MEMBRANE RIGIDITY IN VEGETATIVE CELLS AND SPORES OF *BACILLUS SPECIES*

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ABSTRACT OF THE THESIS

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The Genus *Bacillus* contains Gram-positive, aerobic spore-forming bacteria. Some *Bacillus* species are a big concern for the food industry due to financial losses from spoilage. Relatively little is known about how their spore structures contribute to their physiological characteristics. As an important interface between the cytoplasm and the outer environment, membranes play vital roles in maintaining cell survival and growth. This study was undertaken to determine the membrane rigidity of vegetative cells and spores among *Bacillus* species. *B. subtilis, B. cereus, B. anthracis Sterne, B. mycoides, B. thuringiensis* that were grown and harvested under the same conditions. 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) was used as a fluorescent probe in the measurement of cell membrane rigidity. There were significant differences in the vegetative cell membrane rigidity among these five *Bacillus* species when they were grown on three different media (Luria Broth, Nutrient Broth and Luria Broth Noble Agar). There were also significant differences in the spore membrane rigidity among the three *Bacillus* Species. Significant

differences also existed in the membrane rigidity between the vegetative cell membrane and the spore membrane for *Bacillus subtilis* and *Bacillus anthracis Sterne*. This represents the first report regarding spore membrane rigidity and its relationship to the rigidity of vegetative cell membranes among *Bacillus* species.

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Dedication

I would like to dedicate this thesis to my beloved family for their love, help and support.

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List of Abbreviations

ATCC	American Type Culture Collection
DPA	Dipicolinic acid
FA	Fatty acid
HPP	High Pressure Processing
DPH	1, 6-diphenylhexa-1, 2, 3-triene
THF	Tetrahyrafuran
NB	Nutrient broth
LB	Luria broth
PBS	Phosphate buffered saline
LBNA	Luria broth noble agar
LBGA	Luria broth granulated agar

Chapter I - The Literature Review

I.1. The Genus *Bacillus*

After changing the name of Ehrenberg's (1835) "*Vibrio subtilis*" to *Bacillus subtilis*, Ferdinand Cohn established the genus *Bacillus* in 1872. They are described as endospore-forming, Gram-positive, aerobic rods bacteria. Their unusual ability to form spores caused much attention in the early 1884 and became a key classification characteristic (Harwood 1989). For the endospores in this genus, on account of their high resistance to heat, radiation, desiccation and disinfectants, in particular the spore's ability to attach to processing equipments, some species of *Bacillus* have been troublesome in clinical environment, food production and biotechnology processes (Logan 2012).

Members of the genus *Bacillus* are widely distributed in air, soil and water. They can even be isolated from some extreme environments, such as air at high altitude and deep subterranean sources (Harwood 1989; Kramer and Gilbert 1989; Logan 2012). In addition to the distribution diversity, the metabolic diversity of the genus makes it one of the most commercially important groups of bacteria. Although primarily saprophytes, at least one species can process chemolithotrophic activity in $O_2/CO_2/H_2$ or O_2/CO_2 and two species can fix nitrogen (Harwood 1989). Logan et al (2009) mentioned that during the process of producing fermented food based on leaves and seeds, *Bacillus* species play a vital role in developing the flavor. Strains isolated from the healthy plants also have a role in plant protection and promote the growth of plants by fixing nitrogen (Reva et al. 2002).

Nowadays four types of products are still the main commercial products obtained from strains of *Bacillus spp.* They are enzymes, biochemicals (include food supplement

and flavor enhancer), antibiotics and insecticides (Harwood 1989).

The Genus *Bacillus* encompasses a great diversity of species which lack a common evolutionary history (Bhandari et al. 2013). Bacteriologists are struggling to seek more agreements for their classification. The scheme of Gordon, Smith et al remains preeminent. In 1946, they developed a primary division of the genus into three groups depending on their cellular morphology and physiological properties (Smith et al. 1946; Harwood 1989).

Group 1 contains Gram-positive species that form oval spores that do not appreciably swell the sporangium. It includes *Bacillus subtilis*, *Bacillus cereus* and *Bacillus megatherium*. Group 2 is composed of those with oval spores that distinctly swell the sporangium and include the species *Bacillus circulans*, *Bacillus polymyxa* and *Bacillus macerans*. Bacteria of group 3 are characterized by having swollen sporangia with round spores. It is made up of *Bacillus sphaericus* and *Bacillus pasteurii*. Members of the latter two can be Gram-positive, Gram-negative or variable (Smith et al. 1946; Harwood 1989; Kramer and Gilbert 1989).

Thanks to advances in technology, especially the molecular technology which started in the 1970s, molecular data started to be used to describe the bacteria. In the beginning, the whole gene DNA-DNA hybridization was used as a standard method. Then nucleotide variation in the 16S rDNA and genetic variation in 16S rRNA were used for bacterial taxonomy (Vilas-Boas et al. 2007). All these new methods of analysis caused a huge expansion in the bacterial taxa and its continuous modification. Therefore, *Bacillus mycoides*, *Bacillus thuringiensis* and *Bacillus anthracis* are suggested to be considered as a single species, not a subspecies of *Bacillus cereus* (Vilas-Boas et al. 2007;

Logan 2012).

Currently the phenotypical traits and the sequences of 16 S rDNA are some main factors that used in the classification. The taxonomy of the genus *Bacillus* is still in flux (Harwood 1989; Vilas-Boas et al. 2007).

I.2. Introduction to The Species Used

I.2.a Bacillus subtilis

Bacillus subtilis is a Gram-positive, rod-shaped, spore-forming bacterium. It can be isolated from diverse environments including soil, plant roots and even animals' GI tracts. Similar to most other *Bacillus* species, *Bacillus subtilis* is non-pathogenic, and has been assigned GRAS (Generally Recognized As Safe) status by US Food and Drug Administration (Kunst et al. 1997; Earl et al. 2008; Zweers et al. 2008). The *Bacillus subtilis* species has a long history of safe use. Currently, it is well-known as a source of useful enzymes and as a host for protein production in industry (Kunst et al. 1997; Zweers et al. 2008). Also it is a model organism used in the scientific research. It is highly amenable to genetic manipulation, especially for sporulation. It is used as an excellent model for cell differentiation (Hilbert and Piggot 2004; Iber et al. 2006; Zweers et al. 2008).

I.2.b Bacillus cereus

Bacillus cereus is a Gram-positive, spore-forming, aerobic, facultative anaerobic

food-borne pathogen. Most strains of *Bacillus cereus* are motile by means of pertrichous flagella. Its growth temperature is from 10-48℃, with optimum between 28-35℃ (Kramer and Gilbert 1989; Vilas-Boas et al. 2007).

Bacillus cereus can cause two types of foodborne illnesses: diarrhoeal illness and emetic illness. The diarrhoeal disease is often associated with protein-rich food, including meat, pudding and milk products. It is thought to be caused by ingestion of vegetative cells or spores. The enterotoxins produced by the vegetative cells can lead to the typical symptoms including abdominal pains, watery diarrhea, nausea and vomiting, which will last for 24-48h. The emetic disease is usually associated with the rice dishes. The toxin, called cereulide, is produced in foods prior to ingestion. The symptoms mainly contain nausea and vomiting which occur quickly after digestion of food for 0.5-6h and will last for 6-24h (Abee et al. 2011; Logan 2012). In addition, strains of *Bacillus cereus* sometimes cause systemic and local infections, especially associated with neonates and drug addicts (Vilas-Boas et al. 2007; Turabelidze et al. 2013).

