IDENTIFICATION OF SPOREFORMING BACTERIA IN THE MILK CHAIN AND ITS POTENTIAL SOURCES IN FARM ENVIRONMENT

by

Bismarck Antonio Martínez Téllez

A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor in Philosophy

Major: Food Science and Technology

Under the Supervision of Research Assistant Professor Andreia Bianchini

Lincoln, Nebraska

April, 2016

IDENTIFICATION OF SPOREFORMING BACTERIA IN THE MILK CHAIN AND ITS POTENTIAL SOURCES IN FARM ENVIRONMENT

Bismarck A. Martínez Téllez, Ph.D.

University of Nebraska, 2016

Advisor: Andreia Bianchini

The dairy industry is recently facing a quality problem due to the presence of sporeformer bacteria in different products. These bacteria are members mainly of the *Bacillus* taxonomic group and due to their abilities to survive pasteurization, can not be controlled by this process technology. Consequently, this bacteria population affects the quality of some products during storage (e.g. fluid milk) and limit their potential markets (e.g. milk powder). Since no technology/interventions are currently available to eliminate this bacteria type once they enter the milk chain, the identification of their entry points and sources is essential information to design potential interventions to control them at early stages. In order to address this issue, this research focused on identifying those "problematic" sporeforming bacteria across the milk chain (from sources at farm level, raw milk, fluid milk and condensed milk) using DNA-based methods (*rpoB* or 16S). Moreover, associations affecting their entrance into the milk chain were also studied, including weather and potential farm practices. The identification of problematic sporeformers suggests that *Paenibacillus* spp. are responsible for spoilage of fluid milk due to their ability to grow and survive the processing conditions encountered during pasteurization and condensation. Other *Bacillus* species found in condensed milk include: *B. claussi*, *B. subtilis*, *Lysinibacillus* sp., *B. safensis*, *B. licheniformis*, *B. sonorensis* and *Brevibacillus* spp. These last three species are capable of growing at thermophilic

temperatures (55 °C) being potential problematic for the milk powder industry. Therefore, contamination of other dairy products could occur when condensed milk is used as ingredient. When evaluating the raw milk at the farm level, no seasonal effect was associated with the prevalence of sporeformers bacteria; however, farms seems to have an effect. Among the sources of problematic strains at the farm, it was found that milking equipment and cow teats are associated with a wide variety of psychrotrophic and thermophilic problematic strains, suggesting that any interventions at farm level should be targeting these areas. By better understanding the potential sources, scientists and farmers could design and implement suitable interventions to decrease sporeformer counts in raw milk improving the quality of dairy products.

ACKNOWLEDGMENTS

I would like to acknowledge my committee members for their expert advices throughout my career at University of Nebraska. I will like to give special thanks to Dr. Andreia Bianchini and Dr. Jayne Stratton for being my direct supervisors during my Doctorate and Master degrees. I would also like to extend my appreciation to Dr. Rolando Flores, Dr. Robert Hutkins and Dr. John Rupnow for their academic support and advices beyond class periods.

I would also like to thanks the farmers and condensed milk plant employees in Nebraska that help me during this project, without their support during samples collection, this project would not be possible. I deeply appreciate the funding entity, Midwest Dairy Association (MDA) for their economic support. I would like to show my appreciation to my lab-mates: Luis E. Sabillón, and especially to Maricarmen Estrada Anzueto.

Finally, I will like to thanks my parents; Nora and Guillermo Martinez, for their support and sacrifices during all these years when persuading my greatest academic achievement. I can never thanks enough to my wife, Yulie E. Meneses, for her unconditional love and support that she offers every single day of my life.

PREFACE

The present doctoral dissertation is focused on providing useful information about the prevalence of sporeformers in the milk chain, specifically in the state of Nebraska. This information is divided in five chapters. Chapter 1 provides a review of the current literature on sporeformers and their importance for the dairy industry. Chapter 2 describes the sporeforming bacteria population collected from a condensed milk plant and the identification of strains with spoilage characteristics. Chapter 3 focuses on the identification of sporeformer population from environmental samples at farm level and the assessment of potential transmission routes into raw milk. Chapter 4 examines the seasonal effect on the prevalence of problematic sporeformers in raw milk throughout the year. Chapter 5 presents a summary of the major findings from previous studies including studies on farms, pasteurized and condensed milk plants. Results of this chapter provided a clear picture of the sporeformers bacteria population in Nebraska, and established the basis to design potential strategies for the effective control of sporeformers in the milk chain.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1.

