granum et al., 1993, Peña et al., 2014). Upper and lower limits for independent variables (temperature and duration) were fixed, based on previous reports and studies (Vinthanage et al., 2017). Spiked raw milk samples were incubated in a refrigerated incubator (FSC-97-990E, Fisher Scientific, Passaic, NJ, USA) at their respective temperatures and durations as shown in table 2.1 to 2.3. Spore-formers and spores were enumerated by using BHI agar as described in above section. To search the direction of an optimum domain, a first-degree model was performed accordingly, the equation can be expressed as:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \varepsilon \quad (1)$$

where x_1 is temperature, x_2 is duration and y is the response (spore-former or spore). The variables x_1 and x_2 are independent variables where the response y depends on them and experimental error term is denoted as ε. Our study includes response surface methodology (RSM) to model and analyze spore-former and spore growth in spiked raw milk samples, influenced by temperature and duration of holding. We assume that the multiplication and outgrowth of spore-formers or spores are influenced by the change in temperature x_1 and duration x_2.

2.2.4.1.2 Applying Second Degree Model

To accurately approximate the true response surface for spore-formers, the fitted first-order model suggested using the second-degree model thus, a series of additional time-temperature combinations were evaluated towards the optimum region called steepest ascent model at 6, 8, 10 and 12˚ C at their corresponding duration 36, 42.6, 49.2 and 55.8 h for vegetative spiked raw milk. Plating and enumeration of spore-formers were performed at these points as described in section 2.4. All the data from above two separate experiments were used to generate the second-degree equation by mean centering and adjusting the effect of covariates. The equation can be expressed as:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x11 + \beta_{22} x22 + \beta_{12} x_{12} + \varepsilon \qquad (2)$$

Where x11 = x1 * x1, x22= x2 * x2 and x12 = x1 * x2

2.2.4.2 Spore spiking

Spores of BL ATCC 6634 were spiked in raw milk samples with spiking levels of approx. 2.0 log cfu/mL. The spore spiked raw milk samples were incubated at 4, 6, 8, 10 and 12˚ C for 0, 24, 36, 48 and 72 h of durations as shown in table 2.3. Standard protocols were followed as described above and microbial analysis was carried out at 0-72 h duration with 24 h interval.

2.2.5 Statistical Analysis, Regression Surface and Contour Plots

Two-sample t-test, multiple linear regression analysis and response surface methodology (RSM) were performed using SAS 9.3 software (SAS Institute Inc., Cary, NC) to observe any significant difference between initial and final vegetative or spore

counts of spiked raw milk. The dependent variable (measured response) was the $\log_{10}$ of colony forming unit per milliliter of *B. licheniformis* spore-formers and spores for specific time-temp. combination. All the experiments were done in triplicates with three replicates. The significance of the model was analyzed, and lack of fit test was performed. Regression equations were used to generate contour plots and the model generated values were compared with that of experimental values.

The objectives of current study were to (1) Understand the influence of temperature and duration of raw milk holding on multiplication and germination abilities of low sporulating *Bacillus* strain (2) To derive regression equation which can be applied at farm and industrial level and based on initial logs these equations will help understand the optimum time temperature combinations that will lead to minimum or least increase in the bacterial load.

2.2.6 Quantification of Spore-formers and Spores

Incubated spiked raw milk samples were enumerated for spore-formers and spores. Sterile PBS solution at pH 7.4 was used for the serial dilution of spiked raw milk to enumerate spore-formers and spores. Sporulation was detected by enumerating samples for spores, spiked raw milk was heated to 80˚ C for 12 minutes, and was then cooled for 10 min in the ice bucket (Kent et al., 2016). The diluted samples were pour plated by using Brain heart infusion agar (BHI). Plates were stored at 30° C for 24-48 h (Khanal et al., 2014) and plates with 25 to 250 colonies were enumerated. Calculation of colony-forming units per milliliter of samples was done using a previous method (Wehr, 2004).

2.3 RESULTS AND DISCUSSION

2.3.1 Growth Curves of Bacillus Strains

The growth curves of both strains are illustrated in Figure 2.1. The growth constant (k) and estimated mean generation time (T_d) for BL ATCC 6634 were 1.180/min and 35.2 min. Therefore, in terms of mean generation time our results agree with previous reports that estimated the mean generation time of *Bacillus* species of 28.7 and 30 mins at 35˚ and 30˚ C (Powell, 1956, Leighton and Doi, 1971). This growth curves were helpful in identifying the mid-exponential phase for two individual strains. Activated culture was spiked in raw milk only after mid-exponential phase was reached, which was at approx. 11 h.

2.3.2 Effect of Independent Variables on Population Dynamics of Vegetative Cells of B. licheniformis

It is known that temperature and duration of storage conditions play a key role for bacterial proliferation in raw milk (Griffiths et al., 1987, Griffiths et al., 1988, Francis et al., 1998, Lafarge et al., 2004, De Jonghe et al., 2011) whereas, Kumari and Sarkar (2014) reported the ability of *Bacillus* species to produce biofilms at 4˚ C isolated from chilling tanks. Among *Bacillus* species of thermoduric psychrotrophs, *B. licheniformi*s and *B. coagulans* are the most predominant species found in raw milk and considered as a potential source of spoilage-causing microbes in milk and its products including pasteurized as wells as UHT milk products (Johnston and Bruce, 1982, Francis et al., 1998, Barbano et al., 2006, Leitner et al., 2008, Buehner et al., 2014). Recent studies (Vithanage et al., 2016, 2017) confirmed that the abused temperature conditions during

refrigerated storage of milk processing can significantly impact the microbiological quality of milk, due to increase in total bacterial counts and several other factors. Despite this, there are no reports, that investigated the effect of these factors on population dynamics of *Bacillus* species when spiked in raw milk.

To identify the population dynamics of *B. licheniformis* stored at refrigeration conditions for a specific duration, this study analyzed its growth behavior in terms of multiplication, germination and sporulation at different time-temperature combinations ranging from temperatures, 4.0 to 12.0˚ C & duration 0 to 72 h. The present study involves enumeration of total spore-formers and spores of *B. licheniformis* at specific time-temperature combinations spiked in raw milk at the rate of log 4.0 cfu/mL and their growth patterns are shown in table 2.1, respectively. In this study, we have observed that during 12˚C storage of spiked raw milk, more extensive multiplication of spore-formers occurred when compared with low-temperature storage (Table 2.1). Santos et al. (2003) compared two storage temperatures (0.5 & 6˚C) for pasteurized fluid milk and observed lower bacterial counts when fluid milk was stored at a temperature of < 6˚ C.

Spore-former enumeration (table 2.1) showed no significant difference between counts at 0 h for 4 and 8˚ C, thus confirms that the initial spiking levels were significantly similar for all replicates. Though the mean log values of spore-formers were highly significant between 4 and 8˚ C after 72 h of storage. In terms of outgrowth of microbes in milk during low-temperature storage, the present study results agree with the previous studies (Datta and Deeth, 2003, Lafarge et al., 2004, Barbano et al., 2006, Leitner et al., 2008). The study performed by Leitner et al., (2008) observed that the milk collected from farm bulk milk silos and dairy tanks, possess significant difference in bacterial

counts after 48 h of storage. Therefore, it is well documented that milk quality is influenced by the storage conditions and the potential leading cause is, continuous bacterial growth. In the present study, the total vegetative counts in raw milk spiked with B. *licheniformis* ATCC 6634 were 3.80 ± 0.05 and 3.92 ± 0.19 log cfu/mL for 4 and 8˚ C at 0 h of spiking (Table 2.1). We have observed, the number of total vegetative counts varied over a same range of temperature after 72 h of storage and these differences were found significant. Our results are consistent with Vithanage et al. (2016) that reported a significant increase of bacterial counts in 2-3 days of holding at ≥ 6˚ C or 4 days at 4˚ C.

For temperatures 4 and 8˚ C, about 0.29 and 0.50 log increase were observed after 72 h in spiked raw milk previously inoculated with vegetative cells of *B. licheniformis* at 3.80 and 3.92 logs, respectively (Table 2.1). Similarly, a much higher multiplication of vegetative cells was observed for milk stored at higher degrees for longer durations. After comparing initial inoculum level of vegetative cells, we observed 1.12 - 3.25 log increase in spiked raw milk stored at 10 and 12˚ C after 49.2 and 55.8 h, respectively. On the other hand, no significant increase in spore numbers was observed after 72 h of inoculation for milk stored at 4 and 8˚ C. Therefore, this is an important observation demonstrating the multiplication behavior of vegetative cells of *B. licheniformis* at low-temperature storage. Abused temperature conditions in farm silos and dairy plant, may lead to a greater multiplication of such thermoduric psychrotrophs that can be further carried over beyond pasteurization. Therefore, may serve as a source of post-pasteurization contamination leading to several quality issues in final milk products. Our study agrees with the previous study done by Grosskopf and Harper (1974), reported the multiplication of *B. licheniformis* and other *Bacillus* species in pasteurized milk and milk products stored at

4˚ C for four weeks. Current trends of holding raw milk for an extended time at refrigeration temperatures may exacerbate the significance of *B. licheniformis* for food and dairy industry.

2.3.3 Effect of Temperature and Holding Time on Population Dynamics of B. licheniformis Spores

It is assumed that, during the holding of raw milk, spores possess the ability to germinate and produce vegetative cells and can again form spores under stressed conditions in farm and dairy silos. Depending on temperature and storage duration under unfavorable environmental conditions these spore-formers may sporulate to produce large numbers of spores. Numerous studies have evidenced the growth of vegetative cells at refrigeration temperatures during milk processing but none of the studies explored spore behavior in raw milk, stored at low temperatures. Scott et al. (2007) reported the predominant sporulation sites as evaporators and plate heat exchanger during whole milk powder manufacturing but did not investigated raw milk in farm or dairy silos. If hygienic conditions are not followed, then 10^2 to 10^6 cfu/mL of spores in raw milk is contributed by, silage, transportation, animal health and environment (Te Giffel et al., 2002). The heat stable spores of *Bacillus* can survive milk processing thus, germinate and outgrow to elevated levels in final products such as milk powders, cheese and infant food formulas (Becker et al., 1994, Rukure and Bester, 2001). Therefore, to identify the population dynamics of *B. licheniformis*, spores were held at refrigeration conditions for 72 h of duration, this study analyzed the ability of spores to germinate at various low temperatures ranging from (4.0-12.0˚ C) for 0 – 72 h of duration. Thus, pre-prepared

spores of *B. licheniformis* ATCC 6634 were spiked (log 2.0 cfu/mL) in raw milk and their growth pattern during low temperature of holding is shown in table 2.3, respectively.

In present study, we have observed that during 12˚ C incubation of spore spiked raw milk, more extensive germination of spores occurred when compared to 4.0 and 6.0 ˚ C of storage (Table 2.3). Table 2.3 represents, decrease in spore numbers from 2.48 ± 0.03 and 2.45 ± 0.02 to 2.00 ± 0.02 and 1.73 ± 0.02 at 10 and 12˚ C after 72 h of incubation. Similarly, we observed a slight increase in vegetative cell population from 3.17 ± 0.01 and 3.13 ± 0.02 to 3.87 ± 0.01 and 3.93 ± 0.00 at 10 and 12˚ C after 72 h of holding. Thus, our results represent the ability of spores to germinate at higher refrigeration temperatures.

2.3.4 Influence of Covariates (pH and dissolved oxygen)

From Table 2.1 and 2.2, we can clearly observe the increase in pH with increase in duration of storage. As per previous studies (Bosworth et al., 1922, (Bosworth et al., 1922, Cárdenas-Fernández et al., 2012) few strains of *Bacillus* species possess the ability to produce ammonia that can significantly increase the pH of milk with duration. Similarly, a previous study (Chantawannakul et al., 2002) have evaluated that, the increase in pH during fermentation process by *Bacillus* species is possibly due to proteolysis and ammonia release. They have also observed that the difference in temperature conditions influence the difference in pH changes. The present study agrees with previous reports and confirms that increase in pH are more prominent at higher temperatures of storage. Therefore. this increase in pH from 0 to 72 h of storage of

spiked raw milk at 4 and 12˚ C indicates the ammonia release due to higher activity of *Bacillus* protease. Whereas, we have not observed any significant change in dissolved oxygen before and after storage of spiked raw milk samples at low temperatures for 72 h for both spiking experiments. Thus, our results indicate that DO and pH may not be a factor influencing the population changes during raw milk holding.

2.3.5 Optimization of Temperature and Holding Time for Raw Milk Storage by Response Surface Methodology (RSM)

Two sample t-test and multiple linear regression model was performed, and we observed positive influence of independent variables (temperature and duration) on the dependent variable (vegetative cell counts) whereas, no significant difference was observed in spore numbers after 72 h of incubation. A slight increase in counts of vegetative cells (*B. licheniformis*) was observed at 4˚ C in spiked raw milk samples, during 72 h of storage. Similar trends were observed at 8°C after 72 h of storage. The multiple linear regression model was found significant for vegetative cell counts for vegetative spiked raw milk samples. Based on the requirements of experimental design, two mathematical models (first and second-degree model) were fitted to the time-temperature combination data (log cfu/mL) of *B. licheniformis* vegetative cells. Similarly, data from raw milk spiked with spores was analyzed using two sample t-test and multiple linear regression model. We observed a log-cycle reduction in spore numbers with simultaneous increase in temperature and duration of holding.

2.3.6 Regression Models of Response

2.3.6.1 Log Increase in Sporeformers - Applying First and Second-Degree Model on Vegetative Spiked Raw Milk Data. Three temperatures and duration levels were evaluated to approximate polynomial (first-order model) for two dependent variables, explaining their effect on population dynamics of *B. licheniformis* in terms of multiplication and outgrowth. Table 2.1 represents the experimental design consisting response variable (y), total number of spore-formers and spores (log cfu/mL) enumerated for each set of experiment. Based on Eq. (1), the data was analyzed, and the first order equation is given as follows:

$$Y\hat{} = 4.08786 + 0.11874\ (x1) + 0.19629\ (x2) \quad (3)$$

According to Eq. (3), all the factors have positive effects and were significant. Interestingly, we observed increase in spore-formers counts with increase in temperature and duration of storage. We observed that a unit increase in temperature and duration lead to significant increase in vegetative cell counts. Therefore, temperature and duration are significant independent variables, which can affect the growth rate, leading to an increase in vegetative cell counts (dependent variable). The influence of storage temperature was more pronounced and significant than that of duration of storage. Interestingly, the spore numbers did not change throughout the storage conditions while the increase in spore-formers numbers were observed. This inferred that *B. licheniformis* possess the ability to multiply and grow at lower temperatures and their growth is influenced with the unit increase in temperature and duration of holding whereas, no sporulation was observed at lower temperature of storage after 72 h for *B. licheniformis* ATCC 6634.

In summary, the first order model (preliminary) indicated and suggested to rearrange all the variables to search direction of the optimum region. Therefore, based on the above-obtained model equation, Eq. (3), to analyze the optimum domain with more accuracy, the steepest ascent experiment (data shown in table 2.2) was conducted and the relocation of a central point was done as follows: Temperature 6˚ C and Duration 36 h. Further new levels of temperature and duration combinations (additional points), were chosen as factors (Table 2.2, N5 to N13), after analyzing steepest ascent experiment. enumerating spore-formers to optimize the independent variables. The second-degree equation was obtained from the data shown in table 2. Three, second-degree equations were generated from vegetative spiked raw milk data. Equation 4 explains the effect of temperature and duration of holding vegetative spiked (log 4.0 cfu/mL) raw milk by adjusting the effect of pH and DO on a response is as follows:

$$\text{Log Veg. cells} = 4.22749 + 0.09379(x1) + 0.01006(x2) + 0.00177(x12) + 0.04365(x11) - 0.00017558(x22) \quad (4)$$

Regression equation 3 explains the response of log-cycle increase of vegetative cells of *B. licheniformis* 6634 influenced by holding temperature and duration in vegetative spiked raw milk.

2.3.6.2 Log Shift in Spores to Spore-formers, Spore Spiking Evaluation. The response variable (log-cycle reduction of spores) with the mean value (n=9) were obtained using data as shown in table 2.3. After applying quadratic centered regression model, second-order polynomial equations were generated explaining the effect of independent variables with and without adjusting for the effect of pH and DO on the response variable.