*I.2.c **Bacillus mycoides***

Bacillus mycoides did not receive much attention because it is not as harmful to human as *Bacillus cereus* and *Bacillus anthracis* are *(Turchi et al. 2012)*. It is a Gram-positive spore-forming species which is widely distributed in soils (Goodwin and Roy Jr 1994). It is similar to *Bacillus cereus* groups in physiological character except for growth. When growing on the solid media, *Bacillus mycoides* will form rhizoid colony. It is not motile (Todar 2011; Logan 2012).

I.2.d Bacillus thuringiensis

Bacillus thuringiensis is a well-known Gram-positive, insect pathogen. It owns this ability to the production of a group of crystalline protein called endotoxins during sporulation, which target on specific groups of insects (lepidopteran, coleopteran pests etc.). After the insects digest the endotoxin and spores, the protein will dissolved in the midgut of insect larva and cause damage to it. Besides, the spores may even geminate and the resultant cells grow in the midgut, and then the insect will stop feeding and die (Vilas-Boas et al. 2007; Logan 2012).

Currently, the strains of *Bacillus thuringiesis* have huge successes in biological control programs, accounting for approximately 90% of the world's microbial pest control agents marketed. It is widely used on major crops, including maize, cotton, soybean, tomato et al (Vilas-Boas et al. 2007).

I.2.e Bacillus anthracis Sterne

As the etiological agent of the disease anithrax, *Bacillus anthracis* is a spore-forming bacterium. Its spores have high resistance. They can survive in a variety of environments (such as boiling water, freezing or even suspension in the alcohol) and persist for a long period (Vilas-Boas et al. 2007).

Anthrax is diagnosed in three forms based on the infection route: cutaneous, gastrointestinal and inhalation. It can be initiated by the invasion of spores into the body of the host and mainly affects herbivores. Actually all mammals are susceptible to the anthrax. Humans can incidentally become infected the anthrax bacterium when they have

made contact with disease farm animals or contaminated animal products (Vilas-Boas et al. 2007; Todar 2011).

There are two main virulence factors in *Bacillus anthrax*. One is an antiphagocytic poly D-glutamic acid capsule that mediates the invasive stage of the infection. The other is production of three proteins (multicomponent anthrax toxin) which facilitates the toxigenic stage. They are all coded on two plasmids called pXO1, pXO2. If one of them is missing, the bacteria cannot fully produce all of its virulence factors which results in reduced ability to cause illness. The pXO2 plasmid forms the capsule and pXO1 codes the gene for anthrax toxin (Vilas-Boas et al. 2007).

Because it lost the plasmid pXO2, *Bacillus anthracis Sterne* lost its ability to produce a capsule. Compared with normal wild-type strains, the *Sterne* strain is relatively avirulent, but still has the ability to stimulate a protective immune response.

The strain 34F2 of *Bacillus anthracis Sterne* is widely used for the formulation of livestock vaccines against anthrax worldwide. No human anthrax infection has been reported due to it and the strain has an excellent safety record (National Center for Emerging and Zoonotic Infectious Diseases ; Turnbull 1991).

I.3 Vegetative Cell Membranes

I.3.a The Structure and Function of Cell Membrane

As a fundamental structure of the cell, the cytoplasmic membrane performs a vital role in separating cells from the external environment. It acts as an interface between the cellular cytoplasm and the external environment. In addition to the boundary function, the

cytoplasmic membrane also has the ability to provide communication between outside and inside. The membrane is important in energy generation and maintaining ion and solute gradients. The cytoplasmic membrane allows numerous diverse compounds (such as water and numerous gases) and ions to cross the bacterial cytoplasmic membrane by diffusion and active transport. In addition, the cytoplasmic membrane also stabilizes membrane proteins. This is essential for the functioning of embedded enzymes. In summary, an intact plasma membrane is a must for all microbial cells to their continued survival and growth (Russell et al. 1995; Trevors 2003; Vincent et al. 2004; Mykytczuk et al. 2007).

Our current understanding of the membrane structure is based on the fluid mosaic model raised by Singer and Nicholson (Singer and Nicolson 1972). From this model, the bacterial cytoplasmic membrane is composed of lipid bilayers with embedded integral and peripheral proteins, even with some proteins that transverse the membrane. There can be carbohydrates molecules attached to the protein or lipids, and extend outward from the membrane (Trevors 2003; Mykytczuk et al. 2007).

This model has been modified to provide a better understanding of membranes (Engelman 2005). The primary lipids in the membrane are the polar glycerophospholipids, glycerol is the backbone of it. In aqueous solution or in an appropriate environment, the membrane is maintained by the dominant force called the hydrophobic interaction. In order to avoid hydrophobic exposure, the bilayer is formed into a closed structure: the head groups oriented externally and the lipid acyl chains oriented to the interior. In this structure, the lipids have considerable motional freedom and in a lamellar, liquid crystalline state. This is the normal state of the membrane (Beney and Gervais 2001;

Trevors 2003; Mrozik et al. 2004; Engelman 2005). The membrane lipids contain saturated and unsaturated fatty acids. There are other structures of the acyl chains, such as branched (*iso*, *anteiso*, hydroxyl fatty acid) (Cho and Salton 1966; Mrozik et al. 2004).

I.3.b Membrane Rigidity

Rigidity is one of the most important parameters of the cytoplasmic membrane (Trevors 2003; Mrozik et al. 2004). The rigidity is most frequently defined as the optimal state of the membrane. Actually it is a term that encompasses the lipid order, structure, microviscosity as well as membrane phase, lipid shape, packing and curvature (Rilfors et al. 1984).

Membrane rigidity changes as a result of the physical and chemical interaction of membrane lipids and the environment factors such as temperature, pH, pressure and ions. The bacterial response to different environmental perturbation is a multi-factorial process and the cytoplasmic membrane is the primary response mechanism (Mrozik et al. 2004; Mykytczuk et al. 2007).

The membrane will modify itself to maintain it in a fluid state. There are many studies addressing these modifications. They show that when responding to a change in temperature, there will be many changes on the fatty acid component of lipid for some bacterial. Sometimes changes in the head-group composition also occur (Russell et al. 1995; Mrozik et al. 2004).

I.3.c Lipid Order Influence Membrane Rigidity

Regardless of the contributions from the protein and lipid-protein interaction, we need to focus on the key characteristics of bacterial lipids for modulating the membrane rigidity.

First, the longer chain fatty acids will give the membrane a more rigid structure, because longer chains increase the acyl chain interactions by further penetrating into the bilayer. The lipid length alternation can only be observed in the growing cells.

Secondly, the ratio of saturated to unsaturated fatty acids in the membrane is the primary mechanism for regulating the membrane rigidity. The saturated fatty acids can pack tightly to optimize the van der Waals interaction and give the membrane a rigid structure. Both the *cis* ad *trans* unsaturated fatty acids will disrupt the chain interaction due to their own kinked structure. In some conditions, bacteria can isomerize unsaturated fatty acids from the *cis* to the *trans* conformation, which occurs when the cell stop growing and dividing. After isomerization, the *trans* fatty acids act more like saturated fatty acids, packing in a more ordered manner.