CHAPTER 2.

CHAPTER 3.

CHAPTER 4.

CHAPTER 5.

LIST OF FIGURES

CHAPTER 1.

CHAPTER 2.

CHAPTER 3.

CHAPTER 4.

CHAPTER 5.

CHAPTER 1. SPOREFORMERS, A RECENTLY RECOGNIZED PROBLEM IN THE DAIRY INDUSTRY

CHAPTER 1. SPOREFORMERS, A RECENTLY RECOGNIZED PROBLEM IN THE DAIRY INDUSTRY

1. INTRODUCTION

Milk is an important agricultural commodity that is susceptible to microbial spoilage due to neutral pH, presence of sugars available for bacterial metabolism, proteins and fat. Even though important improvements in the dairy industry have been successfully developed to improve the shelf-life of dairy products, from farm level to processing, further extending the shelf life of these products is still an industry goal. In fact, dairy processors and producers look forward to extend the shelf life of dairy products aiming to expand their markets, nationally and internationally.

Historically, Gram negative bacteria and lactic acid bacteria have been responsible for milk spoilage causing milk to acidify at farm level (Samaržija* et al., 2012). Trying to overcome this problem, farmers and dairy processors developed a cold storage procedure that is now widely applied at farm levels, being at this point easy to preserve the quality of the raw milk by reducing the bacterial growth rate. Psychrotrophic microorganisms became then the limiting factor, which was overcome by increasing sanitization procedures and more frequent pick-up time schedule for the milk at the farm (Samaržija* et al., 2012). According to the Pasteurized Milk Ordinance (PMO) in the United States (FDA, 2011), raw milk should be cooled below 10°C within 4 hours of starting the milking protocol; and it should be below 7°C within two hours after milking is finished. Storage temperature of ≤4 °C is recommended, especially when milk will be stored for as long as 2 days, to reduce the growth of psychrotrophic bacteria (Chambers, 2002).

Nowadays, the new microbial challenge is the presence of sporeforming bacteria. The main reason why these sporeformers are a problem is their ability to survive the pasteurization process (i.e.72°C for 12s). Among microorganisms associated with milk, at least two important taxonomic groups: aerobic bacteria belong to the *Bacillus* group and their anaerobic counterparts *Clostridium* group. Both groups are Gram positive bacteria that in certain conditions have the ability to form a spore, a structure that resists extreme conditions including heat, pressure, among others. Another important characteristic is that both sporeforming bacteria groups have the ability to produce quality defects in dairy products when germination occurs.

Clostridium spp. are associated with quality defects, mainly caused by lipolysis and gas production during maturation of certain cheeses, affecting economically this dairy sector. In fact, it has been described that two different species of this genus named *C. tyrobutyricum* and *C. beijerinckii* are the main ones associated with cheeses with defects (Le Bourhis et al., 2007).

The *Bacillus* genus is a widely diverse group of microorganism, and it has been well known for its uses in the pharmaceutical industry. This genus is widely distributed in multiple environments; therefore, it has been considered by microbiologist as ubiquitous organisms. This chapter will overview the current taxonomy of this genus and its challenges in classification, the spore-formation process, their ability to survive during processing of dairy products, and finally its role in affecting the quality and safety of dairy products.

1.1 Taxonomy classification of *Bacillus* group

Endo-sporeformer bacteria are taxonomically located in the Bacteria kingdom, and part of the Firmicutes phylum. The *Bacillus* group is a widely diverse taxonomic group, and some of the strains show phenotypic and metabolic diversity such as: high temperature, high saline concentrations, resistance to acidic conditions, among others (Maughan and Van der Auwera, 2011). These characteristics allow them to survive in different environmental conditions, granting them a status of ubiquitous organisms, often found in soil, freshwater, saline water, plant, animals and even air (Maughan and Van der Auwera, 2011).

Historically, the *Bacillus* group was first described by Ferdinand Cohn and Robert Koch in the 19th century and include two main species such as *B. anthracis* and *B. subtilis* (Maughan and Van der Auwera, 2011). It was assumed that most of aerobic sporeforming bacteria were part of this group. Later, scientists developed a description based on morphology and some other characteristics which lead to a classification of 22 different species (Fritze, 2004).