Log spores = 0.51015 – 0.02722(x1) + 0.00291(x2) – 0.00145(x12) – 0.01262(x11) – 0.00001(x22) (5)

Regression equation 5 explain the response of log-cycle change in spore population of *B. licheniformis* 6634 influenced by holding temperature and duration in vegetative spiked raw milk. According to equation 5, we observed that a unit increase in temperature resulted into decrease in spore population by 0.03 $\log_{10}$ spores/mL whereas, the holding duration positively influenced the increase in spore population and thus lead to increase in spores by 0.003 $\log_{10}$ spores/mL. We observed a very low probability value ($P < 0.0001$) for the above equations, demonstrating that the model is highly significant. Higher computed F-values indicated that the treatment differences are highly significant, therefore, the models were found to be adequate. The above equations (3-5) helped us develop contour plots across the holding temperature and duration of holding (Fig. 2.2 A & B). The current trends of holding raw milk for an extended duration at refrigeration temperatures also enhances the significance of sporeformers such as *B. licheniformis* for food and dairy industry. Regression equation 5 explains the response of log-cycle increase of *B. licheniformis* influenced by temperature and duration of holding vegetative spiked raw milk, including the effect of adjusted pH. Part of this work (Awasti et. al, 2017) was also presented in American Dairy Science Association, 2017 in Pittsburg, PA

2.3.7 Analysis of Variance (ANOVA)

The analysis of variance (ANOVA) was analyzed for above quadratic models by using R programming and we observed the R values were close to 1 indicating a high degree of correlation between predicted and observed values. We observed a very low

probability value ($P < 0.0001$) for above equations, demonstrating that the model is highly significant. Higher computed F-values indicated that the treatment differences are highly significant, therefore the above models were found to be adequate.

2.3.8 Optimum Time Temperature Conditions Generated using Contour Plots

Regression equations were applied to generate contour plots to estimate the population shift during raw milk holding. To identify the optimum levels of each independent variable with least or no change in log numbers of vegetative cells (spore-formers) and spores, contour plots were used to determine time-temperature combinations where 1.0 log increase in population can be observed. According to a previous study (Buehner et al., 2014), raw milk collected from Midwest dairy farms, usually consisted of 2.61 to 2.76 log cfu/mL sporeformers and 1.08 to 2.06 log spores/mL thus, our study includes comparative numbers for initial spiking levels. It is important to note that the product defects are detected when the concentration of microorganisms reaches up to the level of approx. log 7.0 cfu per mL of milk (Shehata et al., 1971, Janštová et al., 2006). As the maximum spiking levels of vegetative cells and spores of *B. licheniformis* is approx. 4.0 and 2.0 logs, therefore even 1.0 log increase in population was identified as a cutoff point to decide the optimum parameters.

2.3.9 Comparing the Experimental Values with Model Generated Predicted Values

Contour plots in figure 2.2 generated from equation 4 explain the increase in response variable as a function of temperature and duration by using the mean values. According to the experimental values approx. 1.0 log increase in the vegetative cell population after holding milk at 10˚ C increased the vegetative population within 50 h.

As per predicted values, if raw milk is predominated by a low sporulating strain then, milk could be held at 8˚ C or below up to 72 h or 9˚ C up to 48 h without 1.0 log increase in sporeformer population. Although holding milk at 10˚ and 12˚ C will increase the low sporulating strain population within 42 and 35 h respectively. The experimental values were similar to that of regression model predicted values that were generated using second-degree polynomial regression equation. Since the actual experiments were done only for certain time-temperature combinations, but the analysis of variance (ANOVA) analyzed for above quadratic models by using R programming showed that the R values (coefficient of determination) were close to 1, indicating a high degree of correlation between predicted and observed values. Therefore, this increases our confidence in applying contour plot generated predicted values, as the fitted values were comparable with actual values. This increases the scope to create predicted values for vegetative cell population growth at a wide time-temperature range, which can be easily generated using regression equations. According to the model predicted values, if the raw milk holding temperatures were 10˚ or 12˚ C then 1.0 log increase can be seen at 70 and 60 h of holding. From vegetative spiking results, we observed that the spore populations did not change much due to low sporulation ability of BL ATCC 6634 in raw milk. Thus, the current study observed that the change in population dynamics is influenced by strain predominance, multiplication and germination behavior, and variations in temperature and duration during raw milk holding. Overall, based on strain predominance, sporulation and germination behavior, if raw milk is predominated by a low sporulating strain then the contour plots suggested using time-temperature combination of 8˚ C or below for 72 h. In view of this, holding milk at time-temperature of 8˚ C for not more than 72 h will

not allow the increase in population by 1.0 log cfu/mL, and will keep the population towards vegetative side, which can be easily inactivated using thermal treatments such pasteurization. The contour plots being proposed in this study could readily be used even over an extended time-temperature range of storage to predict the behavior of sporeformers during raw milk holding in bulk silos at production farms and dairy plants.

2.4 CONCLUSIONS

After statistical analysis, no significant increase in spores was observed for vegetative spiked raw milk samples held at 72 h of incubation for all refrigeration temperatures. Whereas, we have observed a significant increase in vegetative (spore-formers) counts after 72 h of incubation at all the temperatures. Similarly, an increase in vegetative cells was observed when spores were spiked due to potential germination of spores and multiplication of vegetative cells. Thus, we can infer that, when raw milk is spiked with a standardized dose of *B. licheniformis,* there is a significant shift of spores to vegetative cells at low temperature of storage for 72 h due to potential germination of spores and multiplication of spore-formers under specified conditions. *Bacillus licheniformis* thus may possess the ability to multiply and germinate in raw milk samples at low temperatures, whereas, the sporulation behavior was not observed under the specified conditions. Therefore, the above generated contour plots can help the industry understand the use of optimum time-temperature combinations, resulting in least or no population shift of spore-formers.

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Table 2.1. Experimental design with real and coded values and log cfu/mL of spore-formers and spores present in spiked raw milk as a function of temperature and time

Experiment*	Temperature (°C)	Time (h)	Spore-formers (log cfu/mL)[1,2]	Spores (log cfu/mL) [1,2]	Covariates†	
					pH	DO
N1	4.0(-1)	0.0(-1)	3.80 ± 0.05^{aC}	0.30 ± 0.11^{aA}	6.7 ± 0.02	20.4 ± 0.04
N2	4.0(-1)	72.0(+1)	4.09 ± 0.12^{aB}	0.70 ± 0.08^{aA}	6.9 ± 0.01	19.0 ± 0.12
N3	8.0(+1)	0.0(-1)	3.92 ± 0.19^{aC}	0.42 ± 0.15^{aA}	6.7 ± 0.01	20.3 ± 0.19
N4	8.0(+1)	72.0(+1)	4.42 ± 0.12^{bA}	0.48 ± 0.18^{aA}	7.1 ± 0.01	18.8 ± 0.12
N5	6.0(0)	36.0(0)	4.09 ± 0.10^{aB}	0.67 ± 0.14^{aA}	6.9 ± 0.01	18.6 ± 0.09
N6	6.0(0)	36.0(0)	4.07 ± 0.05^{aB}	0.56 ± 0.19^{aA}	6.9 ± 0.01	19.0 ± 0.04
N7	6.0(0)	36.0(0)	4.15 ± 0.17^{aB}	0.73 ± 0.15^{aA}	6.9 ± 0.02	18.9 ± 0.16
N8	6.0(0)	36.0(0)	4.15 ± 0.05^{aB}	0.60 ± 0.11^{aA}	6.9 ± 0.02	18.7 ± 0.07
N9	6.0(0)	36.0(0)	4.21 ± 0.05^{aB}	0.67 ± 0.07^{aA}	6.9 ± 0.02	18.9 ± 0.05

* Nine sets of experiments are conducted, and three trials are performed in triplicates (N = 3 × 3)

† Covariates (pH and dissolved oxygen) analyzed for all design points

Coded values are presented between brackets; values in table represents means ± standard errors of each trial performed in triplicates

[1] Lowercase superscripts represents significance level checked for same temperatures

[2] Uppercase superscripts represent significance level checked for durations for all temperatures

Table 2.2: Experimental design after analyzing steepest ascent model and additional points with log cfu/mL of spore-formers present in spiked raw milk as a function of temperature and time

Experiment	Independent variables		Dependent Variables	Covariates	
	Temperature (°C)	Time (h)	Vegetative (log cfu/mL)	pH	DO (%)
N1	6	36	4.13 ± 0.10	6.9 ± 0.02	19.1 ± 0.09
N2	8	42.6	4.29 ± 0.10	6.9 ± 0.02	18.2 ± 0.10
N3	10	49.2	4.99 ± 0.10	6.7 ± 0.02	17.9 ± 0.21
N4	12	55.8	7.05 ± 0.27	6.5 ± 0.02	17.7 ± 0.27
N5	4.6	45	3.87 ± 0.01	6.83 ± 0.02	19.03 ± 0.17
N6	5	30	3.83 ± 0.02	6.8 ± 0.00	19.7 ± 0.11
N7	5	60	4.24 ± 0.01	6.76 ± 0.00	19.06 ± 0.08
N8	7	30	4.22 ± 0.46	6.83 ± 0.00	19.13 ± 0.08
N9	7	60	4.93 ± 0.00	6.78 ± 0.00	18.63 ± 0.14
N10	7.4	45	4.77 ± 0.01	6.80 ± 0.00	19.07 ± 0.03
N11	6	23.8	4.38 ± 0.07	6.89 ± 0.00	18.9 ± 0.15
N12	6	66.2	4.78 ± 0.01	6.81 ±0.00	19.06 ± 0.18
N13	6	45	4.55 ± 0.01	6.83 ± 0.00	19.26 ± 0.03

Values in table represents means ± standard errors of each trial performed in triplicates.

Table 2.3: Influence of temperature and duration of holding on germination behavior of spores (log cfu/mL) in raw milk.

	4˚ C		6˚ C		8˚ C		10˚ C		12˚ C	
Duration	Spores	Veg	Spores	Veg	Spores	Veg	Spores	Veg	Spores	Veg
0 h	2.38 ± 0.01^{Aa}	3.15 ± 0.00^{dA}	2.39 ± 0.01^{aA}	3.12 ± 0.02^{cA}	2.45 ± 0.01^{Aa}	3.09 ± 0.02^{cA}	2.48 ± 0.03^{aA}	3.17 ± 0.01^{dA}	2.45 ± 0.02^{aA}	3.13 ± 0.02^{eA}
24 h	2.36 ± 0.01^{aC}	3.17 ± 0.00^{cBC}	2.38 ± 0.01^{aC}	3.14 ± 0.02^{bcC}	2.43 ± 0.01^{aB}	3.12 ± 0.02^{cC}	2.49 ± 0.02^{aA}	3.22 ± 0.01^{dAB}	2.41 ± 0.01^{abBC}	3.27 ± 0.01^{dA}
36 h	2.35 ± 0.01^{aC}	3.18 ± 0.00^{bcB}	2.37 ± 0.01^{aBC}	3.17 ± 0.02^{abcB}	2.43 ± 0.01^{aA}	3.19 ± 0.03^{bcB}	2.41 ± 0.01^{aAB}	3.30 ± 0.00^{cA}	2.37 ± 0.01^{bBC}	3.34 ± 0.02^{cA}
48 h	2.33 ± 0.02^{aB}	3.20 ± 0.01^{bB}	2.35 ± 0.02^{aB}	3.22 ± 0.03^{abB}	2.42 ± 0.01^{aA}	3.25 ± 0.06^{bB}	2.22 ± 0.02^{bC}	3.60 ± 0.04^{bA}	2.08 ± 0.01^{cD}	3.66 ± 0.03^{bA}
72 h	2.32 ± 0.02^{aA}	3.23 ± 0.01^{aD}	2.34 ± 0.01^{aA}	3.24 ± 0.02^{aD}	2.36 ± 0.01^{bA}	3.40 ± 0.03^{aC}	2.00 ± 0.02^{cB}	3.87 ± 0.01^{aB}	1.73 ± 0.02^{dC}	3.93 ± 0.00^{aA}

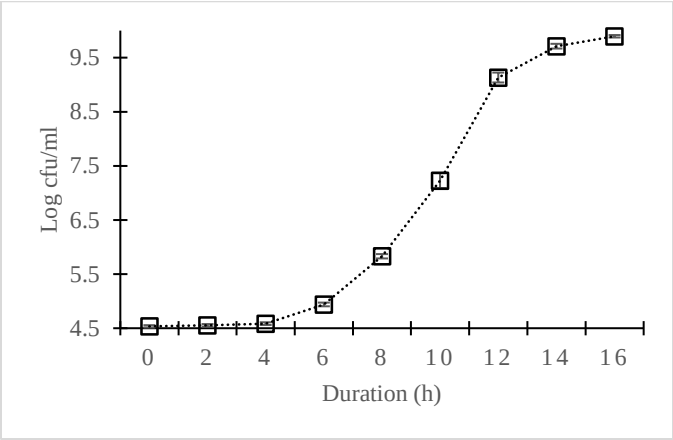
* Nine sets of experiments are conducted, and three trials are performed in triplicates (N = 3 × 3)

Coded values are presented between brackets; values in table represents means ± standard errors of each trial performed in triplicates

Lowercase superscripts represents significance level checked for same temperatures and different duration (along column, separately done for spores and vegetative cells)

Uppercase superscripts represent significance level checked for same durations for all temperatures (along row, separately done for spores and vegetative cells)

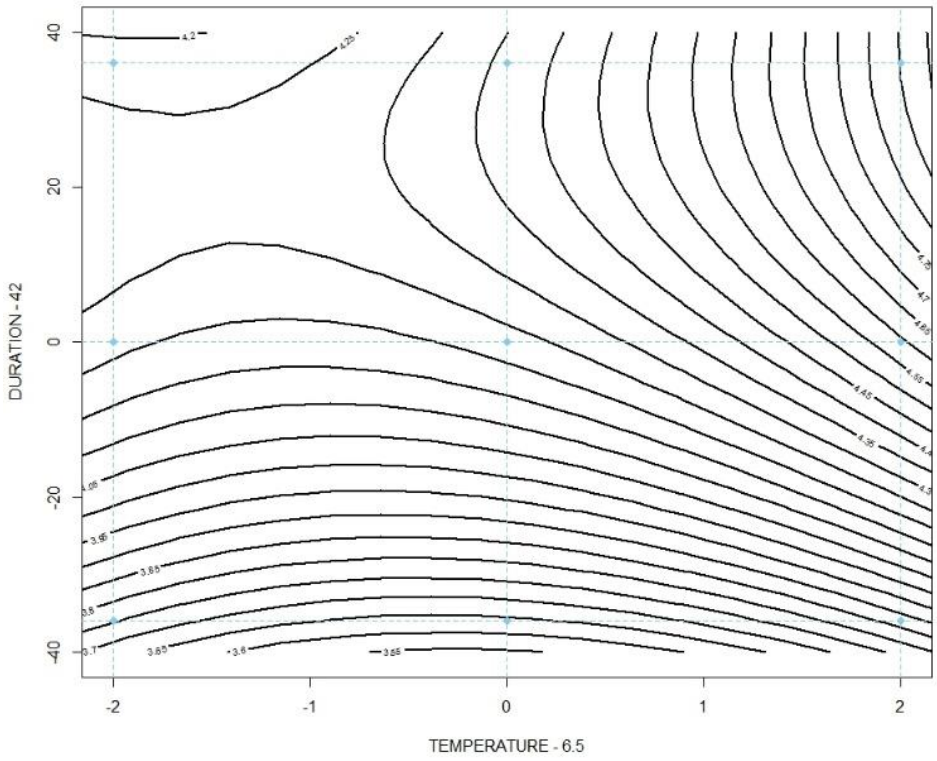
Figure 2.1: Growth curve of *B. licheniformis* ATCC 6634