Thirdly, different types of branching also have an effect on membrane rigidity. Cyclopropanoic fatty acids most occur in Gram-negative bacteria, while methyl branching most commonly found in Gram-positive bacteria. Both branched and cyclic fatty acids can slide past each other and do not form crystalline structures between the acyl chains, leading to the increase of the membrane rigidity (Denich et al. 2003; Trevors 2003; Mykytczuk et al. 2007).

I.4 Fluorescent Probes

I.4.a Introduction

The use of fluorescent probes is a hot topic in the field of biology and medicine. It has enormous application potential. Acting as a "molecular reporter", the probe can show information on the state of adjacent environs via their fluorescent signals. The signal is analyzed by appropriate instruments to transfer the signals into different kinds of fluorescent parameters, such as fluorescent intensity, quantum yield, emission spectrum, polarization etc. Many of them are related by physical equations that can serve as a check of the correctness of the experimental procedure (Slavík 1996).

I.4.b Advantages and Characteristics of Fluorescent Probes

Firstly, probes are stable and easily obtainable. They all have specific absorption and emission spectra, and are easily detectable: weak signals can generally be detected.

Secondly, fluorescent probes can be repeatedly excited and detected. Instruments for detection include spectrofluorometers, flow cytometers, fluorescent microscopes and so on. Most of these instruments have the corresponding software to do the data analysis.

Thirdly, probes can be incorporated into intact cells without disrupting the cytoplasmic membrane and do not interfere with the event being measured. The probes can be used in living bacterial cells at all phases of their growth.

Fourth, no pretreatment of cells is needed for uptake of the probe into the cells. Only small concentrations of bacterial cells are needed per analysis.

Finally, fluorescent probes allow cytoplasmic membranes to be studied under

diverse incubation conditions, environmental conditions, in the presence or absence of toxicants and over wide range of temperatures (Trevors 2003; Vincent et al. 2004; Mykytczuk et al. 2007).

I.4.c The Fluorescent 1, 6-diphenyl-1, 3, 5-hexatriene Probes

Most research on the bacterial cytoplasmic membranes has used 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) and its analogue 3-(4-(6-phenyl)-1, 2, 5-hexatrienyl phenylpropionic acid (DPH-PA) as fluorescence probes (Trevors 2003).

DPH is an extremely hydrophobic, symmetrical probe. It can penetrate into the membrane's hydrophobic region, orienting itself parallel to the fatty acid side chain (Adler and Tritton 1988). The spontaneously intercalation of DPH into the membrane is accompanied by an increase of its fluorescence. It has a significant shift in excitation wavelength from 358 nm to its fluorescent emission at 428 nm (Vincent et al. 2004; Hurjui et al. 2012).

I.5 Fluorescence Polarization Analysis

I.5.a Introduction

Based on the fluorescence parameter used, fluorescence provides three ways to measure the rigidity of the cytoplasmic membrane. They are bleaching of dyes after a short strong laser, polarization measurement and exhibited excimer fluorescence. Depending on the concept of a wobbling motion of the dye in a cone-restricted space,

fluorescence polarization is measured to collect information on the rotational mobility of the probe in the membrane-incorporated fluorescent dyes. The technique can also be applied for the measurement of cytosol viscosity (Slavík 1996).

I.5.b The Measurement Procedure

In order to achieve the fluorescence polarization analysis, a spectrofluorometer equipped with polarizers, a cuvette holder with temperature control and sample stirrer are used (Denich et al. 2003; Trevors 2003; Mykytczuk et al. 2007). A xenon lamp is usually used as a light source. It can produce the excited beam. After going through the excitation polarizer, the excitation beam will be vertically polarized before passing through the sample, then the excited beam is collected and directed to the emission polarizers. When two emission polarizers are used, one is oriented in the vertical direction and other in the horizontal direction. When passing through the photomultiplier tubes, the emitted light is amplified. Finally, the computer software does the data analysis (Trevors 2003; Mykytczuk et al. 2007).

The principle of the whole measurement process can be described in that fashion. The depolarization of the excitation light is used to determine the rotational motion of DPH. Then the rotational motion of the DPH is used to determine the membrane state. The equation of Shinitzky and Barenhoz defines the fluorescence polarization ratio (P). $P= (I_{VV}-I_{VH}\,G) / (I_{VV}+I_{VH}\,G)$, where I_{VV} and I_{VH} stand for the light intensities emitted in the vertical and horizontal directions relative to the beam of excitation (Steiner 1984; Trevors 2003; Mykytczuk et al. 2007). The G is the grating factor. It is instrument-dependent and accounts for the correction of the photomultipliers' different sensitivity to

polarized light in the vertical and horizontal directions. It is proportional to sample turbidity and can be compensated for by using low cell numbers (Trevors 2003; Mykytczuk et al. 2007).

The cytoplasmic membrane rigidity value is just the inverse value of the probe polarization ratio. Anisotropy (r) and polarization (P) are both expressions for the same phenomenon and they can be interchanged by the equation: r=2P/(3-P) (Steiner 1984; Trevors 2003). Previously, the polarization result was the parameter used most frequently. However, the anisotropy value is currently the more frequently used parameter because it is considered simpler (Steiner 1984).

As the depolarization of the fluorescence probe only provides an over view of the entire cytoplasmic membrane, we cannot determine the membrane rigidity of specific membrane locations by this method (Trevors 2003; Mykytczuk et al. 2007).

I.6 Spores

I.6.a Introduction

Spores of various *Bacillus* species are formed during sporulation, a process that is initiated in response to the starvation of one or more nutrients (Driks 2002). These endospores are metabolically dormant, and contain little or no high-energy compounds such as NADH and ATP. There is no or little detectable metabolism of exogenous or endogenous compounds (Setlow 1994; Black et al. 2007). In the absence of enzyme action and metabolism, the dormant spore cannot recover from damage to macromolecules such as protein and DNA. Consequently, the spores need multitude mechanisms to protect macromolecules from damage. In addition, spores are found that

are extremely resistant to heat, radiation and chemicals which can rapidly kill vegetative cells (Setlow 2006). These two remarkable features of spores allow them to survive for long periods, even millions of years (Vreeland et al. 2000).

I.6.b Spore Structure

Compared with growing cells, it is not surprising that the spore has a very different layer structure. From the outside to the inside, they are: exosporium, coats, outer membrane, cortex, germ cell wall, inner membrane and central core (Figure 1).

As the outmost layer of the spore, the exosporium is made of proteins and carbohydrate.

The spore coats, which may consist of several different layers, have an important role in the spore resistance to some chemicals, and protects spores from lytic (lysozyme-like) enzymes.

Beneath the coat layer is the outer membrane. It is the essential structure in spore formation. But it is not likely act like a significant permeability barrier in dormant spores and removal of it seems have little effect on the spore resistance properties and dormancy.

The cortex appears to be extremely important for the formation of a dormant spore and for maintaining the low water content of the spore core. The cortex is composed of peptidoglycan (PG), similar structure of the vegetative cell PG, but it has some specific modifications.