Advances in molecular biology, physiology and biochemical testing, allowed scientists to provide the first characterization of those species in this genus. Unfortunately, some of the biochemical techniques were difficult to standardize among laboratories, being a limitation for its implementation; and therefore, these techniques were not widely adopted by the scientific community. In any event, these methods do not necessary provide accurate evolutionary data that could be used for relationship purposes. In fact, in 1980, different experts in microbial taxonomy defined more species into the *Bacillus*

group. They described at least 31 species and provided the addition of new genera in this group (Fritze, 2004). Some of these new genera include: *Sporolactobacillus, Sporosarcina,* and *Thermoactinomyces*.

Therefore, it was not until the development of 16S RNA/DNA sequencing that scientists finally could determine clearly the relationship between some *Bacillus* species. It was discovered at that time that phenotypical characteristics (first used to categorize them as species) did not necessary correlate with genotypic profile, resulting in the big challenge that is the taxonomic classification of this group. These new techniques also led to the separation of organism in more genera, as well as different allocations in the taxonomy tree for some strains (Fritze, 2004). Indeed, the *Paenibacillus* genus was only proposed in 1994 by the use of PCR and molecular techniques. Currently, after other taxa were added to this new list of sporeformers, a total of 18 genera are counted called part of the *Bacillus* group; among those: *Alicyclobacillus, Brevibacillus, Geobacillus, Lysinobacillus, Ureibacillus* and *Vigibacillus* (Postollec et al., 2012).

Even though the use of 16S RNA/DNA is considered a key standard method for the new taxonomy, some strains are too closely related; being this technology insufficient to separate them (Maughan and Van der Auwera, 2011). In fact, it is considered that two bacterial species are different, when they usually show a 3% divergence or greater using 16S RNA/DNA portion, being this value a conservative cutoff in most taxonomic groups (Cohan, 2002). A more recent study suggested that at least 116 species were differentiated using the 16S RNA of *Bacillus* species from 7510 sequences using a cutoff value of 97% identity (or 3% divergence) (Maughan and Van der Auwera, 2011). A brief

overview of those species contained in this genus are shown in Figure 1; with about 59 species.

On the contrary, well-known species that were easily separated by phenotypic characteristic (such as *B. thuringiensis*, *B. cereus* and *B. anthracis*) may not have divergence (larger than 3%) using their genetic material; and in fact, only 1 or 2% divergence was found using 16S RNA portion (Vilas-Boas et al., 2007). Therefore, if a small cutoff value is used to differentiate this genus, an overwhelming number of species could be generated. There is still not a consensus in the scientific community at this moment, regarding the definition of species level in the *Bacillus* genus.

Beyond the taxonomic issues encountered with this genus (as described), there are at least two groups to which the scientific community pays more attention: *Bacillus subtilis* group and *Bacillus cereus sensu lato* (Fritze, 2004). These two groups contain a widely variety of microorganism in the pharmaceutical industry, such as *Bacillus subtilis*; and the group of pathogenic bacteria, including *B. cereus* (for humans), *B. anthracis* (for bovine), and *B. thuringiensis* (for insects).

Bacillus cereus sensu lato is complex taxa and has been described containing the following species: *B. cereus sensu stricto, B. mycoides, B. pseudomycoides, B. thuringiensis, B. weihesterphanesis* and one of the most recently added *B. cytotoxicus* (Guinebretière et al., 2013, Dréan et al., 2015). This *B. cereus sensu lato* contains highly similar strains by the 16S rRNA gene sequences and it has been determined that horizontal transfers of plasmid genes further complicate their differentiation (Fritze, 2004, Dréan et al., 2015). This taxa is of importance for the scientific community because

at least 3 species have hemolytic capabilities and Penicillin-resistance (*B. cereus*, *B. thuringiensis*, and *B. mycoides*) (Fritze, 2004). Some of the *Bacillus* species found in milk are part of this taxonomic group including *Bacillus weihenstephanensis* with importance in the fluid milk industry due to its ability to grow under refrigeration temperature (6°C) (Ivy et al., 2012). This organism will be described in a greater detail in this manuscript and its potential to produce illness which is not clear at this point.