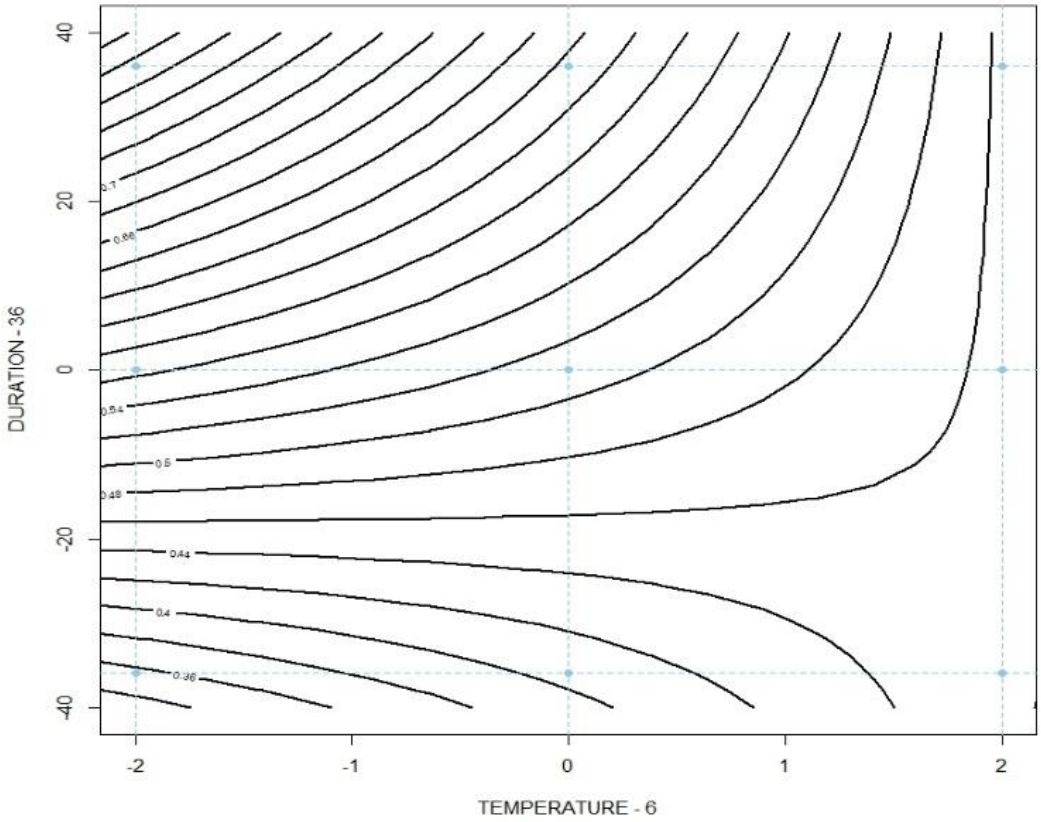
Values presented are Mean ±SE: □ represents growth curve of *B. licheniformis* ATCC 6634 at 32˚ C

Figure 2.2: Contour plots showing *B. licheniformis* ATCC 6634 (A) vegetative cell and (B) spore population (log_{10} cfu/mL) in response to varying degrees of temperature and duration of holding vegetative cells (4.0 log cfu/mL) spiked raw milk

A



B


DURATION - 36
TEMPERATURE - 6
40
20
0
-20
-40
-2
-1
0
1
2
0.7
0.66
0.54
0.5
0.48
0.44
0.4
0.36

Chapter 3

Influence on Population Dynamics of High Sporulating Strain During Raw Milk Holding at Different Refrigeration Temperatures – A Case Study

ABSTRACT

As *Bacillus* species such as *B. licheniformis*, is widely reported spore-former in raw milk and other milk products. The organism, being most predominant in Midwest region of United States, is considered a challenge during milk processing due to spore forming ability of few strains. In the first phase of our study (Chapter 2), we noticed that the strain BL ATCC 6634 was a slow spore producing strain Therefore, it would be of interest to understand the growth dynamics of rapid sporulating strain during raw milk storage at low temperature in farm and plant silos. The current study was conducted to observe the changes in population of vegetative cells and spores of *B. licheniformis* ATCC 14580, spiked in raw milk samples at about 4.0 and 2.0 log cfu/mL respectively. The milk samples spiked with vegetative cells and spores were separately incubated at 4, 6, 8, 10 and 12˚ C for up to 72 h and enumeration was performed after every 24 h interval. Standard protocols were followed for microbial analysis. Spore enumeration was done by heating the spiked milk samples at 80˚ C for 12 min prior to plating on Brain Heart Infusion agar. Three trials, in replicates of three were conducted, and the data were analyzed using two sample t test, analysis of variance, and first order regression model. In vegetative cell spiking study of *B. licheniformis* ATCC 14580, 1.0 log shift was observed only after 80 h at 8˚ C, with increased sporulation potential at lower temperature of storage and vegetative spiking study showed spore formation at different storage temperatures. Evidence of some parallel germination was observed for this strain

at 8˚ C or higher when raw milk samples were spiked with spores. The experimental values obtained for sporeformers and spore counts were validated with contour plots generated values. Overall, for raw milk samples predominated by a readily sporulating strain, raw milk could be held at 8˚C for 80 h. Sporulation behavior, germination and multiplication ability, and temperature and duration of holding raw milk influenced the population dynamics of *Bacillus* species. Keeping holding temperature below 6˚ C for less than 48 h will keep the cell population cell changes below 1.0 log. In addition, under these storage conditions, the population would remain as vegetative cells that are easily inactivated by pasteurization. The contour plots, so generated, would help predict the population shifts, and define optimum holding conditions for raw milk before further processing.

3.1 INTRODUCTION

Bacillus species are major contaminants in food and dairy industry and have been responsible for milk and dairy products spoilage (Seale et al., 2015). Various quality and shelf stability issues caused by *Bacillus* species are bitty cream, sweet curdling, off flavor, flat sour, and ropiness (Berkeley, 2002, Heyndrickx and Scheldeman, 2008, Burgess et al., 2010). Previous studies reported the presence of thermoduric psychrotrophs in raw milk and determined that a significant proportion of shelf life problems in milk and dairy products are linked to *Bacillus* species (Francis et al., 1998, Sharma and Anand, 2002). According to the Grade ‘A’ Pasteurized Milk Ordinance (PMO; FDA, 2017), after completion of the first milking cycle, raw milk is recommended to be cooled to 10˚ C (50˚ F) or less in four hours or less, and to 7˚ C (45˚ F) or less,

within two hours after the completion of milking. After completion of each milking, many a time, due to economic and organizational reasons, raw milk is stored at low temperatures in the farm for long hours, and several times the supplied milk is not processed immediately. Since after milking, raw milk is cooled to 8˚ C or less, however, abused temperature (>8˚ C or more) conditions during milking intervals and transportation can significantly impact the microbiological attributes of raw milk leading to the outgrowth of microbial population (Lafarge et al., 2004, Salustiano et al., 2009). Holding raw milk for up to 96 h at temperatures higher than 6˚C (O'Connell et al., 2016) can impair its chemical, physical as well as microbiological quality and thus, reduces its suitability for processing various dairy products (Barbano et al., 2006, Leitner et al., 2008). After *Pseudomonas*, *Bacillus* is the second predominant species frequently isolated from refrigerated raw milk (Sørhaug and Stepaniak, 1997, Vithanage et al., 2016). The major bacilli that contaminate and spoil milk and dairy products are *B. cereus, B. subtilis, B. licheniformis, B. stearothermophilus, B. coagulans* (Robinson, 2002), *B. sporothermodurans* (Pettersson et al., 1996, De Jonghe et al., 2008), *Brevibacillus bortelensis, Paenibacillus* (Scheldeman et al., 2006, Huck et al., 2007), and *Anoxybacillus flavithermus* (Scott et al., 2007). Among these species, *B. licheniformi*s has frequently been encountered in milk powders, and pasteurized, as well as, UHT milk products (Barbano et al., 2006, Leitner et al., 2008, Buehner et al., 2015, Miller et al., 2015) it was also identified as the most predominant species in Midwest dairy farms (Buehner et al., 2014). These psychrotolerant spore-forming *Bacillus* species are regarded as a major challenge, as many of these organisms can withstand pasteurization and can germinate and outgrow in final product (Ivy et al., 2012, Masiello et al., 2014). Based on

the strain specificity, these *Bacillus* species can multiply, germinate or may form spores under stressed conditions such as temperatures below and above their optimum range, acid shock, and other environmental conditions (Griffiths et al., 1987, Griffiths et al., 1988, Francis et al., 1998, Lafarge et al., 2004, De Jonghe et al., 2011). When bacilli cells are stressed, they respond to stress by converting themselves into spores, which help them resist those stresses and remain viable for a longer period of duration (Setlow et al., 2001, Henriques and Moran, 2007). If adequate hygienic conditions are not practiced at farm level, then as high as 10^2 to 10^6 cfu/mL of spores may be encountered in raw milk (Te Giffel et al., 2002). These heat-stable *Bacillus* spores could survive milk processing steps, and at optimum conditions they germinate and cause spoilage (Becker et al., 1994, Rukure and Bester, 2001, Chen et al., 2004), due to the production of enzymes (proteases and lipases) and acid (Chen et al., 2004). Bacterial build-up during storage of raw milk may lead to maintenance of ‘persistence cycles' of thermodurics that may lead to microbiological quality issues such as ‘bacterial or spore-spikes' in the final products (Hayes et al., 2001). Numerous studies have evidenced the growth of vegetative cells at refrigeration temperatures during milk processing (Kent et al., 2016) but none of the studies explored the sporulation behavior in raw milk, stored at low temperatures. Regardless of all the above aspects, the influence of length of storage and temperature on germination and sporulation behavior of different sporeformers, and their ability to form spores during raw milk holding at the farm and plant silos is not understood clearly. The variable sporulating ability of this genus has been indicated in *Bergey’s Manual of Systematic Bacteriology* (Logan et al., 2009). According to few studies under unfavorable or stressed conditions a few strains of *Bacillus* can sporulate to produce large numbers of

spores and thus can withstand high thermal treatment such as pasteurization to serve as a potential contaminant. Taking an example of *B. licheniformis*, we hypothesized that different strain of the organism could behave differently in terms of germination, multiplication, and sporulation in raw milk held at refrigeration temperatures for longer durations. The problem of bacterial outgrowth could be addressed early on during milk processing by optimizing raw milk holding conditions. Therefore, understanding the influence of time-temperature combinations that favor minimum multiplication or sporulation of sporeformers by using process optimization strategies, could help control bacterial load in raw milk. In the present study, a shift or increase in population was considered when the bacterial population increased by approx. 1.0 log cfu/mL from initial spiked levels. Regression equations were generated to develop contour plots to optimize raw milk holding conditions to keep the population towards the vegetative side with least multiplication and sporulation.

3.2 MATERIALS AND METHODS

3.2.1 Sourcing of Bacterial Strains and Growth Conditions

This study included a *Bacillus licheniformis* ATCC 14580 (BL ATCC 14580) cultures purchased from the American Type Culture Collection of Microorganisms (ATCC). Brain Heart Infusion (BHI; BD Difco^{TM}) media was used to grow and incubate culture at 37˚ C. The culture was preserved for future use by using protocol suggested by (Perry, 1998). The pellets of actively grown cultures were prepared at mid-exponential phase by centrifuging at 4500 × g for 30 min. Phosphate buffer saline (PBS), pH 7.4, was used to suspend pellets, and the cultures were preserved in 1.8 mL cryogenic vials

(CRYOBANK™ – Copan Diagnostics Inc., CA, USA). For further use, the cryovials were stored at -80˚ C in a deep freezer (NuAire ultralow freezer, NuAire Inc. MN, USA).

3.2.2 Developing Growth Curves, Dose Standardization, and Formation of Spores

Growth curve of *Bacillus* strain was developed using standard protocols (Fig. 3.1). For dose standardization, activated cultures of *B. licheniformis* was centrifuged after the respective time intervals and was separately suspended in 1.0 mL of phosphate buffered saline (PBS). Different concentrations of the culture were obtained by adjusting the OD (approx. 0.3) at 600 nm (Spectronic 200, Fisher Scientific, Passaic, NJ, USA) and pour plating was done to validate the viable counts. Plates were incubated at 37˚ C for 24–48 h, and plates with 25–250 colonies were enumerated for calculating total counts (Wehr and Frank, 2004). Endospores were prepared by using a method as recommended by Khanal et al. (2014). Approximately 1.0 mL of actively growing broth culture of BL ATCC 14580 was spread-plated on the BHI plates. The plates were incubated at the appropriate incubation temperatures for up to 15 d. To monitor the level of sporulation, spore staining was performed occasionally throughout the incubation period and after achieving 90% of sporulation, spores were harvested using a similar technique described by Wang et al. (2009).

3.2.3 Sample Collection, and Challenge Studies

Challenge studies were conducted using aseptically collected raw milk from South Dakota State University (SDSU) dairy farm. The raw milk samples were transported in a cooler and stored at 4˚ ± 0.5˚ C, until used. Raw milk samples (500 mL

each) were spiked separately with vegetative cells (approx. 4.0 log cfu/mL) and spores (approx. 2.0 log cfu/mL) of *B. licheniformis* ATCC 14580. Spiked raw milk samples were stored in a refrigerated incubator (FSC-97-990E, Fisher Scientific, Passaic, NJ, USA) at the respective temperatures and durations for two strains (Fig. 2 and 3). Vegetative cells and spores were enumerated using BHI agar as described below.

3.2.4 Overall Experimental Design of Challenge Studies

Two factors were studied to understand the influence of sporulating behavior on population dynamics of *B. licheniformis* namely: temperature and duration of holding. Additional parameters such as pH (Fischer AE150, Fischer Scientific, Passaic, NJ, USA) and dissolved oxygen (Dissolved Oxygen Meter Pen, Fischer Scientific, Passaic, NJ, USA) were used as covariates, and were recorded for all time-temperature combinations during our study. For the population dynamics, the challenge studies were conducted by spiking raw milk samples with vegetative cells of BL ATCC 14580, and the data thus obtained were supported by conducting spore spiking experiments of both the strains.

3.2.5 Vegetative Cell Spiking for Studying Population Dynamics.

Raw milk samples (500 mL each) were spiked with approx. 4.0 log cfu/mL of vegetative cells of BL ATCC 14580 strains. Spiked raw milk samples were incubated at 4, 6, 8, 10 and 12˚ C for 72 h durations, and samples were collected at 24 h intervals. The samples were analyzed for vegetative and corresponding spore population after holding them at specific temperatures. Sporulation was detected by enumerating after heating raw milk to 80˚ C for 12 minutes, followed by cooling (Kent et al., 2016). Appropriately,

diluted samples were pour plated using Brain heart infusion (BHI) agar. The vegetative cell spiking data were regressed using quadratic regression model to understand the influence temperature and duration of storage and regression equations so obtained, were applied to develop contour plots.

3.2.6 Spore Spiking for Studying Population Dynamics.

In a parallel study, spores of BL ATCC 14580 were spiked in raw milk samples at spiking levels of approx. 2.0 log cfu/mL. The spore spiked samples were incubated at 4, 6, 8, 10 and 12˚ C and standard enumeration protocols were followed, as described above, and microbial analysis was carried out at 0-72 h duration, at 24 h intervals.

3.2.7 Regression Analysis

Our study included multiple linear regression analysis to model and analyze sporeformers and spore growth in spiked raw milk samples, as influenced by sporulation potential of strains, and temperature and duration of holding. We assumed that multiplication and outgrowth of sporeformers or spores are influenced by the tendency to form spores, and changes in temperature (x1) and duration (x2) of holding.

First, the following first-degree model was fitted:

$$y = \beta 0 + \beta 1x1 + \beta 2x2 + \varepsilon (1)$$

where x1 is temperature, x2 is duration and y is the response (spore-former or spore). The variables x1 and x2 are independent variables, where the response y depends on them and experimental error term is denoted as ε. The adequacy of the first-order

model was assessed using residual analysis. Then the second-order model was fitted to account for curvatures. The second-order model with an interaction term used is given by:

$$y = \beta 0 + \beta 1x1 + \beta 2x2 + \beta 11x11 + \beta 22x22 + \beta 12x12 + \varepsilon \quad (2)$$

where, x11 and x22 are quadratic terms, namely x11= x1 + x2, x22= x2 + x2 and x12 is an interaction between temperature and duration x12= x1 + x2. The independent variables were mean centered to alleviate the multicollinearity problem.

Additional statistical analysis was performed using SAS 9.3 software (SAS Institute Inc., Cary, NC) to see any significant difference between initial and final vegetative cells or spore counts of spiked raw milk. The dependent variable (measured response) was the $\log_{10}$ of colony forming unit per milliliter of *B. licheniformis* vegetative cells and spores for specific time-temperature combinations. All the experiments were done in triplicates with three replicates. The significance level of the model was analyzed, and a lack of fit test was performed. Contour plots were generated from regression coefficients using R programming (Studio, 2012) and predicted response values from the fitted regression were analyzed and compared with that of experimental values.