The germ cell wall seems to play no role in spore resistance. It will not degrade during the germination process and becomes the cell wall of the outgrowing spores.

Several studies have demonstrated that the inner membrane plays a major role in the

spore resistance to many chemicals, especially those can damage the spore's DNA. The extremely low permeability of the inner membrane sometimes does not allow even water molecules to pass through (Setlow 2006; Black et al. 2007). Compared with the lipid composition of the vegetative cell membrane, biophysical analysis shows that it is not the lipid content that confers the inner membrane's remarkable impermeability, but the status of these lipid molecules in the membrane. By analysis of the fluorescence redistribution after photobleaching of lipid probes, it has been demonstrated that the lipids located in the inner spore membrane are largely immobile (Cowan et al. 2004; Setlow 2006; Leggett et al. 2012).

The final spore component is the core. It contains most of spore enzymes as well as the spores' DNA, RNA and ribosomes. There are two unique constituents in the core. One is the pyridine-2, 6-dicarboxylic acid (dipicolinic acid or DPA). DPA plays a vital role in reducing the water content in the core and in protecting the spore DNA from damage. The second unique molecule present in the core is α/β-type small, acid-soluble proteins (SASP).The α/β-type SASP to DNAs change the DNA structure, protecting the spore DNA against moist and dry heat, oxidizing agents and ultraviolet radiation (Setlow 2006; Black et al. 2007).

I.6.b Sporulation

The mechanism of endospore production is similar among many Gram-positive bacteria, including species of the *Bacillus* and *Clostridium* genera (Driks 2002).(Figure 2)

The sporulation cycle consists of seven stages (stage 0-5). Stage 0 is the initiation

of sporulation. Stage Ⅰ is defined as an apparent condensation of the replicated chromosome to form an axial filament. Stage Ⅱ is a specialized cell division in which a septum formed. During this stage, the smaller forespore compartment forms the cellular core of the spore, whereas the mother cell is mortal; Stage Ⅲ is engulfment of the forespore; the mother cell septal membrane continues to grow and migrates around the prespore compartment. Stage Ⅳ is the formation of the electron-transparent cortex. Stage Ⅴ is the formation of the spore's coats. Stage Ⅵ is maturation during which spore's full heat resistance properties are developed. Stage Ⅶ is the release the mature spore (Russell 1982; Moir et al. 1992).

I.6.c Germination

Starvation causes the sporulation process of *Bacillus species*. when the conditions are again favorable for growth, the spore will germinate and go through outgrowth, eventually converting back into the vegetative cell (Setlow 2003). During the germination process, the spore will ultimately lose its metabolic dormancy and environmental resistance (Moir et al. 2002). The germination process can be triggered by nutrients (generally single amino acids, purine nucleoside or sugars) and non-nutrient agents, termed germinants (Moir et al. 2002; Setlow 2003; Moir 2006).

The whole process consists of three parts: activation, germination and outgrowth. During the germination period, the germinant first binds to the receptor located in the spore's inner membrane. Then the spore's core releases its dipicolinic acid (DPA) and cations. These components are replaced by water. Next the spore's peptidoglycan cortex

is hydrolyzed by abundant lytic enzymes. Finally, it leads to the completion of cortex hydrolysis and expansion of the germ cell wall (Moir et al. 2002; Moir 2006; Setlow 2006). The whole germination process is essentially a biophysical process that does not need any macromolecular synthesis (Moir 2006).

I.6.d Spores as Agent of Disease and Spoilage

Being one of the most resistant forms of living organism, the bacterial spore has been investigated since its discovery (Carlin 2011). The most health-related spores are from the species of *Bacillus* and *Clostridium*. The three major food poisoning spore-forming pathogens often mentioned are *Clostridium botulinum, Clostridium perfringens* and *Bacillus cereus*. Besides these food poisoning species, there are still many non-pathogenic spore-forming species which can cause significant economic spoilage to food producers (Brown 2000; Black et al. 2007).

The contamination routes of spores to food can be varied. The direct way is through the soil. As the processed foods are the combinations of multiple ingredients, each can bring their own spore-forming bacteria. Some common ingredients such as spices, milk powder and flours have very high spore concentrations. In addition, food processing facilities can also be the source of spores, owing to the species resistance to the facilities cleaning operation and the spores' ability to adhere to processing equipment (Carlin 2011).

Contamination by *Bacillus cereus* may come from cows, raw milk, the transport and further storage of the milk, or even the packing materials. So the control of spore contamination should focus on performing safe food manufacture procedures (Andersson

et al. 1995). Greater understanding of the resistance characteristic spores will help improve their food safety regulation.

I.7 New Trends and Hazards in Food Industry

Nowadays, consumers are increasingly interested in the health benefits associated with the consumption of food. They are demanding the high-quality, fresh-tasting, low-salt, preservative-free food which have high convenience and require minimal preparation time. All these demanding lead the food industry to focus on the production of minimally-processed, ready-to-eat food (Juneja et al. 2003).

Thermal pasteurization and sterilization were traditionally used in the past. They are broadly effective and relative inexpensive method. It has high efficacy against food-borne pathogens and food-spoilage microorganisms, generally at the expense of its sensory, food nutrient content as well as limit the type of material used for packaging (Shearer et al. 2000; Lado and Yousef 2002). During the past years, even advances in technology allow the minimal deterioration of the food quality, new thermal technologies such as High-temperature short-time (HTST) pasteurization and ultra-high temperature (UHT) sterilization have been used in the industry. But these technologies still cannot keep the fresh flavor and texture (Lado and Yousef 2002; Tewari et al. 2003).

Due to the great need for a method for inactivating microorganisms that is safe, economical and can keep the flavor and texture, alternative technologies have been investigated. In general, the alternative technologies are called “non-thermal”. All of these technologies are named based on the major processing parameter used in the cell inactivation. There are four novel non-thermal processes that call so much attention

recently. They are High-Pressure Processing (HPP) which exposes the food under a high hydrostatic pressure; Pulsed Electric Field (PEFs); Ultrasonication; Ionizing radiation (Lado and Yousef 2002; Ross IV et al. 2003).

The advantage of alternative technologies is that they can inactivate microorganism at ambient or sublethal temperature which will preserve the food nutrient value, flavor and color. In the meantime, alternative processes can only play the same role as pasteurization, not sterilization. So the resistance of the bacterial spore becomes a big concern. Sometimes in order to inactive the microorganism to some specific degree, high treatment intensities are required. They also influence the functional or sensory of the food. Besides, some technologies just cost too much to be practiced in the industry (Ross IV et al. 2003).

High-pressure processing (HHP) technology as an example of a nonthermal process that has been successfully commercialized (Tewari et al. 2003), There are two major theories to underline the HPP's antimicrobial effect. The first one is *Le Chatelier's Principle*, which mentions that any phenomenon in equilibrium accompanied by a decrease in volume is enhanced by pressure. The second one is the *Isostatic Principle*, which states that the pressure can transmit through the entire sample uniformly and independently of sample size. This is a big difference from the thermal process (Tewari et al. 2003; Black et al. 2007).