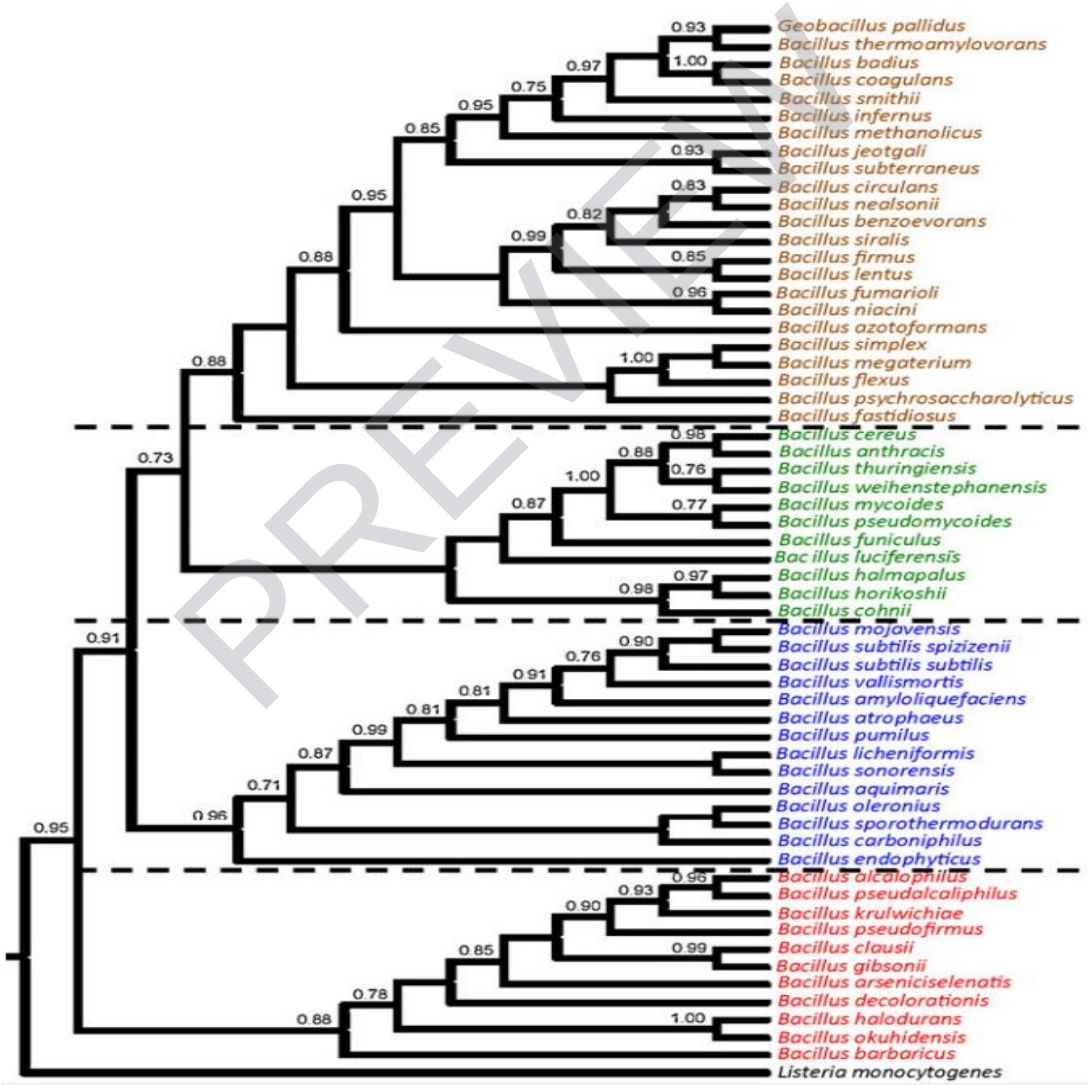

Figure 1. Evolutionary relationships of 59 *Bacillus* species using Maximum likelihood of the 16S rDNA locus portion described and adapted from Maughan and Van der Auwera (2011).

1.2 Subtyping methods for sporeforming bacteria

In order to subtype *Bacillus*, researchers have developed powerful molecular methods that are economically feasible and allow not only for the determination of genus and species, but also characterization at subspecies (or strain) level. As it was previously described, 16S portions within the *Bacillus* genus are extremely homologous; therefore, hyper-variable regions between the *Bacillus* strains are required to differentiate them. These sections are mainly housekeeping genes, such as *rpoB* and *gyrB.* In fact, at this point, the use of subtyping methods is the only option available in order to trace back and understand how these sporeforming bacteria may enter the dairy chain.