3.3 RESULTS AND DISCUSSION

3.3.1 Growth Curves of Bacillus Strains

The growth curves of BL ATCC 14580 IS illustrated in Figure 3.1. The growth constant (k) and estimated mean generation time (T_d) were 1.379/min and 30.1 min. Therefore, in terms of mean generation time our results agree with previous reports that

estimated the mean generation time of *Bacillus* species of 28.7 and 30 mins at 35˚ and 30˚ C (Powell, 1956, Leighton and Doi, 1971). This growth curve was helpful in identifying the mid-exponential phase for two individual strains. The activated culture was spiked in raw milk only after mid-exponential phase was reached, which was at approx. 9 h for BL ATCC 14580.

3.3.2 Raw Milk Holding Conditions Influences the Population Dynamics

To understand the population dynamics of *B. licheniformis* in spiked raw milk, stored under refrigeration conditions, this study analyzed growth pattern in terms of multiplication, germination, and sporulation at different time-temperature combinations ranging from temperatures, 4˚ to 12˚ C and duration 0 to 72 h. Regression model was also developed subsequently.

3.3.2.1 Changes in Vegetative cell and Spore Population. Vegetative cell enumeration showed no significant difference between counts at 0 h for all storage temperatures, thus confirms that the initial spiking levels were comparable for all replicates. Although an average (± SE) vegetative cells of 4.11 ± 0.03 and 4.16 ± 0.02 $\log_{10}$ cfu/mL at 0 h for holding temperatures of 4˚ and 8˚ C showed a significant increase in microbial population after 72 h of holding with average (± SE) of 4.71 ± 0.03 and 4.99 ± 0.01 $\log_{10}$ cfu/mL (Table 3.1). For holding temperature of 12˚ C, more than 1.0 log increase in vegetative cells of S2 from 4.11± 0.04 to 5.25 ± 0.01 $\log_{10}$ cfu/mL was only evident after 72 h of holding (data not shown). A corresponding increase of spore average (± SE) was observed at 4˚ and 8˚ C, which were 1.39 ± 0.03 and 1.55 ± 0.03 $\log_{10}$ spore/mL at 0 h, whereas, 2.10 ± 0.00 and 1.88 ± 0.02 $\log_{10}$ spore/mL after 72 h of holding (Table 3.1).

Thus, current results indicate a higher sporulation potential of BL ATCC 14580 at lower storage temperatures. Although, holding spiked raw milk for 72 h at 12˚ C resulted into the decrease in spore population from 1.54 ± 0.03 to 1.25 ± 0.03 $\log_{10}$ spore/mL, demonstrating expeditious germination of spores at higher storage temperatures into vegetative form. From the above results, it is interpreted that BL ATCC 14580 strain has the potential to sporulated, as well as, germinate at higher refrigeration temperatures. In terms of microbial outgrowth, our results agreed with Vithanage et al. (2016) that reported a significant increase of bacterial counts in milk after 2-3 days of holding at ≥ 6° C or 4 days at 4˚ C. Similarly, a previous study (Santos et al., 2003) reported increased multiplication behavior of bacterial counts in stored pasteurized milk. They compared, two storage temperatures (0.5 and 6˚ C) and observed lower bacterial counts when milk was stored at a temperature of < 6˚ C. The multiplication and outgrowth of *Bacillus* vegetative population in milk during refrigeration storage is consistent with previous studies (Datta and Deeth, 2003, Lafarge et al., 2004, Barbano et al., 2006, Leitner et al., 2008). For raw milk collected from farm bulk milk silos and dairy tanks, Leitner et al. (2008) reported the significant influence of storage duration on bacterial counts after 48 h of storage. The total bacterial counts and psychrotrophs after 48 h of raw milk holding ranged from 4×10^3 to 7×10^6 cfu/mL whereas, in current study increased bacterial counts to 5.25 ± 0.01 log10 cfu/mL was observed at 55.8 h of raw milk holding.

The main aim of spore spiking study was to understand and support the growth trends of *Bacillus* strains as obtained in the vegetative cell spiking study discussed above. The growth pattern of vegetative and spore population after spiking raw milk samples individually with BL ATCC 14580 spores are shown in Table 3.2. Spore enumeration

showed no significant difference between counts at 0 h for all storage temperatures, thus our results confirmed that the initial spiking levels were significantly similar for all the replicates. In present study, spore spiking of BL ATCC 14580 with approx. 2.0 log spores/mL in raw milk, showed a shift in spores to the vegetative population for all storage temperatures with significant decrease in spore population was observed with some germination of spores into vegetative cells. Our results represent, decrease in average (± SE) (Table 3.2) spore population from 2.32 ± 0.02 to 1.93 ± 0.01 $\log_{10}$ spores/mL at 4˚ C after 0 and 72 h of spiking. Whereas, incubating raw milk samples spiked at 8˚ C resulted into decrease in spore population from 2.39 ± 0.02 to 1.52 ± 0.02 $\log_{10}$ spores/mL. With decrease in spore population, a corresponding increase in vegetative population was observed as shown in Table 3.2. The corresponding significant increase in average (± SE) vegetative population at 4˚ C were 3.40 ± 0.01 to 3.93 ± 0.06 $\log_{10}$ cfu/mL after 0 and 72 h of spiking. Similarly, incubating raw milk samples spiked separately with spores, resulted into significant increase in vegetative cells from average (± SE) 3.37 ± 0.02 to 4.04 ± 0.07 $\log_{10}$ cfu/mL after 0 and 72 h of incubation at 8˚ C. Incubating spore spiked raw milk samples at 12˚ C showed a significant decrease in spore numbers from 2.31 ± 0.04 to 1.67 ± 0.01 $\log_{10}$ spore/mL with corresponding significant increase in vegetative cells from 3.36 ± 0.03 to 4.20 ± 0.06 $\log_{10}$ cfu/mL after 0 and 72 h of spiking. Thus, current results confirmed the tendency of spores to simultaneously germinate and add to the overall vegetative cell population at 8˚ C and above. Germination potential of these *Bacillus* spores in raw milk is in agreement with a previous study (Mikolajcik and Koka, 1968). In a recent study Buehler et al., (2018) observed a maximum growth rate of *Bacillus* species of approx. 0.5 to 1.0 log cfu/mL per

day and 0.5 to 1.5 log cfu/mL per day at 4˚ and 6˚ C of holding. Overall, in the present study, we have observed that during 8˚ and 12˚ C incubation of spore spiked raw milk, more extensive germination of spores to vegetative cells occurred, when compared to 4˚ C of storage. By applying two sample t-test and multiple linear regression models, we observed the significant influence of independent variables (temperature and duration) on the dependent variable (Log_{10} vegetative cell and spore) after 72 h of incubation.

3.3.3 Influence of Covariates (pH and Dissolved Oxygen)

We did not observe any significant changes in pH and dissolved oxygen during storage of spiked raw milk samples at low temperatures for 72 h for all spiking experiments. Thus, our results indicate that pH and dissolved oxygen may not be factors influencing the population changes during raw milk holding under the conditions of storage used in this study.

3.3.4 Application of Regression Equations and Contour Plots in Depicting the Population Changes during Raw Milk Holding

Based on the requirements of experimental design, two mathematical models (first and second-degree model) were fitted to the time-temperature combination data of individual spore forming strains of *B. licheniformis* in raw milk. After applying regression model on vegetative spiked data of both strains, we observed that a unit increase in temperature and duration lead to increase in vegetative cell counts (Equation 3). Therefore, temperature and duration are significant independent variables, which can influence the *Bacillus* growth in raw milk, leading to an increase in vegetative cell counts

(dependent variable). From coefficient values in equation 3, the influence of storage temperature was more pronounced and significant than that of the duration of storage.

Log Veg. cells = 4.52500 + 0.04109(x1) + 0.01250(x2) + 0.00103(x12) + 0.00076918(x11) + 0.00001103(x22) (3)

Regression equation 3 explains the response of log-cycle increase of vegetative cells of *B. licheniformis* 14580 influenced by holding temperature and duration in vegetative spiked raw milk.

Log S2 spores = 1.63681 – 0.04945(x1) + 0.00335(x2) – 0.00205(x12) – 0.00533(x11) – 0.00001582(x22) (4)

Regression equation 4 explain the response of log-cycle change in spore population of *B. licheniformis* 14580 (S2) influenced by holding temperature and duration in vegetative spiked raw milk. According to equation 4 we observed that a unit increase in temperature resulted into decrease in spore population by 0.05 $\log_{10}$ spores/mL whereas, the holding duration positively influenced the increase in spore population and thus lead to increase in spores by 0.003 $\log_{10}$ spores/mL. We observed a very low probability value ($P < 0.0001$) for above equations, demonstrating that the model is highly significant. Higher computed F-values indicated that the treatment differences are highly significant, therefore, the models were found to be adequate. The above equations (3 & 4) helped us develop contour plots across the holding temperature and duration of holding (Fig. 3.2 A & B). The current trends of holding raw milk for an extended duration at refrigeration temperatures also enhances the significance of sporeformers such as *B. licheniformis* for food and dairy industry.

Present study provides regression equations that can be applied at farm and dairy facilities to help predict the optimum conditions that will result into minimum increase in sporeformer population during raw milk holding. Before applying these equations, few assumptions considered were (1) the initial sporeformers population of approx. 4.0 logs and (2) strain variability; the predominance of either low or rapidly sporulating strain in raw milk.

3.3.5 Optimum Time Temperature Conditions Generated using Contour Plots.

Regression equations were applied to generate contour plots to estimate the population shift during raw milk holding. To identify the optimum levels of each independent variable with least or no change in log numbers of vegetative cells (spore-formers) and spores, contour plots were used to determine time-temperature combinations where 1.0 log increase in population can be observed. According to a previous study (Buehner et al., 2014), raw milk collected from Midwest dairy farms, usually consisted of 2.61 to 2.76 log cfu/mL sporeformers and 1.08 to 2.06 log spores/mL thus, our study includes comparative numbers for initial spiking levels. It is important to note that the product defects are detected when the concentration of microorganisms reaches up to the level of approx. log 7.0 cfu per mL of milk (Shehata et al., 1971, Janštová et al., 2006). As the maximum spiking levels of vegetative cells and spores of *B. licheniformis* is approx. 4.0 and 2.0 logs, therefore even 1.0 log increase in population was identified as a cutoff point to decide the optimum parameters.

3.3.5.1 Comparing the Experimental Values with Model Generated Predicted Values.

Contour plots in figure 3.2A generated from equation 3 explain the increase in response

variable as a function of temperature and duration by using the mean values. According to the experimental values approx. 1.0 log increase in the vegetative cell population was observed in less than 56 h of holding at 12˚ C, whereas, the experimental values showed an increase in vegetative population by 1.0 log at 10˚ C only after 72 h of holding although, for lower temperatures of holding 8˚ C and below, a log increase was not evident up to 72 h. (Figure 3.2A). Experimental values for sporeformers were found in agreement with that of regression model predicted values. The experimental values were similar with that of regression model predicted values that were generated using second-degree polynomial regression equation. Since the actual experiments were done only for certain time-temperature combinations, but the analysis of variance (ANOVA) analyzed for above quadratic models by using R programming showed that the R values (coefficient of determination) were close to 1, indicating a high degree of correlation between predicted and observed values. Therefore, this increases our confidence in applying contour plot generated predicted values, as the fitted values were comparable with of actual values. This increases scope to create predicted values for vegetative cell population growth at a wide time-temperature range, which can be easily generated using regression equations. According to the model predicted values, if raw milk is predominated by a readily spore producing strain then, raw milk could be held at 8˚ C or below for up to 72 h, although a corresponding increase in spore population was evident only at lower temperatures of holding such as 4˚ and 6˚ C. Whereas, if the raw milk holding temperatures were 10˚ or 12˚ C then 1.0 log increase can be seen at 70 and 60 h of holding. Whereas, from Table 3.1 it is evident that BL ATCC 14580 can readily produce spores during low temperatures of holding, thus a shift in vegetative cells to

spores can be seen if the raw milk population is dominated by high sporulating strain such as in the current study. From above results, it is interpreted that strain BL ATCC 14580 have the potential to sporulate, as well as germinate, at higher refrigeration temperature.

Thus, current study observed that the change in population dynamics is influenced by strain predominance, sporulation and germination behavior, and variations in temperature and duration during raw milk holding. Overall, based on strain predominance, sporulation and germination behavior, if raw milk is predominated by a rapidly sporulating strain then it could be held at 8˚ C or below for not more than 72 h. In view of this, if raw milk held at these conditions, holding milk at these temperatures will not allow the increase in population by 1.0 log cfu/mL, and will keep the population towards vegetative side, which can be easily inactivated using thermal treatments such pasteurization. In terms of strain variability, this strain was compared with that of BL ATCC 6634 in a study presented (Awasti et al., 2018) in American Dairy Science association (ADSA) held at Knoxville, TN. The contour plots being proposed in this study could readily be used even over an extended time-temperature range of storage to predict the behavior of sporeformers during raw milk holding in bulk silos at production farms and dairy plants.

3.4 CONCLUSIONS

Findings from the current study suggest that *B. licheniformis* ATCC 14580 can readily produce spores during refrigeration storage of skim raw milk. *B. licheniformis* ATCC 14580 can multiply, sporulate, and germinate in raw milk during refrigeration

storage, and thus may significantly influence the keeping quality of raw milk. Control of these spoilage-causing organisms, before processing stages itself, is essential to produce final products with lower spore and sporeformer counts and extended shelf life attributes. Our study came up with regression equations that can be applied to predict the behavior of high sporulating strain population for specific temperatures and durations of raw milk holding. In the case of presence of high sporulating strains, holding raw milk at time-temperature of 8° C for no more than 72 h would ensure that the population does not change by 1.0 log cfu/mL. Such optimized milk holding conditions would prove useful for the dairy industry, and thus in future can be used as a strategy for shelf life determination of raw milk. Further studies need to be conducted to include more sporeformers and their strains to create more robust models. Although the current study is based on just one readily sporulating strain which provides an evidence on abilities of *Bacillus* strain to multiply, germinate or sporulate during raw milk holding. Based on this the further studies can be expanded to look at more sporeformers and their strains. For future studies, application of these regression models should be combined with product processing techniques such as filtration, evaporation and drying that could help target the overall decrease in common sporeformers and spores in the end products.