Currently, the major concern of this technology is its sporocidal effect aimed at the spores of different foodborne pathogens (Tewari et al. 2003): The study of *Bacillus subtilis* spores demonstrated that the HPP will induce the spore germination. Applied under moderately high pressure (MHP, 50 to 300 MPa), it will activate the nutrient

receptors of the spore to induce the germination process. When used at very high pressures (VHP, 400-800 MPa), the induction process starts with the release of Ca-DPA. We only know that the mechanisms of MHP and VHP are different, and should be studied further. What is more, several studies found that there are “superdormant” spores that can survive HPP. These super pressure-resistant spores can cause the “tail” of the lethality kinetics and become a potential hazard in the food industry (Lado and Yousef 2002; Black et al. 2007).

I.8 Significance

Arguably the vital processing parameters in control of the food poisoning and spoilage are the lowering the water activity by preservative solutes, pH and temperature. As all of those parameters influence the structure of the membrane, to study the physiology of food-associated bacteria’s response to the food preservation means to find how it adapts its membrane composition.

Since lipids are the major component of membrane and most changes in lipid are responsible for maintenance of the particular phase behavior in membrane bilayers, a better understanding of the mechanism and regulation of lipid changes will provide essential information. This information can help in improving a more effective method aimed at targeting food-associated bacteria. It can give us some basic hints for designing novel methods to protect foods. Also this information will help improve the efficiency of the novel non-thermal processing technology.

There is a clear need more comparative data is needed on the response to changes in membrane composition change. To our knowledge, there is a paucity of information on

study the cytoplasmic membrane polarization values for vegetative cells and spores, let alone, comparing these values among the *Bacillus* genus. This thesis provides fundamental information on this topic.

Chapter II-Hypothesis and Objectives

We hypothesize that membrane rigidity will be influenced by the culture medium, species of bacteria, and whether the membrane is located in a spore or a vegetative cell. This hypothesis will be tested by addressing four objectives.

Objectives:

1. To determine the influence of media on the rigidity of vegetative cells.
2. To study the difference in vegetative cell membrane rigidity among species.
3. To identify the difference in spores membrane rigidity among species.
4. To determine whether spores and vegetative cells have similar membrane rigidity.

Chapter III-Materials and Methods

III.1. Bacterial Cultures and Growth Condition

Bacillus subtilis ATCC 6633, *Bacillus mycoides* ATCC 21929, *Bacillus cereus* ATCC 7004 and *Bacillus thuringiensis* ATCC 13366 were all purchased from American Type Culture Collection (ATCC, Mannassas, VA). *Bacillus anthracis* Sterne was a gift from Dr. Darcy Haynes, Center for Food Safety and Applied Nutrition, College Park, MD.

All strains were maintained on the Nutrient Agar (Difco) slants at 4 ℃as working stocks and renewed monthly for a maximum of 6 months (at which point the new working stocks were from the spores produced by the original cultures).

III.2. Media and Reagents

All strains were cultured on four different media: Nutrient Broth (NB, Difco); Luria Broth (LB, Difco); Luria Broth Granulated Agar (LBGA), which was prepared by adding 1.5% granulated agar (Difco) into the Luria Broth; Luria Broth Noble Agar (LBNA), which were different from the LBGA by adding 1.5% noble agar (Difco) instead of granulated agar. All broth and agar were autoclaved at 125 ℃ for 15 minutes before use.

DPH (1, 6-diphenyl-1, 3, 5-hexatriene), THF (tetrahydrofuran), Lysozyme and Phosphate Buffered Saline (PBS) were purchased from Sigma.

III.3. Vegetative Cell Culture Preparation

The growth curve of each strain was build up based on the data measured by a spectrometer (SmartSpec ^{TM} 3000, Bio-ad, CA) at OD $_{600}$ nm.

Each strain was inoculated (3% v/v) into different media and incubated at 37 ℃ overnight, then repeated the same process once to achieve the pre-active process. After pre-activation, cells were inoculated (3% v/v) into each medium and incubated at 30 ℃ with shaking (200 rpm). The vegetative cells were harvested when the strains reached the stationary phase. This time was different for each strain.

III.4. Spore Preparation

Sporulation Process: after pre-activating each strain in LB at 37 ℃ overnight, Phosphate Buffered Saline (PBS) was used to dilute the cells 10^3 fold. Aliquots (100uL) of the diluted cell solution were plated on LBGA and incubated at 30 ℃. Phase-contrast microscopy was used to observe cell sporulation daily, when ~ 90% of the spores were phase dark, sterilized PBS was added to each plate and the endospores were harvested from the surface of LBGA by a sterilized bent glass rod.

Spore Purification: Oak Ridge style Nalgene centrifuge tubes with caps with O-rings were used to centrifuge the harvested spore solutions at 10,000 × g for 10 minutes at 4℃. Care was taken to discard the supernatant. The pellet was resuspended in 25 ml of cold sterile distilled water containing 0.01% lysozyme and held at 4℃ for 48h. The spores were then centrifuged twice at 5,000 × g for 20 minutes and then resuspended in sterile distilled water. The final spore stock contained 95% phase-

bright spores. The purified spores were stored at 4 ℃.

III.5. Steady-state Fluorescent Anisotropy Measurement

The anisotropy value, also called the membrane rigidity, is based on the emitted intensity of DPH measured by a Perkin-Elmer Luminescence Spectrometer (PE LS50B, CT). The spectrofluorometer used the excitation wavelength at 360 nm to excite the probe. The emitted light was measured at 430 nm through a polarizer which received signals in both vertical and horizontal directions. The grating factor (G) used was 1.27. The slit width for excitation and emission was set at 2.5 nm.

Whole-cell Anisotropy: The cell suspension was centrifuged at 8000 × g at 4 ℃ for 10 minutes (JA-17 rotor), the supernatant was discarded and the pellet was washed using PBS. This was repeated and PBS used to resuspend the cell pellet to A_{450}= 0.26~0.30. DPH was added to the cells to achieve the final concentration of 10^{-6} M and incubated at 30 ℃ for at least 45 minutes. Unlabeled cells were used as blanks to generate the base line values.

To determine the anisotropy of spore membranes: the protocol was similar to that used for whole cells. Spores were harvested directly from the agar surface, purified and suspended in PBS to A_{450}= 0.26~0.30. DPH was added and the spores incubate at 30 ℃. Unlabelled spores were used as blanks.

III.6. Statstical Analysis

A one-way analysis of variance (ANOVA) and t-test were performed using SPSS software.

All polarization values are means and standard deviations from triplicate experiments.

Chapter IV - Results

IV.1. Growth and Sporulation of *Bacillus* Species

Considerable effort was spent in an attempt to identify a medium in which *B. subtilis*, *B. anthracis*, *B. cereus*, *B. thuringiensis* and *B. mycoides* would all sporulate. These efforts were successful; all of the strains grew well in liquid media, with LB being the best. However, neither LB nor NB supported sporulation (Table 1). Because each strain reached a different stage of growth at a given time, the stationary phase of growth was used to measure the membrane rigidity of vegetative cells.