The *rpoB* gene is present as a single-copy in prokaryotes, and has been used to characterize close related species (Case et al., 2007). This gene encodes a section of β-subunit of RNA polymerase, which is a crucial enzyme in the bacterial transcriptional process, being responsible for mRNA, rRNA and tRNA (Adékambi et al., 2009). Among the housekeeping genes present in bacteria, the *rpoB* gene has been described as a good ecological chronometer that could be used for bacterial phylogenetic analysis (Adékambi et al., 2009). This subtyping method has been recently developed for *Bacillus* strains found in the milk chain and it has been used in order to track-down contamination (Durak et al., 2006). In fact, the same research group showed that after analyzed dairy *Bacillus* strains, they obtained better yields and quality on their results, with the *rpoB* when compared to other housekeeping genes (*gyrB*, *tuf* and *cpn60*) (Durak et al., 2006).

The *gyrB* gene has also been used to subtyping *Bacillus* strains. This gene encodes DNA gyrase enzyme (subunit B) and plays an essential role in replication, presenting high-

resolution power in *Bacillus pumilus* and *Bacillus safensis* (Liu et al., 2013). This gene is found in a single-copy, as well as *rpoB* gene in prokaryotes cells. Therefore, low copies per cell are a limitation factor for the use of both genes. Some studies on *Bacillus* spp. have used *gyrB* gene, but this analysis is not often used to study milk sporeforming bacteria present in dairy environments. Consequently for comparison purposes with previously reported sequences in databases, this gene might provide limited results.

2. SPORE-FORMATION PROCESS

In order to fully understand these microorganisms, it is necessary to know the functionality of spores and its structure. *Bacillus subtilis* is a Gram-positive, catalase-positive bacterium with the ability to produce endospores, and it has been well studied as sporulation model for the *Bacillus* group. As a brief introduction of this process, starvation induces endospores formation; which is primarily sensed by carbon, nitrogen, and phosphorous sources (Piggot and Hilbert, 2004). This section will describe the complex process of sporulation, the spore structures and its functionalities, concluding with the genes and regulators involved with spore formation.

2.1 Sporulation process

The sporulation process starts when a bacterium form a pre-divisional cell, which contains at least two complete chromosomes (Wang et al., 2006). The first step in the sporulation process is called “axial filament”, in which two chromosomes align across the bacterium cell. Then a division inside of the cell (septum) is formed, in one of the polar positions (Figure 2- I) (Stragier and Losick, 1996). This division is asymmetrically near to one pole; the mother section of the division is called sporangium. The second section is called pre-spore (forespore-smaller section) (Figure 2-II). Scientists have been discussing that the sporulation septum is similar, but not identical to a cell division; the latter contained a thinner peptidoglycan layer that separates the two compartments (Stragier and Losick, 1996). After this division is performed, the mother cell engulfs the pre-spore section (after approx. 1 hour) and keeps it inside of the mother cell. Due to this step in spore formation, early scientists named as endospore (spore-inside). During the later stages, other changes include the production of small acid soluble proteins (SASP) in the forespore site and the substitution of water for calcium dipicolinate (DPA) occur (Wang et al., 2006). Other membranes are formed on top of the engulfed forespore, including cortex, inner and outer layer (Figure 2-III-IV-V-VI) (Stragier and Losick, 1996). After a maturation period, 6-8 hours, mother cell is lysed and the spore is now free (Figure 2- VII) (Popham, 2002).

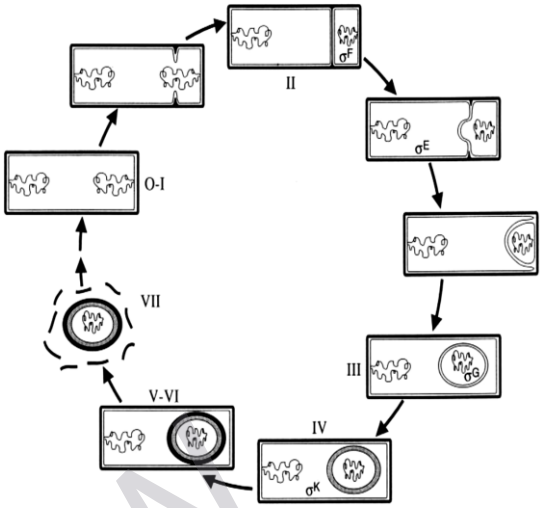

Figure 2. Sporulation process. From Stragier and Losick (1996)