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Table 3.1: Influence of temperature and duration of holding on germination and sporulation behavior of spiked sporeformers (log cfu/mL) of *B. licheniformis* ATCC 14580 in raw milk

	4˚ C		6˚ C		8˚ C		10˚ C		12˚ C	
Duration	Veg	Spore	Veg	Spore	Veg	Spore	Veg	Spore	Veg	Spore
0 h	4.11 ± 0.03^{cA}	1.39 ± 0.03^{cA}	4.11 ± 0.02^{dA}	1.49 ± 0.03^{cA}	4.16 ± 0.02^{dA}	1.55 ± 0.03^{cA}	4.11 ± 0.02^{dA}	1.58 ± 0.02^{aA}	4.11 ± 0.04^{dA}	1.54 ± 0.03^{aA}
24 h	4.20 ± 0.03^{cC}	1.52 ± 0.01^{bA}	4.27 ± 0.04^{cBC}	1.52 ± 0.03^{cA}	4.34 ± 0.01^{cB}	1.53 ± 0.01^{cA}	4.31 ± 0.01^{cB}	1.55 ± 0.02^{abA}	4.44 ± 0.01^{cA}	1.41 ± 0.01^{bB}
48 h	4.48 ± 0.01^{bE}	2.04 ± 0.01^{aA}	4.63 ± 0.01^{bD}	1.87 ± 0.01^{bB}	4.73 ± 0.02^{bC}	1.77 ± 0.03^{bC}	4.84 ± 0.01^{bB}	1.47 ± 0.02^{bcD}	5.09 ± 0.01^{bA}	1.29 ± 0.03^{cE}
72 h	4.71 ± 0.03^{aE}	2.10 ± 0.00^{aA}	4.82 ± 0.01^{aD}	1.98 ± 0.03^{aB}	4.99 ± 0.01^{aC}	1.88 ± 0.02^{aB}	5.09 ± 0.01^{aB}	1.39 ± 0.05^{cC}	5.25 ± 0.01^{aA}	1.25 ± 0.03^{cD}

Values in table represents means ± standard errors of three trials performed in triplicates

Lowercase superscripts represents significance level checked for same temperatures and different duration (along column, separately done for spores and vegetative cells)

Uppercase superscripts represent significance level checked for same durations for all temperatures (along row, separately done for spores and vegetative cells)

Table 3.2: Influence of temperature and duration of holding on germination behavior of spiked spores (log cfu/mL) of *B. licheniformis* ATCC 14580 in raw milk

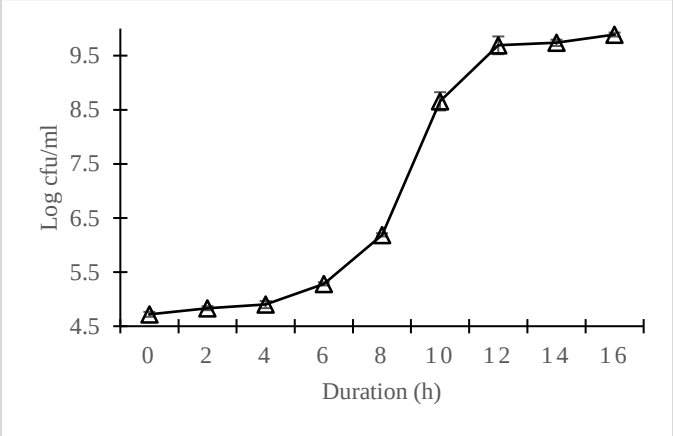
	4˚ C		6˚ C		8˚ C		10˚ C		12˚ C	
Duration	Veg	Spore	Veg	Spore	Veg	Spore	Veg	Spore	Veg	Spore
0 h	3.40 ± 0.01^{dA}	2.32 ± 0.02^{aA}	3.39 ± 0.01^{dA}	2.32 ± 0.03^{aA}	3.37 ± 0.02^{dA}	2.39 ± 0.02^{aA}	3.40 ± 0.02^{cA}	2.39 ± 0.02^{aA}	3.36 ± 0.03^{cA}	2.31 ± 0.04^{aA}
24 h	3.53 ± 0.01^{cD}	2.28 ± 0.02^{aA}	3.57 ± 0.00^{cD}	2.24 ± 0.03^{aAB}	3.63 ± 0.01^{cC}	2.26 ± 0.07^{aA}	3.73 ± 0.01^{bB}	2.01 ± 0.08^{bBC}	3.81 ± 0.03^{bA}	1.79 ± 0.09^{bC}
48 h	3.68 ± 0.01^{bD}	2.20 ± 0.02^{bA}	3.73 ± 0.00^{bD}	2.12 ± 0.02^{bA}	3.79 ± 0.02^{bC}	1.97 ± 0.05^{bB}	3.86 ± 0.02^{bB}	1.86 ± 0.02^{bcBC}	3.92 ± 0.01^{bA}	1.80 ± 0.01^{bC}
72 h	3.93 ± 0.06^{aB}	1.93 ± 0.01^{cA}	3.98 ± 0.07^{aAB}	1.78 ± 0.03^{cB}	4.04 ± 0.07^{aAB}	1.52 ± 0.02^{cD}	4.14 ± 0.08^{aAB}	1.79 ± 0.00^{cB}	4.20 ± 0.06^{aA}	1.67 ± 0.01^{bC}

Values in table represents means ± standard errors of three trials performed in triplicates

Lowercase superscripts represents significance level checked for same temperatures and different duration (along column, separately done for spores and vegetative cells)

Uppercase superscripts represent significance level checked for same durations for all temperatures (along row, separately done for spores and vegetative cells)

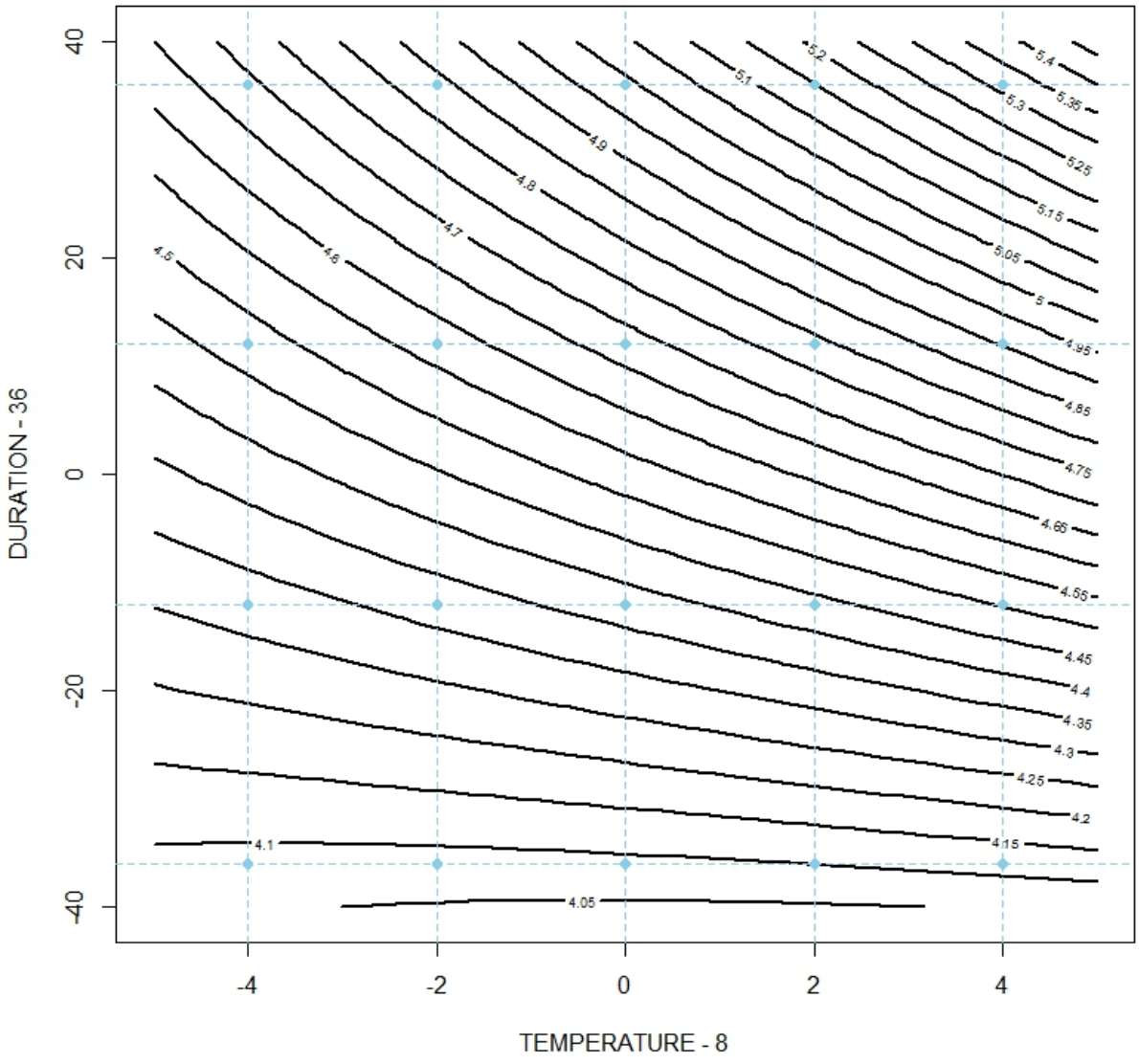
Figure 3.1: Growth curve of *B. licheniformis* ATCC 14580



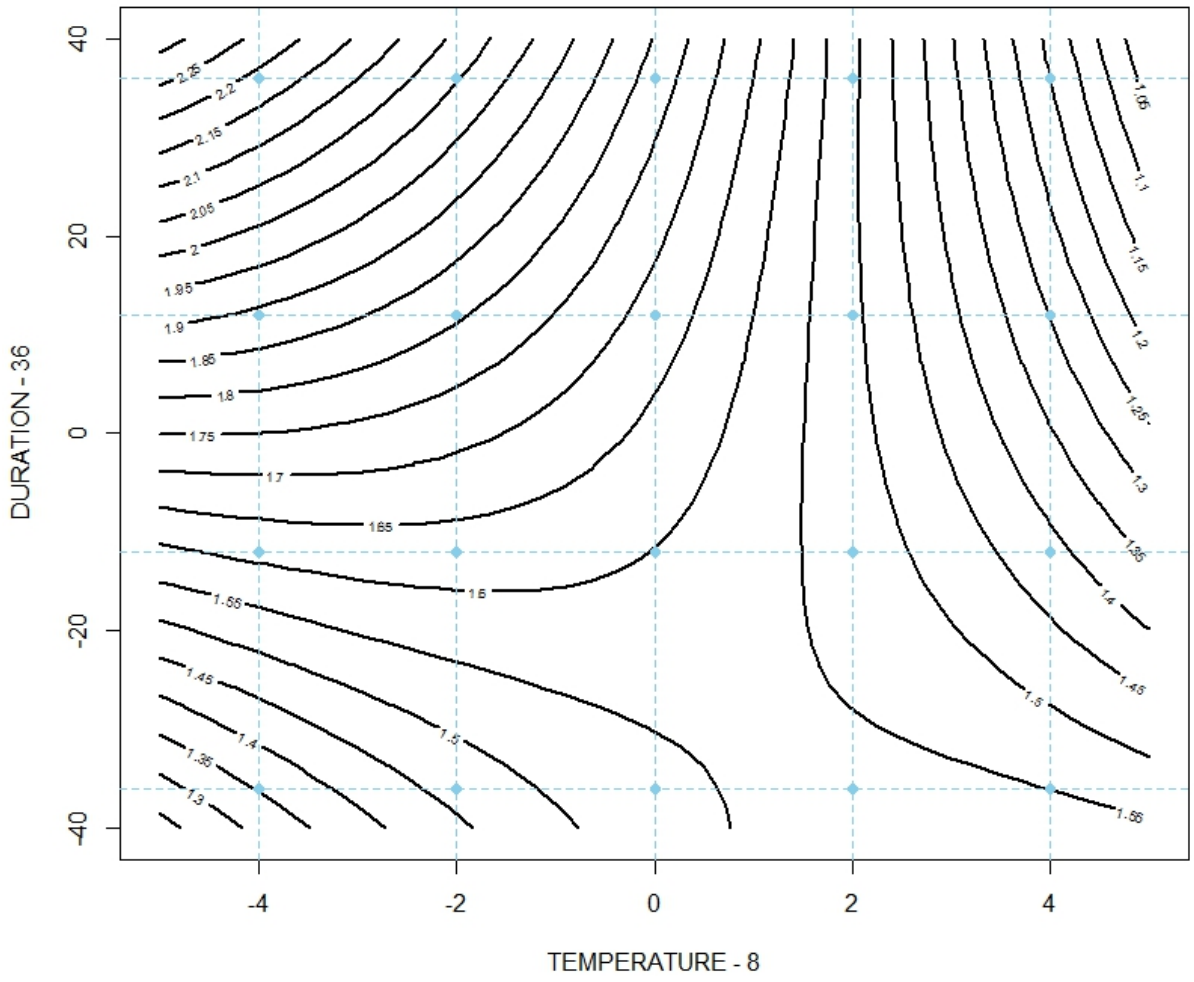
Values presented are Mean ±SE: △ represents growth curve of *B. licheniformis* ATCC 14580 at 37˚ C

Figure 3.2: Contour plots showing *B. licheniformis* ATCC 14580 (A) vegetative cell and (B) spore population (log_{10} cfu/mL), in response to varying degrees of temperature and duration of holding vegetative cells (4.0 log cfu/mL) spiked raw milk

A



B


DURATION - 36
TEMPERATURE - 8
40
20
0
-20
-40
-4
-2
0
2
4
2.25
2.2
2.15
2.1
2.05
2
1.95
1.9
1.85
1.8
1.75
1.7
1.65
1.6
1.55
1.45
1.4
1.35
1.3
1.05
1.1
1.15
1.2
1.25
1.3
1.35
1.4
1.45
1.5
1.55

Chapter 4

A Pilot Scale Skim Milk Powder Manufacturing by Controlling Raw Milk Holding Time-Temperature Conditions- A case study

ABSTRACT

B. licheniformis is one of the most prevalent species of spore forming bacteria in the Midwest region of United States dairy environment and is capable of surviving pasteurization and other thermal treatments. Currently, dairy industries are more focused in attaining strategies to reduce sporeformers and spores during milk powder processing run. Therefore, it is important to understand the influence of each processing conditions individually, such as evaporation and drying in terms of sporulation, multiplication and germination. In terms of sporulation and multiplication, results from our previous studies (chapters 2 and 3) identified variable behavior of *Bacillus licheniformis* strains. Amoung two strains, BL ATCC 14580 showed increased sporulation potential during refrigeration storage of raw milk. Spores are resistant towards thermal treatments, therefore current study tracked BL ATCC 14580 behavior during powder manufacturing run at pilot scale. Two treatments were separately conducted. In treatment 1 raw milk was held in silo using PMO recommended conditions and in second silo raw milk was held at optimum conditions at 4˚ C for 72 h (treatment 1) before processing milk into milk powder. Standard protocols were followed to enumerate vegetative cells and spores using brain heart infusion agar. Samples were collected in replicates of three and means were compared using ANOVA. The objective of this study was to understand the influence of raw milk holding conditions, evaporation and drying temperatures on sporulation and germination behavior of *B. licheniformis* strain. The overall final spore and vegetative cell counts in the powders

manufactured under optimum holding condition (4˚ C for 24 h) were found to be significantly lower (0.58 ± 0.04 and 1.82 ± 0.05 log cfu/g) as compared to the conditions likely to practice by dairy plants (2.74 ± 0.03 and 1.03 ± 0.06 log cfu/g). This shows that milk powders with reduced vegetative and spore counts can be prepared by optimizing the raw milk holding conditions. This study is based on just one plant trial with shorter powder processing run of approx. 7 h, therefore, it does not address any changes in the behavior of the organism during longer processing runs. Based on these results, studies can be expanded to look at the survivability of other sporeformers and their strains at pilot scale. For future studies, the application of techniques such as filtration or cavitation could also be included to evaluate the efficiency in elimination or reduction of spores in powder for longer powder processing run.

4.1 INTRODUCTION

The current global export of skim milk powder/nonfat dry milk (SMP/NDM) from the United States is 67,154 tons (aggregate volume as of March 2018), which is 38% more from March 2017 (U.S. Dairy Export Council-Global Dairy market, 2018). The quality of products has, however lead the US to face criticism in recent years from the South East Asian market regarding inconsistencies and tax specifications. There has been a lag in the export of SMP/NDM to major Asian markets such as Philippines, Indonesia and Vietnam, and the major cause is the continuous increasing demand of high specification dairy ingredients that meet their needs, especially low-spore powders.

Skim milk powder manufacturing is a complex process which involves microbial as well as functional changes during all stages of processing. Among spoilage organisms, thermoduric *Bacillus* species are frequently present during all manufacturing steps. Spoilage by *Bacillus*

species has been reported not only in raw milk but also in fluid milk (Magnusson et al., 2006, Scheldeman et al., 2006) and even in milk powders (Buehner et al., 2015). Such spoilage is mostly caused by the heat-stable lipolytic and proteolytic enzymes or post-process contamination of the products during handling (Coorevits et al., 2008). These organisms, essentially in the form of spores, can survive pasteurization, evaporation as well as spray drying temperatures, and are an important source of milk powder contamination (Kalogridou-Vassiliadou, 1992, Scheldeman et al., 2006). *Bacillus* spores are capable of surviving pasteurization treatments, which can reduce 99.9% of the vegetative cells but, provides a suitable environment for the subsequent germination and growth of spores which tend to form biofilms on the surfaces of processing equipment (Palmer et al., 2007). The endospores are formed as a defense mechanism to unfavorable environmental conditions which would hinder its growth/existence in the vegetative state and are resistant to most agents that would normally kill the vegetative cells they are formed from. In New Zealand milk powder plants, seven strains of thermophilic bacilli able to grow at 55˚ C or above have been identified, which included one strain each of *B. subtilis* and *Geobacillus stearothermophilus,* three strains of *Anoxybacillus flavithermus,* and two strains of *B. licheniformis* (Burgess et al., 2009). Similarly, ten *G. stearothermophilus* strains and one strain of *A. flavithermus* were isolated from powder manufacturing plant by Burgess et al., (2014). During milk powder manufacture, few strains of *Bacillus* can grow within the section of processing plant where the temperature for growth is ideal. Regeneration sections of heat exchangers and evaporators are the sections where operating temperature remains between 45 to 75˚ C, which favors the growth of thermophiles resulting up to 10^6 cfu/g in the milk powder (Scott et al., 2007). Three species of bacilli viz. *G. stearothermophilus, B. licheniformis and Anoxybacillus flavithermus* constituted 91.9 % of total bacterial population in milk powder

collected from 18 different countries and screened by a RAPD based survey (Rüeckert et al., 2004). In other studies, Rüeckert et al., (2005) and Rüeckert et al., (2006), reported seven strains of *Bacillus* that commonly occurred in milk powder. These were identified to be *G. stearothermophilus*, *A. flavothermus*, *B. licheniformis* and *B. subtilis* whereas, work done by Buehner et al., (2015) reported *B. licheniformis* as a most prevalent isolated species among Midwest manufacturing companies. NDM/SMP is used as raw ingredients in a variety of products like infant formulae, ice creams, yogurts, bakery, confectionery, etc., and all these foods provide perfect nutrients for the bacteria to proliferate and cause contamination. Therefore, it is important to control spores and sporeformers at initial stages of milk powder processing.