IV.2. Membrane Rigidity of Vegetative Cells Cultured in Different Media

In order to find the influence of medium on the rigidity of vegetative cells, The vegetative cells of all five species were grown on NB, LB, and LBNA and the membrane rigidity of their vegetative cells were examined (Figure 3). There was a trend for the influence of media when the species were compared. The membrane rigidity of vegetative cells grown on LBNA was always higher than that of those cultured on LB liquid medium. Based on the independent t-test result, this difference was statistically significant ($p < 0.05$) for all five species examined. The rigidity of cells cultured in NB was always higher than that of cells cultured in LB. Again, this was of statistical significance in four out of five strains (Table 2).

Through the one-way ANOVA test, for each strain under three different media conditions, the statistical differences were observed in all five species (Table 3).

The importance of the agar used is shown in the comparison of vegetative cell

membrane rigidities (Table 4). There were no significant differences among these species under two different conditions (LBNA and LBGA) except for *Bacillus anthraci*s. The data we get from LBGA have very high standard deviation, especially for *Bacillus mycoides* and *Bacillus thuringiensis.* Because the sporulation process on LBNA was inconsistent, LBGA was used to generate spores for the determination of spore membrane rigidity.

IV.3. Rigidity of Spore Membranes

The membrane rigidities of spore membranes from *B. subtilis*, *B. anthracis* and *B. mycoides* were determined (Figure 4). With a rigidity of 0.31±0.02, spore membranes of *B. subtilis* were more rigid than those of *B. mycoides* or *B. anthracis*. Through the one-way ANOVA test, there were significantly differences among these three species. Table 5 showed that there were significantly differences when compared the *Bacillus subtilis* with other two species. There were no significant differences between *Bacillus anthracis* and *Bacillus mycoides*.

IV.4. Comparison of Membrane Rigidity between Vegetative Cells and Spores

In order to eliminate the influence of the medium on the different species and make the comparison of result more reliable, cells grown in LB were compared with spores generated in LBGA.

The independent t-test (Table 6) demonstrated that for the *Bacillus anthracis* strain and *Bacillus subtilis* strain, there was significant difference (P value <0.05) in the membrane rigidity between the vegetative cells and spores.

Chapter V- Discussion

Since the term “Homeoviscous Adaptation” was raised in 1974 (Sinensky 1974), the primary genetic and chemical processes to maintain lipid homeostasis has been well studied in membranes of vegetative cells. The bacteria have evolved mechanisms to control the formation of fatty acids and to modify the existing fatty acids in order to minimize the energy expenditure and optimize growth. All of these mechanism result in a cell membrane that has a constant rigidity at a given temperature (Zhang and Rock 2008). Our data on membrane rigidity of vegetative cells are comparable to theirs. Fatty acid synthesis is achieved by a conserved set of genes. We can assume that the species carried similar genetic information may have similar mechanisms in maintain the lipid homeostasis. When comparing the same strain under different conditions, our result just supports this: five species from the same genus followed the same trend (specific manner). When comparing the different strains under same medium condition, the different genetic information owned by different species can be a reason to explain the difference observed.

As the whole cells were used in measurements, the contribution of physiological changes should also be taken into consideration when studying the membrane rigidity (Najjar et al. 2007). This suggests that the explanation of some phenomena more complex. In a controlled environment, each species uses the limited nutrients to synthesize its own cell membrane. This can explain the differences among NB, LB and LBNA. Nutrient Broth is composed of beef extract and peptone while the Luria Broth is made of tryptone, yeast extract and sodium chloride. These different components in each medium can provide different kinds of amino acids, vitamins, inorganic components and

fatty acids. These differences can be a reason to explain the medium-dependent differences in rigidity that were observed in this thesis.

The quality of granulated agar is determined solely by its physical properties while the noble agar is the purified agar to make is essentially free from impurities. As the only difference between the LBGA and LBNA is the agar, it may be that the impurity of components in granulated agar influences the membrane rigidity of five species vegetative cells. Based on the observation (Table 4), data obtained from LBGA had very high standard deviation for *Bacillus mycoides* and *Bacillus thuringiensis*. The impurity components in agar may influence the variability of the data.

In a conclusion, medium is a factor that influences the membrane fluidity of vegetative cell of these species. Furthermore, it is thus plausible that the medium influences the membrane fluidity of vegetative cells among the *Bacillus* genus, even among other genera. In the future work, comparing the membrane rigidity data of different strains, the medium used should be carefully controlled.

To find a proper medium that enables a variety range of strains to sporulate is always a demanding task. Previous studies in the culture of *Bacillus subtilis* have suggested that the media composition can regulate the spore-to-cell ratio and suggested that the carbon and nitrogen source may act as a repressor of one sporulation-specific enzyme (Schaeffer et al. 1965).

The relation between the rigidity of the vegetative cell membranes and spore membrane has never been examined among species. The inner spore membrane and outer spore membrane are both derived from mother cells. The inner spore membrane becomes the new plasma membrane after germination (Setlow and Setlow 1980; Cortezzo et al.

2004). In the study of the methylamine transfer in *Bacillus megaterium* spores, the cell wall and spore outer membrane have little or even no permeability barrier while the spore inner membrane is impermeable (Swerdlow et al. 1981; Swerdlow and Setlow 1984). Thus, the inner membrane of spore is thought to play a vital role in the dormant spore's resistance properties. Our attention should focus on the spore inner membrane.

1, 6-Diphenyl-1, 3, 5-hexatriene (DPH) can incorporate itself into the cytoplasmic membrane easily without disturbing the cell membrane. Because of this advantage, DPH is widely used in the membrane rigidity measurement (Trevors 2003). DPH can partition the spore inner membrane and be an accurate reporter of spore inner membrane rigidity (Voss and Montville 2014).

Previous studies on spore have mainly focused on the media's influence on the spore resistance properties, spore's response for different stress (heat, pressure, chemicals) and spore changes during germination. There are few reports on the spore membrane rigidity of dormant spores. Our research provides these data.