Taking an example of *B. licheniformis*, we hypothesized that different strain may behave differently in terms of germination, multiplication, and sporulation in during powder processing run especially during evaporation. If the sporulation and germinations sites are known during powder manufacturing, then those specific conditions can be optimized to reduce sporeformers and spores in final product. To understand the influence of processing conditions, current study analyzed the effect of evaporator and dryer at pilot scale on population dynamics of common sporeformers and spores. As a follow up to our previous study (Awasti et al., 2019), current study aims at comparing powder made from two different treatments, where control includes holding spiked raw milk at 10˚ C for 4 h and then at 7˚ C for up to 72 (as per PMO guidelines, FDA 2017), treatment 1 and 2 includes raw milk holding at 4˚ C for 24 h and 8˚ C for 72 h (Awasti et al., 2019). Milk from all the above-mentioned treatments and a control were HTST pasteurized, evaporated and dried for efficiently inactivating endospores and conveniently processing skim milk to get skim milk powder with low viable counts. Understanding the influence of powder processing conditions that favors either sporulation or germination of spores

can help understand the behavior of *Bacillus* species and help identify the steps responsible for the outgrowth of microbes. Such information can be helpful to control spores and sporeformers by using process optimization strategies.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial Strain Collection and its Growth Condition

This study included two *Bacillus licheniformis* cultures purchased from the American Type Culture Collection of Microorganisms (ATCC). Brain Heart Infusion (BHI; BD Difco™) media was used for both cultures, where *B. licheniformis* ATCC 14580 at 37˚ C. The cultures were preserved for future use by using protocol suggested by (Perry, 1998). The pellets of actively grown cultures were prepared at mid-exponential phase by centrifuging at 4500 × g for 30 min. Phosphate buffer saline (PBS), pH 7.4, was used to suspend pellets, and the cultures were preserved in 1.8 mL cryogenic vials (CRYOBANK™ – Copan Diagnostics Inc., CA, USA). For further use, the cryovials were stored at -80˚ C in a deep freezer (NuAire ultralow freezer, NuAire Inc. MN, USA).

4.2.2 Spore preparation

Endospores of *B. licheniformis* ATCC 14580 were prepared by using the method as described in our previous study (Awasti et al., 2019). Approximately 1.0 mL of actively growing broth culture of each of the spore-formers was spread-plated on the BHI plates. The plates were incubated at the appropriate incubation temperatures for up to 15 d. To monitor the level of sporulation, spore staining was performed occasionally throughout the incubation period and

after achieving 90% of sporulation, spores were harvested using a similar technique described by Wang et al. (2009).

4.2.3 Challenge Studies:

Challenge studies were conducted by aseptically spiking three storage silos containing skim raw milk (1500 lbs. each) with vegetative cells (approx. 4.0 log cfu/mL) of *B. licheniformis* ATCC 14580 at Davis Dairy Plant, South Dakota State University (SDSU). Based on our previous study (Awasti et al., 2019), two out of three silos were held at two different conditions i.e. for treatment 2, the spiked raw milk in tank A was held at 4˚ C for 24 h whereas, for treatment 3, the spiked raw milk in tank C was held at 8˚ C for 72 h before it is pasteurized, evaporated and spray dried to produce skim milk powder. Based on the Pasteurized Milk Ordinance (PMO, 2017) guidelines, control (Tank A, Treatment 1) was held after spiking at 10˚ C for 4 h with an extended holding at 7˚ C for 72 h before processing it to skim milk powder.

4.2.4 Sample Collection Points and Processing Conditions

Figure 4.1 outlines the points from where samples were collected during powder manufacturing at pilot scale. Raw milk (before and after spiking), intermediate (cavitated, pasteurized, evaporated) and powder samples were aseptically collected during all processing steps of treatment and control. Milk was pasteurized at 73˚ C for 15 s by using HTST pasteurizer at Davis Dairy Plant, SDSU. The pasteurized milk was evaporated to obtain approx. 42 to 45% total solids (TS). The temperature of evaporator was set at 65˚ C for both the effects and the flow rate of milk was adjusted accordingly. The evaporated milk samples were dried using Niro drier with outlet temperature of 200˚ C, an inlet temperature of approx. 95˚ C and flow rate of approx.

150-180 L/h. For microbial analysis, samples were collected aseptically in triplicates using sterile containers and were transported in a cooler and stored at 4˚ ± 0.5˚ C, until used. Table 5.1 and 5.2 details the list of sampling points which were collected during the different stages of powder manufacturing including cavitation, pasteurization, evaporation and spray drying using Niro dryer (Davis Dairy Plant, SDSU) with corresponding vegetative cells and spores.

4.2.5 Sample processing and Enumeration of Vegetative cells and Spores

Eleven grams of evaporated milk and powder samples were aseptically transferred to stomacher bag and 99 mL of warm sodium citrate (40˚ to 45˚ C) was used to wash off the sample from weighing dish to the stomacher bag and was blended for 2 minutes in stomacher bag. The samples were analyzed for vegetative and corresponding spore population after collecting them before and after each processing treatment. Spores were enumerated after heating samples to 80˚ C for 12 minutes, followed by cooling (Kent et al., 2016). Appropriately, diluted samples were pour plated using Brain heart infusion (BHI) agar. Plates were incubated at 37˚ C for 24–48 h, and plates with 25–250 colonies were enumerated for calculating total counts (Wehr and Frank, 2004).

4.2.6 Statistical Analysis

Additional statistical analysis was performed using SAS 9.3 software (SAS Institute Inc., Cary, NC) to see any significant difference between initial and final vegetative cells or spore counts of spiked raw milk. All the experiments were done in triplicates with three replicates. The significance level of the model was analyzed using ANOVA.

4.3 RESULTS AND DISCUSSION

4.3.1 Fate of vegetative cells and spores during powder processing

Before spiking, raw milk for all the treatments and control were assessed for the total number of vegetative cells (VC) and spore counts (SC) by using BHI agar pour plate method. The VC in raw milk ranged from 2.27 ± 0.13 to 2.39 ± 0.10 log cfu/mL whereas, SC were 1.18 ± 0.03 to 1.32 ± 0.03 log cfu/mL. Based on previous reports (Vithanage et al. 2016), total plate count in raw milk collected from dairy farms ranged from 1.17×10^4 cfu/mL to 1.34×10^5 cfu/mL. Therefore, we decided to spike raw milk with 4.0 log cfu/mL.

4.3.1.1 Changes in spores and vegetative cell population in treatment 1. Since it is a PMO recommended conditions, thus treatment 1 was considered as a control for this study. In comparison with initial spiking, log mean vegetative cells were significantly different after holding spiked raw milk samples for 4 h at 10˚ C and then for 72 h at 7˚ C (Table 4.1), these results are in accordance with our previous study (Awasti et al., 2019). Whereas, the mean spore count after 72 h at 7˚ C did not show any significant change when compared to spore counts after 4 h at 10˚ C holding (Table 4.2). This behavior of spores is may be due to their germination ability during 72 h of holding which showed a parallel significant increase in vegetative population from 3.69 ± 0.02 to 4.13 ± 0.02 log cfu/mL. After pasteurization and evaporation step, a significant drop in vegetative cells was evident with approx. 2.4 log cfu/mL reduction. Our findings in terms of vegetative counts after pasteurization are in agreement with previous studies (Scott et al., 2007) although the reduction in the current study was slightly higher as compared to <1 log cfu/mL. Several studies have reported an increase in vegetative and spore counts after evaporation (Scott et al., 2007, Murphy et al., 1999) whereas in the current study the

trend of spore increase follows the previous studies results. But for vegetative counts, we observed log reduction of approx. 0.6 logs which were not significantly different from that of pasteurized milk, which further validates the resistance of these strains towards high thermal treatments such as evaporation. Therefore, we suggest that raw milk treatment such as pasteurization and evaporation can lead to the survival of sporeformers and concentration of spores and can influence the quality of end-product. Studies have reported total spore counts in milk powder of approx. 1.10 to 3.40 log cfu/g (Kent et al., 2016) and 3.60 log cfu/g (Buehner et al., 2015). Whereas, for the current study, log mean spore and vegetative counts of the powder prepared from spiked milk resulted in 2.74 ± 0.03 and 1.03 ± 0.06 log cfu/g.

4.3.1.2 Changes in spore and vegetative cell population in treatment 2 and 3. Currently, dairy industries are more focused on attaining strategies to reduce sporeformers and spores during milk powder processing run. Therefore, we came up with an approach of holding raw milk at optimum time-temperature condition before it is processed to end-product (milk powder). To determine best raw milk holding condition at pilot level, VC and SC were compared after separately holding spiked raw milk at 4˚ C for 24 h and 8˚ C for 72 h respectively. Raw milk for Treatment 2 (T2) and Treatment 3 (T3) were spiked with vegetative cells of approx. 4.0 log cfu/mL and were 3.66 ± 0.03 and 3.62 ± 0.04 log cfu/mL at 0 h of spiking. No significant difference was observed between spiking levels of two different treatments. Although after holding raw milk at their respective conditions i.e. T2 for 24 h at 4˚ C and T3 for 72 h at 8˚ C, an increase in VC was evident. After holding, sporeformer population for T2 and T3 significantly increased to 3.84 ± 0.02 and 4.07 ± 0.08 log cfu/mL and their mean values were also significantly different with respect to each other. VC and SC showed totally different population levels and survival patterns. Both survived until the end of the process but, the numbers in milk

powder (end-product) varied between two treatments and a control. After witnessing VC/SC counts of powder samples from T2 and T3, no significant difference between two mean values was observed. Despite of holding raw milk at different conditions, no significant difference was found in VC and SC of milk powder made from two treatments.

Although after evaporation step, we observed an increase in spore numbers for both treatments with a parallel reduction in vegetative population and our results are in agreement with a previous study (Murphy et al., 1999) that also reported a similar trend of survival and growth of microbial population during powder processing run. The results and previous evidence suggest that the number of spores increase basically due to two major reasons; increase in spore concentration along with the concentration of milk, and favorable growth conditions within specific segments of a processing line that supported the sporulation of *Bacillus* strain during powder manufacturing cycles. The microbial population of vegetative and spore counts in powder prepared from treatment 2 resulted in 0.58 ± 0.04 and 1.82 ± 0.05 log cfu/g.

For all treatments, VC population showed reduced levels after pasteurization corresponding to 1.73, 1.66 and 1.26 log reduction respectively. Regardless of using different treatments, the vegetative population showed a similar trend of decline after pasteurization, evaporation and spray drying. Whereas, spore population for T2 and T3 after holding spiked raw milk, resulted in a significant increase from 1.56 ± 0.06 and 1.60 ± 0.01 to 1.79 ± 0.03 and 1.82 ± 0.04 and this increase in SC numbers also agrees with our previous study (Awasti et al., 2019). Whereas, VC/SC population from skim milk powder prepared from treatment 1, 2 and 3 resulted into increase in spore population by 6.2%, 17.4% and 38.2% with overall reduction of vegetative population by 99.9%, 99.9% and 99.7%. After spray-drying, the log means values of VC and SC

from T1, T3 and control ($p > 0.05$) suggested that most of the population in powder existed as spores. On an average, spore counts were found to increase significantly after the evaporation step. Of the two treatments and control powder samples, treatment 2 resulted in significantly lower spore counts then treatment 3 followed by control. Holding milk for 24 h at 4˚ C is an energy efficient process, so the current study suggests using low temperature holding of raw milk to produce a powder with reduced spores and sporeformers.

4.3.1.3 Optimized raw milk holding conditions produces milk powder with lower vegetative cells and spores: The powders prepared using three treatments showed the presence of some spores and vegetative cells, however, the holding time-temp combination of 4˚C for 24 h resulted in lower counts as compared to other treatments and the control. During powder, vegetative cells and spores survived until the end of the processes in all treatments, but their numbers varied. Vegetative cell population showed a decline after pasteurization, evaporation and spray drying, whereas, spore population resulted in significant increase throughout the processing steps. This increase in spore count is similar to a previous study (Murphy et al., 1999). A significant difference between mean values of vegetative and spore counts was observed in the powder samples obtained using treatment 1, 2 and 3. Among the treatments, treatment 2 resulted in lower spore counts as compared to treatment 1 and 3 (Table 4.1 and 4.2).

Log mean spore of the powder prepared after treatment 1 and 2 from spiked milk were 2.74 ± 0.03 and 1.82 ± 0.05 log cfu/g, respectively. Whereas, total vegetative cells were 1.03 ± 0.06 log cfu/g and 0.58 ± 0.04 log cfu/g, respectively. This shows that powder with reduced vegetative cells and spore counts can be prepared by just altering the raw milk holding conditions. After spray-drying, the log mean values of vegetative cells and spore counts from

both treatments ($p > 0.05$) suggested that most of the population in powder existed as spores. Overall, in the current study we did not observe the increased level of microbial numbers as in case of previous studies (Murphy et al., 1999, Scott et al., 2007), that reported thermophilic counts to a level of log 5 and 4.1 cfu/mL, respectively. The possible reason for low microbial build-up in the current study during processing run and, in the end-product may be due to reasons such as 1. The predominance of strain with mesophilic thermoduric characteristics spiked in raw milk, that may allow its survival during pasteurization but restrict its multiplication behavior with parallel spore formation during evaporation under thermal stress, 2. Short-time production cycle of approx. 7 hours (pasteurization to spray drying) may not have allowed biofilm build up and thus have shown the substantial reduction in microbial cells in milk powder. Burgess et al. (2010) reported that despite of low numbers in raw milk, improper CIP and poor sanitization of the dairy plants allow the retention and extensive multiplication of these bacteria within the processing system, especially in the longer processing cycles. This study is based on just one plant trial with shorter powder processing run of approx. 7 h, therefore, it may not explain the behavior of organisms during longer processing run. However, the current study provides a proof of concept that if proper cleaning in place (CIP), sanitation protocols, short powder processing runs are followed accompanied withholding raw milk at optimum time-temperature combinations is helpful in reducing the spores and growth of microbial numbers in milk powder manufacturing plants.

4.4 CONCLUSION

Our study compared the microbial population of different raw milk holding conditions throughout the processing run and concludes that combining optimum raw milk holding

conditions with that of shorter powder processing run can help reduce the spore population to significant numbers. Control of these spoilage-causing spore-forming organisms, during processing stages itself, is essential to produce final products with lower spore and sporeformer counts with extended shelf life attributes. Microbial population (vegetative cells/ spores) from skim milk powder prepared from treatment 2 resulted in reduced spores and vegetative numbers in comparison to treatment 1. Although the current study is based on just one plant trial with shorter powder processing run of approx. 7 h therefore, is a limiting factor which could not able to explain the behavior of organism during longer processing run. Based on these results, further studies can be expanded to look at more sporeformers and their strains at pilot scale. For future studies, the application of techniques such as filtration or cavitation should be included to evaluate the efficiency in elimination or reduction of spores in powder for longer powder processing run.

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Table 4.1: Vegetative counts during skim milk powder manufacturing at pilot scale

	Treatment 1 (Control)[1]	Treatment 2[1]	Treatment 3[1]
Before spiking	2.27 ± 0.13^{d}	2.39 ± 0.10^{c}	2.34 ± 0.14^{c}
After spiking	3.37 ± 0.03^{cA}	3.66 ± 0.03^{bA}	3.62 ± 0.04^{b}
10˚ C after 4 h	3.69 ± 0.02^{b}	-	-
4˚ C after 24 h	-	3.84 ± 0.02^{a}	-
8˚ C after 72 h			4.07 ± 0.08^{a}
7˚ C after 72 h	4.13 ± 0.04^{a}	-	-
After Pasteurization	2.36 ± 0.06^{dA}	2.11 ± 0.04^{cB}	2.41 ± 0.05^{cA}
After Evaporation	1.72 ± 0.07^{dA}	1.67 ± 0.03^{cB}	1.94 ± 0.09^{cA}
Powder	1.03 ± 0.42^{dA}	0.58 ± 0.04^{cB}	0.86 ± 0.16^{cB}

[1] values in table represents means ± standard errors of one trial and samples were analyzed in triplicates

Lowercase superscripts represents significance level checked for same treatment (along column)

Uppercase superscripts represent significance level checked within two treatments for same processing step (along row)

Table 4.2: Spore counts during skim milk powder manufacturing at pilot scale

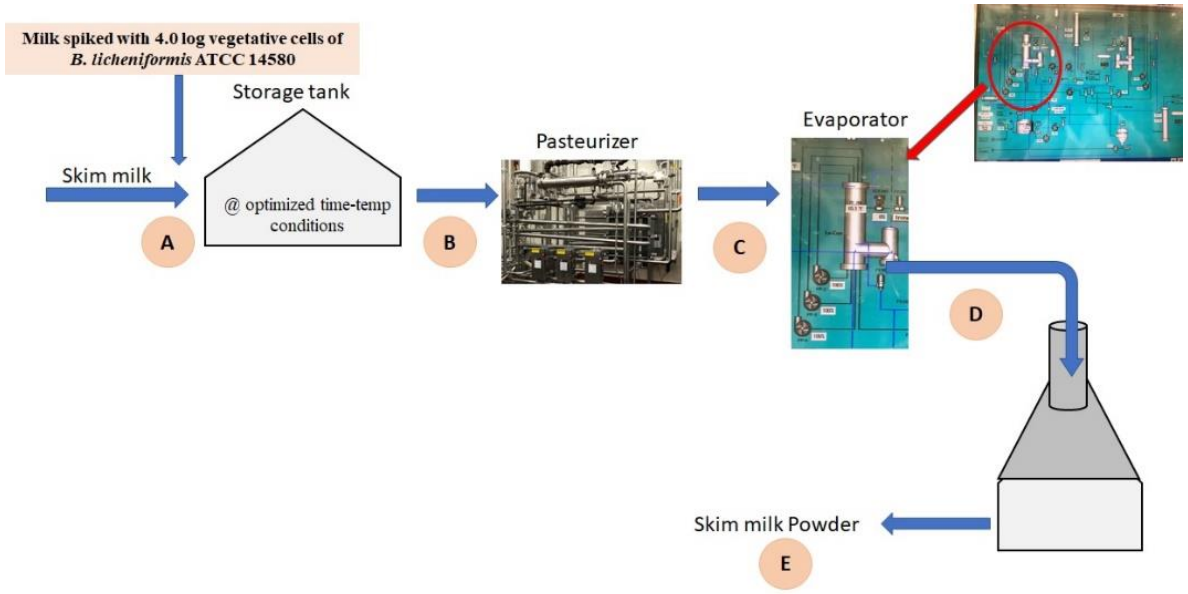
	Treatment 1 (Control)[1]	Treatment 2[1]	Treatment 3[1]
Before spiking	1.32 ± 0.03^{f}	1.19 ± 0.08^{c}	1.18 ± 0.03d
After spiking	2.18 ± 0.01^{e}	1.56 ± 0.06^{b}	1.60 ± 0.01c
10˚ C after 4 h	2.48 ± 0.03^{cd}	-	-
4˚ C after 24 h	-	1.79 ± 0.03^{a}	-
8˚ C after 72 h			1.82 ± 0.04b
7˚ C after 72 h	2.59 ± 0.05^{c}	-	-
After Pasteurization	2.46 ± 0.04^{dA}	1.19 ± 0.08^{cB}	1.31 ± 0.04cd
After Evaporation	2.81 ± 0.02^{aA}	1.88 ± 0.04^{aA}	2.11 ± 0.04a
Powder	2.74 ± 0.03^{bA}	1.82 ± 0.05^{aB}	1.90 ± 0.03b

[1] values in table represents means ± standard errors of one trial and samples were analyzed in triplicates

Lowercase superscripts represents significance level checked for same treatment (along column)

Uppercase superscripts represent significance level checked within two treatments for same processing step (along row)

Figure 4.1: Flow chart representing sampling points during powder processing run for treatment 1, 2 and control



Where,

A represents milk sample from storage tank after spiking,

B represents sample collected after holding,

C represents sample collected after pasteurization,

D represents sample collected after evaporation,

E represents sample collected after Drying

Chapter 5

Ratiometric Fluorescence Spectroscopy - A Novel Technique for Rapid Detection of Bacterial Endospores- A proof of concept

ABSTRACT

The current spore detection methods rely on cultural techniques, having limitations of time, efficiency, and sensitivity. Spore coat contains calcium dipicolinic acid (CaDPA) as a major constituent and can serve as a biomarker for bacterial endospores. We report a rapid and sensitive technique for the detection of bacterial endospores by using ratiometric fluorescence-based sensors. This method is based on the detection of CaDPA that enhances the luminescence of lanthanide ion, when complexed with a semiconducting polymer. A CaDPA standard curve was generated at an excitation-emission wavelength of λ_{275}-λ_{544} by using Synergy 2 fluorescence spectrophotometer. Intensity was recorded after chelating semiconducting fluorescent polyfluorene (PFO) dots with terbium ions, sensitized by different volumes of CaDPA (0.1μM). The standard curve so generated showed a linear relationship ($R^2 = 0.98$) in the experimental concentration range of 2.5 nM to 25 nM of CaDPA, with corresponding intensity (a.u.) of 545 to 2130. Endospores of aerobic spore former, *Bacillus licheniformis* ATCC 14580 were produced at 37˚ C for 15 days, on Brain Heart Infusion agar. The efficiency of sporulation was evaluated by spore staining and plating techniques. Total CaDPA content in spores was estimated after suspending reducing concentrations of spores (logs 9.0 through 1.0 cfu/mL, at one-log intervals) in HPLC grade water and raw skim milk samples. In HPLC grade water, for higher spore spiking levels such as 9.2±0.03,

8.4±0.05, 7.1±0.13 and 6.3±0.02 logs, the corresponding mean CaDPA values observed from the standard curve were 9.4, 7.2, 6.2 and 5.3 nM. Whereas, for lower levels of 4.2±0.05, 3.1±0.04, 2.0±0.11 and 1.36±0.09 logs, we observed 3.8, 3.3, 2.2 and 1.3 nM, mean CaDPA content. Whereas, for raw skim milk spiked with *B. licheniformis* ATCC 14580 spores, the mean CaDPA content on spores detected were approx. 2.5, 3.8 and 5.0 nM for spiking levels of 5.21, 6.39 and 9.47 log cfu/mL respectively. Trials were conducted in replicates of three and means were compared. Trials conducted using HPLC grade water indicated a linear relationship of the CaDPA content of endospores with that of the endospore counts and the standard curve of CaDPA concentration. Whereas, for raw milk spiked samples a reduced fluorescence detection was observed and was approx. five times lower as compared to the spiked samples of HPLC grade water. The reduced fluorescence ability in raw milk can be due to the turbidity of the solution or interference of proteins, amino acids and other ions of milk. This study provides a proof of concept for a potential application of this technique to rapidly detect bacterial endospores in the dairy and food industry. Further studies are required to remove the inference of ionic components in milk in order to rapidly detect spores in other dairy product matrices such as cheese, whey proteins, and powders.

5.1 INTRODUCTION

Spores are very resistant microbial structures that survive adverse conditions and can germinate when conditions are favorable. Detecting bacterial spores in dairy and food processing environments, water, dairy and food matrix can help reduce spoilage and shelf life issues in the final product. Aerobic sporeforming *Bacillus* species are known as a major contaminant in food and dairy industry (Seale et al., 2015). *Bacillus*, being a

second pre-dominant species, is frequently isolated from raw milk and responsible for a total 25% shelf life issues of milk and milk products in the United States (Sharma and Anand, 2002). Detection and enumeration of bacterial endospore concentrations are time-consuming tasks. Therefore, timely detection of sporeformers before milk processing is vital, to identify the source of contamination and strategies to reduce or control *Bacillus* build up. A number of methods has been developed during past decades including plating techniques, molecular and optical methods. The most frequently used methods of quantification of spores are microscopy and plate culture counting methods, which are slow, tedious and may take up to 72 hours for the results to be available (Sharma and Prasad, 1992, Rosen et al., 1997). Whereas, molecular methods usually require costly reagents and requires sample processing time before analysis. Thus, there remains a need for a simple and cost-effective method that can be used for rapid identification of aerobic bacillus spores in food and dairy matrix. From the past two decades, optical method for detection and enumeration of spores based on dipicolonic acid (DPA) have occupied a great deal of attention. Several techniques involving Raman spectroscopy, electrochemical methods and other spectrofluorometric method have been developed for detection of DPA (Zang et al., 2005, Tan et al., 2014). Most of the previous methods evaluated dipicolonic acid (DPA) as a spore marker (Rosen et al., 1997, Bell et al., 2005) with a detection limit of 2 nM DPA. Whereas, a study done by Hindle and Hall (1999) quantified spores by monitoring spore germination and exuded DPA and were able to detect up to 10^4 *B. subtilis* spores/ml.

In a hope to develop a rapid, sensitive and accurate method to quantify spores in a food matrix, the current study investigated CaDPA concentration in *Bacillus* spores using

ratiometric fluorescence technique. As a follow up to a previous study (Li et al., 2013), current investigation applied an optical method for detecting bacterial spores based on the detection of calcium dipicolinate (CaDPA), an important biomarker and major component of bacterial spores. Enhanced emission peak with bright luminescence was observed at 544 nm upon binding of DPA with lanthanide ion (Tb^{3+}), whereas emission peak at 439 nm remained stable without addition of DPA and thus served as an internal reference. This technique showed improved detection, sensitivity when spores were spiked in HPLC grade water, while spore spiked skim milk sample showed reduced fluorescence. Meanwhile, this study provides a proof of concept for the application of this technique to rapidly detect *Bacillus* spores in the dairy and food industry.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of Polymerdots (Pdots)

Functionalized Pdots was prepared by using the nanoprecipitation method as described in a previous study (Li et al., 2013). The stock solution with a concentration of 1 mg/mL was prepared by dissolving semiconducting polymer PFO (poly (9,9-dioctylfluorene)) and functional polymer PSMA (poly (styrene-co-maleic anhydride) in tetrahydrofuran (THF) respectively. PFO and PSMA were mixed and diluted with THF to produce a solution mixture with a PFO concentration of 50 μg/mL and a PSMA concentration of 10 μg/mL. The mixture was sonicated (frequency of 40-50 kHz, power 85% for 35 min at 25˚ C) by using bath sonicator to make a homogenous solution. After sonication 5 mL of this solution mixer was quickly added to 10 mL HPLC grade water. THF was removed by nitrogen stripping followed by filtration through a 0.2 μm filter.

The resulting functionalized Pdot dispersions are clear and stable for months without signs of aggregation.

5.2.2 Preparation of CaDPA

CaDPA solution was prepared by neutralizing reaction where a 3.3-mmol quantity of the DPA and an equal molar amount of calcium hydroxide ($Ca(OH)_2$) were separately dissolved in 50 mL of HPLC grade water and then the DPA solution was neutralized by dropwise addition of $Ca(OH)_2$ aqueous solution. The reaction solution was stored at 5˚ C for 48 h, followed by filtration and evaporation (115˚ C for 2 min).

5.2.3 Validation of the CaDPA Detection by Sensor

For CaDPA sensor detection, the reaction mixture was prepared by adding terbium chloride of 100 µl (0.1 mM TbCl3) to an aqueous solution of pre-prepared functionalized PFO dots (31.74 µl) to produce a solution with a Pdot concentration of about 80 pM and terbium concentrations of 1 µM, respectively. The solution was agitated for 5 min by using agitator. Then luminescence sensing experiments of the terbium chelated PFO dots was performed by adding different volumes of CaDPA (0.1 µM) to the terbium chelated Pdots solutions. Fluorescence spectra were measured using synergy 2 fluorescence spectrometer. First, a standard curve was made by using the different concentration of CaDPA at luminescence intensity of 544 nm (Figure 5.1 A) and then, ratios of intensities were plotted at I_{544}/I_{439} to generate CaDPA calibration curve (Figure 5.1 B). The next step was to validate the protocol by spiking spores in HPLC grade water. Once the validation was proved, a similar protocol will be followed to determine and quantify the spore present in raw skim milk samples.

5.2.4 Bacterial Strain Collection and Spore Preparation

This study included *Bacillus licheniformis* ATCC 14580, purchased from the American Type Culture Collection of Microorganisms (ATCC). *Bacillus licheniformis* ATCC 14580 was grown and incubated at 37˚ C in Brain Heart Infusion (BHI; BD Difco™). Endospores were prepared by using a method as suggested in our previous study Awasti et al. (2019). Approximately 1.0 mL of actively growing broth culture of the *B. licheniformis* was spread-plated on the BHI plates. The plates were incubated at the appropriate incubation temperatures for up to 15 d. To monitor the level of sporulation, spore staining was performed occasionally throughout the incubation period and after achieving 90% of sporulation, spores were harvested using a similar technique described by Wang et al. (2009). The endospores were preserved for future use by using protocol suggested by (Perry, 1998). For further use, the cryovials were stored at -80˚ C in a deep freezer (NuAire ultralow freezer, NuAire Inc. MN, USA).

5.2.5 Estimation of Total CaDPA Content of Spores Spiked in HPLC Grade Water and Skim Raw Milk Samples

HPLC grade water was purchased from Fisher's scientific whereas, skim raw milk samples were collected from Davis dairy plant (SDSU, SD, USA). The reducing concentrations of spores (logs 9.0 through 1.0 cfu/mL, at one-log intervals) were spiked separately in HPLC grade water and raw skim milk samples. The spiked samples were added to the reaction mixture by adding different volumes in it and were analyzed using synergy 2 fluorescence spectrometer. The samples were excited at a wavelength of UV range i.e. λ_{275} and fluorescence were read at two different intensities I_{544} and I_{439}. The ratios

of two intensities were separately plotted for both the samples (Figure 5.2 A and B). The mean CaDPA content in spore spiked HPLC grade water and raw skim milk samples were quantified using CaDPA calibration curve (Figure 5.1 A and B).

5.3 RESULTS AND DISCUSSION

Our strategy to quantify a total number of spores in ion-free water and raw milk is based on detection CaDPA content by using ratiometric fluorescence techniques, which were previously described by Li et al. (2013). Functionalized PFO dots were prepared using a fluorescent semiconducting polymer PFO and functional polymer PSMA as mentioned above. The terbium-dipicolinic acid (Tb-DPA) complex and PFO dots can be at the same time excited with wavelength of 275 nm without affecting the luminescence of each other. The detection of CaDPA content using fluorescence spectroscopic technique depends on common absorption peak (~275 nm) of CaDPA and semiconducting polymer PFO. In the current study long-pass filter was placed in front of detector in order to remove any interference from excitation at 275 nm as observed by previous report (Li et al., 2013).

5.3.1 CaDPA Detection and Calibration Curve

According to the previous study (Li et al., 2013) the sensitivity of CaDPA sensor was evaluated and their results showed a significant luminescence response of the sensor when CaDPA in the aqueous solution was excited at 275 nm. The emission spectra of PFO dots and CaDPA did not showed any interference with each other and their major emission peaks were at 439 nm and 544 nm. The current study validated the results from previous study by plotting the luminescence intensity with increasing CaDPA concentration. In terms of excitation and emission of CaDPA sensor, our results are in agreement with a

previous study (Li et al., 2013). CaDPA was detected and plotted at excitation-emission (λ_{275}- λ_{544}) wavelength by using Synergy 2 fluorescence spectrophotometer (Fig. 5.1A). A linear relationship ($R^2 = 0.98$) exist in our experimental CaDPA concentration range of 2.5 nM to 25 nM with corresponding intensity (a.u.) of 545 to 2130 nm.