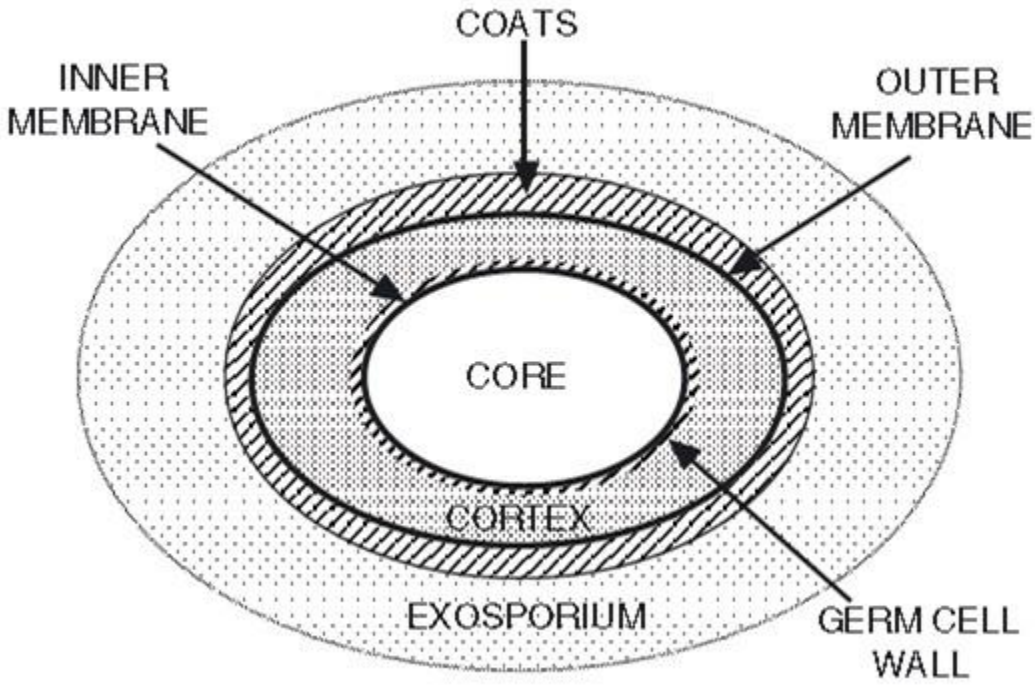
All the membrane rigidities of five species were determined at optima temperature. All reported data in our work fall within the physiological rang and functional membrane (Najjar et al. 2007). Comparing with former membrane rigidity data of *Bacillus subtilis* strain (Voss and Montville 2014), our data is quite similar: the membrane rigidity of vegetative cells is around 0.20 and the spore membrane rigidity is around 0.28~0.33. The membrane of spore is more rigid than the vegetative cell. The higher rigidity is consistent with Setlow's data for *Bacillus subtilis* spore: lipids in the spore's inner membrane also appear to be largely immobile (Cowan et al. 2004).

Chapter VI- Conclusion

This research is the first to address the translational rigidity of spore membranes. Overall, the rigidity of spore membranes is dissimilar from the rigidity of vegetative cell membranes. This contrasts with their lack of lateral mobility as reported by Setlow. There were significant differences among the rigidities of vegetative cells from the five *Bacillus* species when they were grown on different media. Such media dependency has never been reported before and must be considered in future studies. In some species, here were also differences in rigidity between spore and vegetative cell membranes.

This research provides foundational rigidity values and correlates them with specific biological systems. It cannot, however, address the mechanism(s) behind these differences. Further studies will be required to provide a broader view on this topic. As the main force for altering membrane rigidities is their membrane composition, further studies on the fatty acid profiles of spore and vegetative cell membranes would provide better correlations. Ultimately, this knowledge could be used to alter the membrane composition of membranes *in vitro* and determine if it is the underlying mechanism of rigidity. This knowledge then might be applied to food systems, using specific ingredients to alter membrane properties and functions in food systems.

Figure 1: Spore Structure



The picture is not draw to scale. The labeled spore layer and the size of various layer vary significantly between spore of different species, especially for exosporium. Image source: P. Setlow, 2006.

Figure 2: The Sporulation Cycle

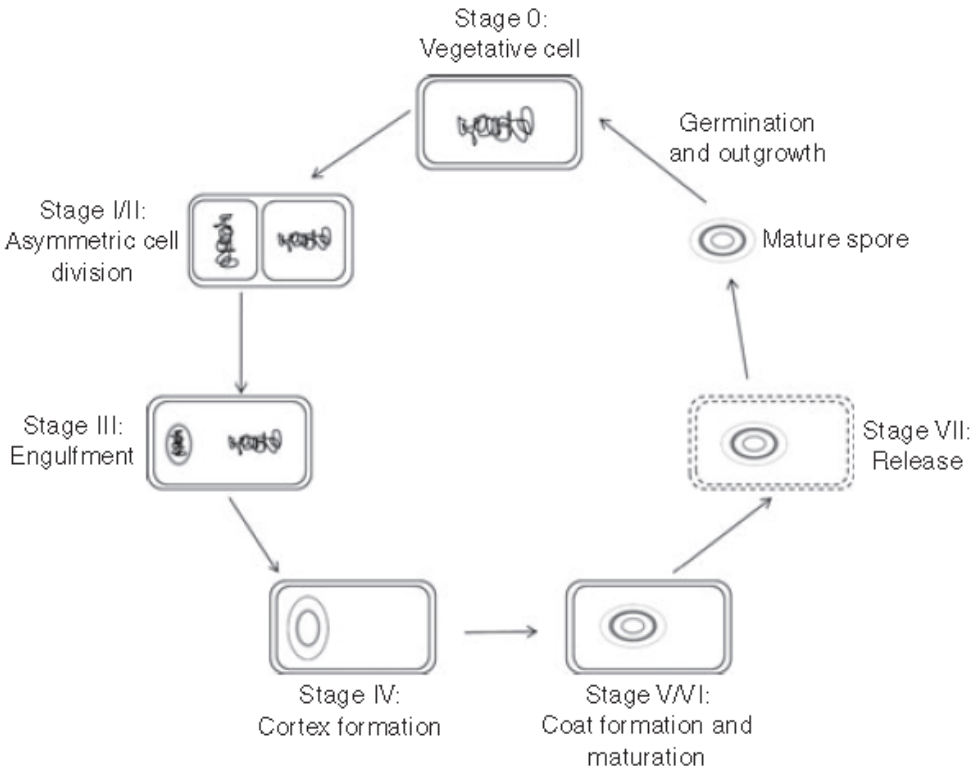


Image source: M.J. Leggett, et.al, 2012

Figure 3: Membrane Rigidity of Vegetative Cells Cultured using Different Media.

Error Bars = ± Standard Deviation.

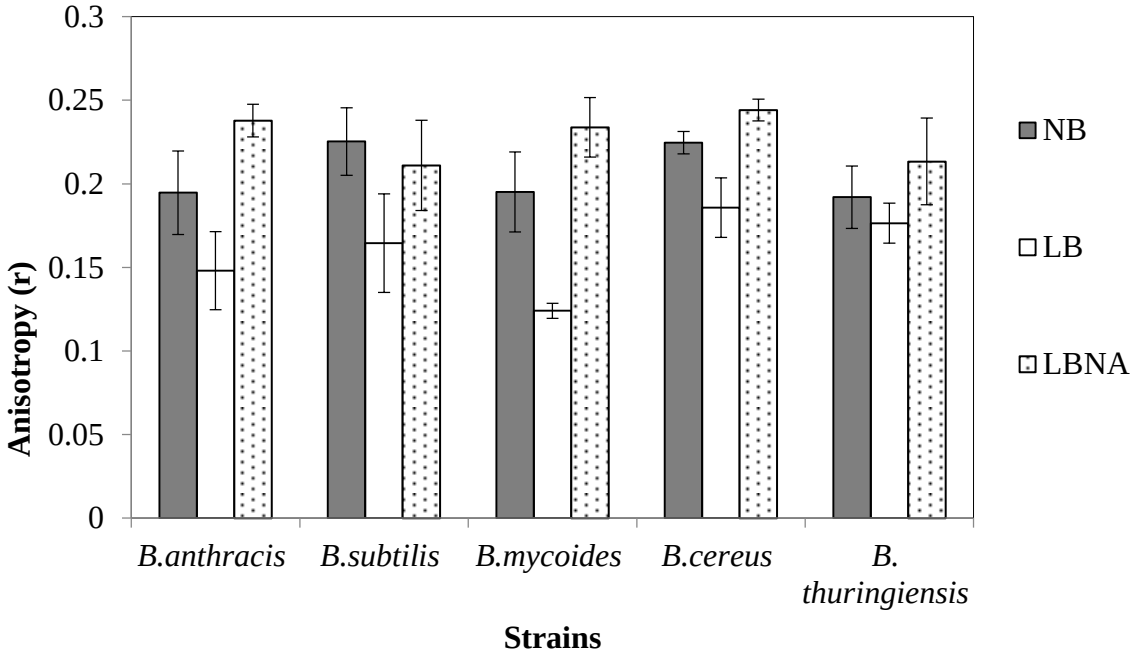


Figure 4: Spore Membrane Rigidity of *Bacillus subtilis B. anthracis*, and *B.mycoides*

Error Bars = ± Standard Deviation.