Figure 1B explains the ratiometric calibration plot (I_{544}/I_{439}) of Pdot sensor as a function of CaDPA concentration. The ratios of two emission intensities were the highest emission peaks of Tb^{3+} excited in the presence of DPA and PFO dots. In terms of sensor sensitivity, our results agree with the previous report and limit of detection observed was approx. 0.2 nM. Figure 5.1B allowed us to create a link between CaDPA concentration and ratiometric intensity which was further used to calibrate total CaDPA content on spores spiked in HPLC grade water and raw skim milk samples.

5.3.2 Estimation of Total CaDPA content of spores spiked in HPLC grade water

Excitation of spore spiked HPLC grade water at 275 nm after adding it to reaction mixture of Pdots resulted into luminescence at two different intensities that is I_{544} and I_{439}. The ratios of these two intensities were plotted against spiked log spores cfu/mL, as shown in Figure 5.2. The graph so obtained was compared with calibration curve (Figure 5.1B) of CaDPA (ratiometric graph) to quantify the total concentration of CaDPA on spores. We observed that for higher spore spiking levels such as 9.2 ± 0.03, 8.4 ± 0.05, 7.1 ± 0.13 and 6.3 ± 0.02 logs, the corresponding mean CaDPA values observed from the standard curve were >25, >25, 25 and 20 nM. Whereas, for lower levels of 4.2 ± 0.05, 3.1 ± 0.04, 2.0 ± 0.11 and 1.36 ±0 .09 logs, we observed 15, 12, 2 and <2 nM, mean CaDPA content. Trials conducted using HPLC grade water indicated a linear relationship of the CaDPA content

of endospores with that of the endospore counts, and the standard curve of CaDPA concentration. The mean CaDPA content on spores ranged from approx. >25 nM to <2 nM. In a current study Liu et al., (2019) were able to detect the concentration range of DPA from 0.5 nM to 2.5nM using carbon dots as a binding polymer. With respect to our experiment, enhanced sensitivity of this technique was due to presence of more DPA binding sites on carboxyl and amide groups of carbon dot. Therefore, after comparing these two studies we conclude that, sensitivity of probe can be enhanced by increasing DPA binding sites.

5.3.3 Estimation of Total CaDPA content of spores spiked in raw milk

To demonstrate the robustness of this detection method, we conducted spore detection in raw milk samples. Different logs count of *B. licheniformis* ATCC 14580 spores were added into the reaction mixture of Pdots solution. The mean CaDPA content on spores detected were approx. <2, ~2.5 and 5.0 nM for spiking levels of 5.21, 6.39 and 9.47 log cfu/mL respectively (Figure 5.2 B). We were not able to detect intensities below the spiking levels of 5.21 log cfu/mL. We observed a reduced fluorescence detection was approx. five times lower as compared to the spiked samples of HPLC grade water. Recently (Yilmaz et al., 2018), a combination of Eriochrome Black T (EBT)- Eu^{3+} has been applied to detect DPA. The sensitivity of system for detection of *Geobacillus stearothermophilus* spores was as low as 2.5×10^5 and is in accordance with our spore detection limit in raw milk. The reduced fluorescence ability in raw milk can be due to the turbidity of the solution or interference of proteins, amino acids and other ions present in milk. Sensor with combined attributes of calorimetric and fluorescent detection of DPA using (EBT)- Eu^{3+} is emerging as new technique, due its enhanced sensitivity and detection and thus can be used

in combination with our technique. To improve the ability of fluorescence sensor, further strategies should be combined with that of other techniques by using binding ions such as Eu^{3+}, carbon dots (with carboxyl and amide ligands) and enzymes technology to remove interfering components such as proteins and other ions.

5.4 CONCLUSIONS

In summary, we have demonstrated that spores in raw milk can be detected and quantified by using ratiometric fluorescent detection technique. This study provides a proof of concept for a potential application of this technique to rapidly detect bacterial endospores in the dairy and food industry. Preliminary studies with *Bacillus licheniformis* spores in raw milk samples showed that about 5.21 log spores/mL can be determined with our method. Further studies are required to remove the inferencing ionic components in milk in order to improve the efficiency of the sensor to rapidly detect spores in other dairy product matrices such as cheese, whey proteins, and powders.

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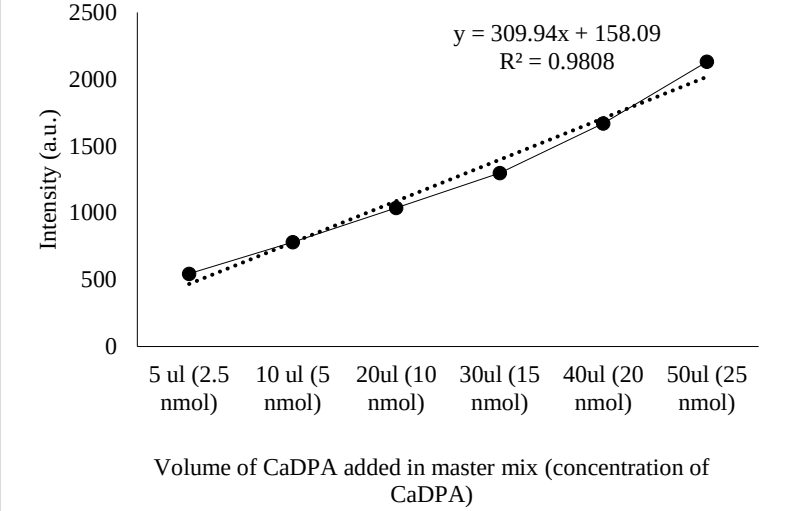
Figure 5.1: (A) Plot of luminescence intensity change (544 nm) of Pdots sensor with increasing concentration of CaDPA; (B) ratiometric calibration (I_{544}/I_{439}) of the Pdot sensor as a function of CaDPA concentration.

A



B

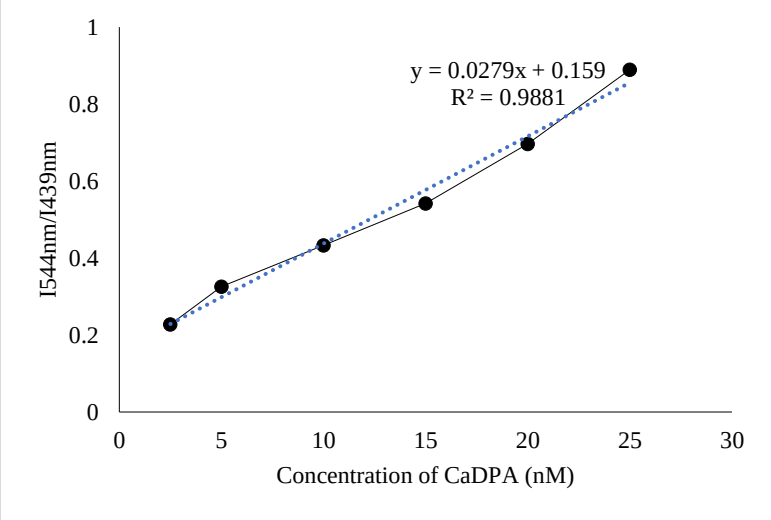
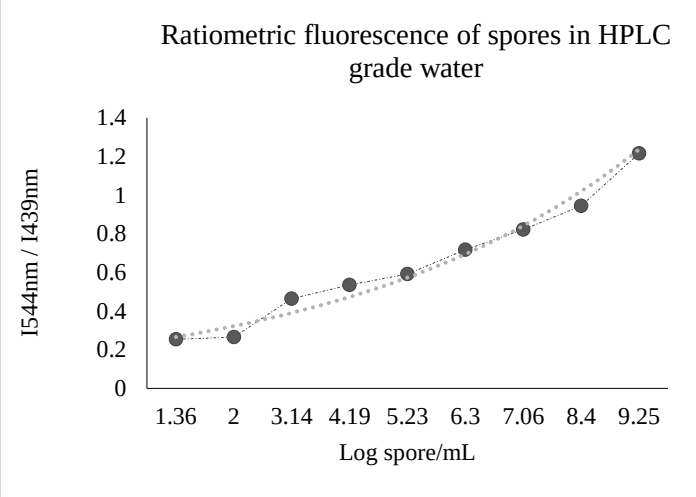
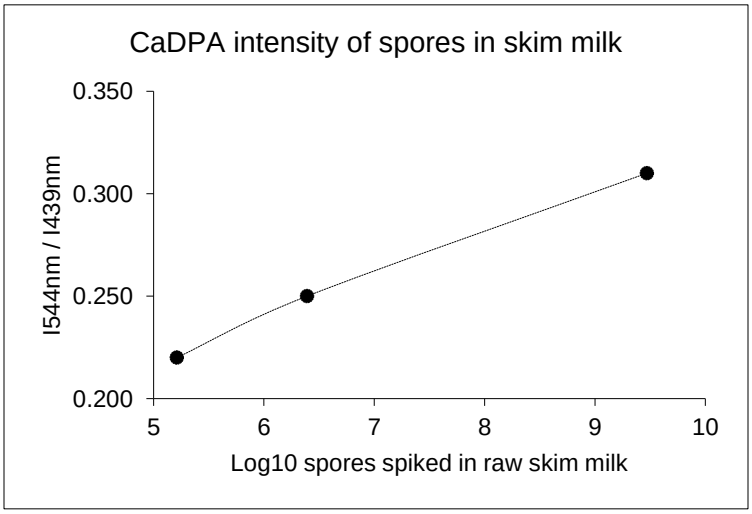


Figure 5.2: Ratiometric calibration plot (I_{544}/I_{439}) of spore spiked (A) in HPLC grade water; (B) in skim milk samples using Pdot sensor as a function of CaDPA concentration

A



B



Overall Summary

Results from refrigeration holding of raw milk suggested that for vegetative cell spiking study of *B. licheniformis* ATCC 6634 (S1), cell population changes remained below 1.0 log up to 72 h at 8˚ C. Whereas, for *B. licheniformis* ATCC 14580 (S2), 1.0 log shift was observed only after 80 h at 8˚ C, indicating greater multiplication potential of S1 as compared to S2. As S2 was a readily sporulating strain, the vegetative spiking study showed spore formation at different storage temperatures. Evidence of some parallel germination was observed for this strain at 8˚ C or higher, when raw milk samples were spiked with spores. Sporulation behavior, germination and multiplication ability, strain variability, and temperature and duration of holding raw milk influenced the population dynamics of *Bacillus* species. In the presence of equivalent numbers of both types of sporulating strains in raw milk, despite of strain variability, holding the milk at 8˚ C for not more than 72 h would keep any cell population changes below 1.0 log. As holding raw milk for up to 72 h at 8˚ C would not allow the increase in population by 1.0 log therefore, further studies were done using raw milk holding time-temperature of 4˚ C for 24 h as an optimum condition to enumerate the influence of these conditions during powder processing steps. In addition, under these storage conditions, the population would remain as vegetative cells that are easily inactivated by pasteurization.

Based on results from two studies, we evaluated that amoung two strains, BL ATCC 14580 produces more spores in raw milk held at lower temperatures. Therefore, further studies were done using high sporulating strain of this species. In terms of survivability, germination, multiplication and sporulation, behavior of BL ATCC 14580 was evaluated during skim milk powder manufacturing at pilot scale. Skim milk powder

prepared using PMO recommended (treatment 1) and optimum holding (treatment 2) conditions at the pilot scale showed different survival patterns of vegetative cells and spores during each processing step. Among two treatments, treatment 2 resulted in lower spore counts when compared to treatment 1. The overall final spore and vegetative cell counts in the powders manufactured under optimum holding condition (4˚ C for 24 h) were found to be significantly lower (0.58 ± 0.04 and 1.82 ± 0.05 log cfu/g) as compared to the conditions likely to practice by dairy plants (2.74 ± 0.03 and 1.03 ± 0.06 log cfu/g). This shows that milk powders with reduced vegetative and spore counts can be prepared by optimizing the raw milk holding conditions. Overall, we did not observe the increased level of microbial numbers and the possible reasons for the low microbial build-up during processing run and in end-product is may be due to two reasons i.e. 1. The predominance of strain with mesophilic thermoduric characteristics spiked in raw milk, that may allow its survival during pasteurization but restrict its multiplication behavior during evaporation under thermal stress, 2. Short-time production cycle of approx. 7 hours (pasteurization to spray drying) may not have allowed biofilm build up and thus have shown a substantial reduction in vegetative cells in milk powder. Although this study was based on just one plant trial with shorter powder processing run of approx. 7 h therefore, is a limiting factor which could not able to explain the behavior of organism during longer processing run. Thus, a current study provides a proof of concept that if proper cleaning in place (CIP), sanitation protocols, short powder processing runs are followed accompanied with holding raw milk at optimum time-temperature combinations is helpful in reducing the spores and growth of microbial numbers in milk powder manufacturing plants.

For ratiometric fluorescence studies, standard curve so generated showed a linear relationship ($R^2 = 0.98$) in the experimental concentration range of 2.5 nM to 25 nM of CaDPA, with corresponding intensity (a.u.) of 545 to 2130 nm. We report a validation of rapid and sensitive technique for the detection of bacterial endospores by using ratiometric fluorescence-based sensors. In HPLC grade water, for higher spore spiking levels such as 9.2±0.03, 8.4±0.05, 7.1±0.13 and 6.3±0.02 logs, the corresponding mean CaDPA values observed from the standard curve were >25, >25, 25 and 20 nM. Whereas, for lower levels of 4.2±0.05, 3.1±0.04, 2.0±0.11 and 1.36±0.09 logs, we observed 15, 12, 2 and <2 nM, mean CaDPA content. Whereas, for raw skim milk spiked with *B. licheniformis* ATCC 14580 spores, the mean CaDPA content on spores detected were approx. 2.0, 2.5 and 5.0 nM for spiking levels of 5.21, 6.39 and 9.47 log cfu/mL respectively. Preliminary studies with *Bacillus licheniformis* spores in raw milk samples showed that about 5.21 log spores/mL can be determined with our method. For raw milk spiked samples, reduced fluorescence detection was observed and was approx. five times lower as compared to the spiked samples of HPLC grade water. The reduced fluorescence ability in raw milk can be due to the turbidity of the solution or interference of proteins, amino acids and other ions of milk. Further studies are required to remove the inference of ionic components in milk in order to rapidly detect spores in other dairy product matrices such as cheese, whey proteins, and powders. Moving forward, to improve the sensitivity of sensor, ratiometric technique can be combined with other methods such as calorimetric, protein digestion (by using proteolytic enzymes) and protein/ peptide separation (using filtration technology).

Conclusions

The current study concludes that *B. licheniformis* strains differ in the ability to multiply, sporulate, and germinate in raw milk during refrigeration storage and drying temperatures, and thus may significantly influence the keeping quality of raw milk and milk powder. Control of these spoilage-causing organisms, before processing stages itself, is essential to produce milk powder with lower spore and sporeformer counts and extended shelf life attributes. Our study came up with regression equations that can be applied to predict the individual strain population numbers for specific temperature and duration of raw milk holding. This increases our ability to create predicted values for vegetative cell population growth at a wide time-temperature range, which can be easily generated using regression equations. Even in the case of mixed types of sporulating strains, holding raw milk at a common time-temperature of 8˚ C for no more than 72 h would ensure that the individual population does not change by 1.0 log cfu/mL. The present study used regression generated time-temperature combinations based on contour plot prediction of raw milk holding and powder prepared showed reduced levels of sporeformers and spores. Thus, the current study demonstrates that different strains of *B. licheniformis* may have variable potential to multiply and sporulate during refrigerated holding but using optimized conditions generated through regression models can help reduce the spore and sporeformer population to significant levels. Using such optimization techniques at farm and plant scale would prove useful for the dairy industry, and thus in future can be used as a strategy for shelf life determination of raw milk and other milk products. Based on these results, further studies can be expanded to look at other sporeformers and their strains. The application of regression models may also be combined with product processing techniques such as

filtration and cavitation, especially during longer powder processing runs, to produce powders or dairy products with improved microbial quality. In addition, this study also provides a proof of concept for a potential application of ratiometric fluorescence spectroscopy technique to rapidly detect bacterial endospores. Further studies are required to standardize the protocol for application in milk and related dairy products.