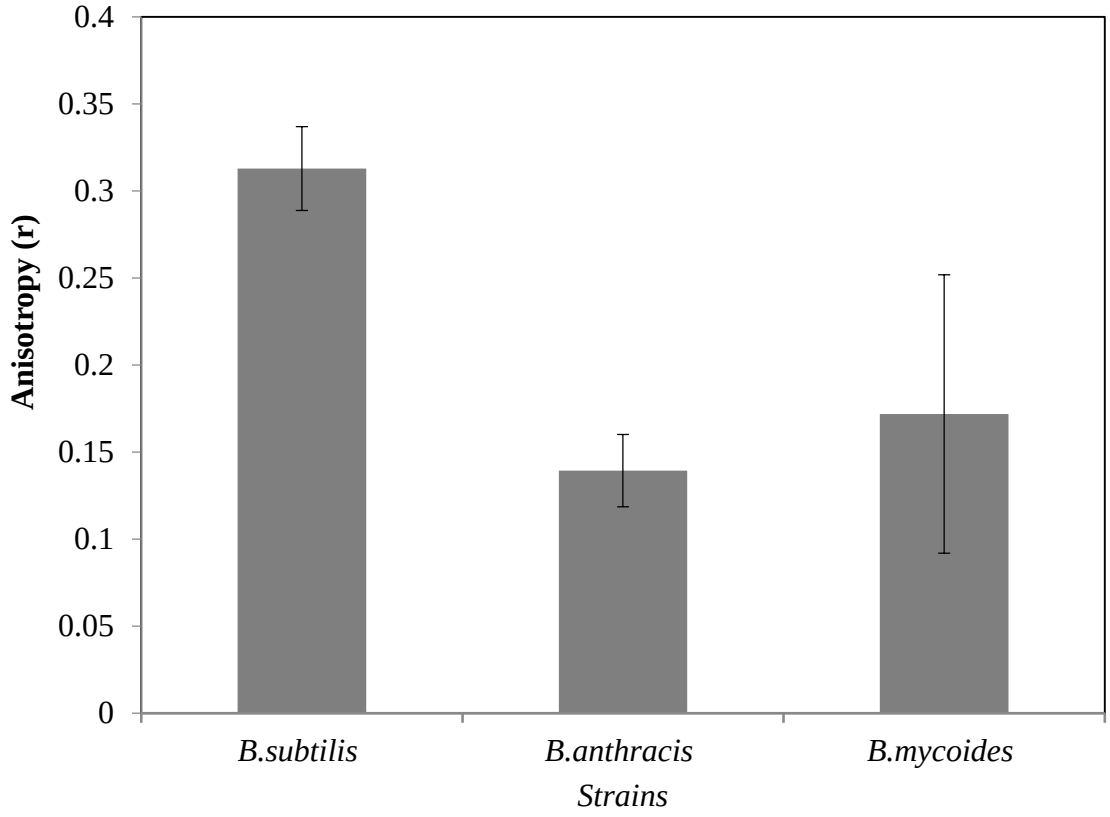


Table 1: Ability of Different Species to Sporulate in Different Media

	Sporulation Viability(+/-)				
	B.anthracis	*B.subtilis*	*B.mycoides*	*B.cereus*	*B.thuringiensis*
NB	–	–	–	–	–
LB	–	–	–	–	–
LBGA	+	+	+	+	+
LBNA	+	+	+/-	+/-	+/-

Table 2: Independent T-test Result for Membrane Rigidity of Vegetative Cells Cultured on LBNA, LB or NB

Strains	Independent T-test P value	
	LBNA vs. LB	LB vs. NB
B. anthracis	0.00	0.00
B. subtilis	0.02	0.00
B. mycoides	0.00	0.00
B. cereus	0.00	0.00
B. thuringiensis	0.01	0.09

P value<0.05 mean there is significant difference.

Table 3: Membrane Rigidity of Vegetative Cells Cultured Using Three Different Media and One-way ANOVA Test Result

Strains	Anisotropy (r) ± S.D			ANOVA (p value)
	Nutrient Broth	Luria Broth	Luria Broth Noble Agar	
B. anthracis	0.19±0.02	0.15±0.02	0.24±0.01	0.00
B. subtilis	0.23±0.02	0.16±0.03	0.21±0.03	0.00
B. mycoides	0.20±0.02	0.12±0.00	0.23±0.02	0.00
B. cereus	0.22±0.01	0.19±0.02	0.24±0.01	0.00
B. thuringiensis	0.19±0.02	0.18±0.01	0.21±0.03	0.01

P value<0.05 mean there is significant difference.

Table 4: The Membrane Rigidity for Vegetative Cells Grown on LBNA and LBGA

Strains	Anisotropy (r) ± S.D	
	Luria Broth Noble Agar	Luria Broth Granulated Agar
B. anthracis	0.24±0.01	0.17±0.01
B. subtilis	0.21±0.03	0.23±0.02
B. mycoides	0.23±0.02	0.15±0.08
B. cereus	0.24±0.01	0.19±0.06
B. thuringiensis	0.21±0.03	0.21±0.03

Table 5: One-way ANOVA: Post Hoc Multiple Comparisons (turkey test) of Spore Membrane Rigidity

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.17359*	.02447	.000	.1111	.2360
	3.00	.14104*	.02447	.000	.0786	.2035
2.00	1.00	-.17359*	.02447	.000	-.2360	-.1111
	3.00	-.03255	.02681	.460	-.1010	.0359
3.00	1.00	-.14104*	.02447	.000	-.2035	-.0786
	2.00	.03255	.02681	.460	-.0359	.1010

*. The mean difference is significant at the 0.05 level.

Group 1:*Bacillus subtilis*; Group 2:*Bacillus anthracis* Group 3:*Bacillus mycoides*

Table 6: Comparison of Membrane Rigidity of Vegetative Cells and Spores among Three Species and Independent T-test Result

Strains	Anisotropy (r) ± S.D		Independent t-test
	Vegetative cell*	Spore*	P value
Bacillus subtilis	0.21±0.03	0.31±0.02	0.00
Bacillus anthracis	0.24±0.01	0.14±0.02	0.00
Bacillus mycoides	0.23±0.02	0.17±0.08	0.12

*Vegetative cells were inoculated in LB (Luria Broth), 30 degree.

*Spores were sporulated on LBGA (Luria Broth Granulated Agar), 30 degree.

P value<0.05 mean there is significant difference.

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