Effect of Electric Field on Growth Kinetics of Yogurt Starter Cultures, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*

THESIS

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By

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Abstract

The effect of electric field on microorganisms has been a topic of research since the late 1800's. The vast majority of research relating to electric field since then has been on the inactivation of microorganisms. Although there were many claims to the lethal effects of electricity, it wasn't until the 1960's, when pulsed electric field was first used to inactivate microorganisms, that these claims were validated. With most of the attention on inactivating microorganisms, how electric field interacts with cells as well as the possibility of stimulation of growth was overlooked. It has only been in the past 30 years or so that valid, verifiable research has been conducted in the area of stimulating microbial growth through the application of electric fields. Fully understanding how electric fields stimulate microbial growth could have huge implications in the food industry, especially the fermented food industry. More research is needed to determine how electric fields interact with microorganisms and what conditions cause a stimulating effect.

The objective of this research was to evaluate the effects of various electric fields, including moderate electric field (MEF) and pulsed electric field (PEF), on the growth kinetics of various lactic acid bacteria in order to expand upon research that was previously limited to only *Lactobacillus acidophilus*.

Common yogurt starter cultures, *Lactobacillus bulgaricus* and *Streptococcus thermophilus,* were subjected to MEF during fermentation in de Man Rogosa Sharpe (MRS) broth. The electric field strength remained constant throughout all experiments at 1 V/cm. Two vessels were created to allow for side by side fermentation comparison. One vessel was ohmically heated by passing a current through the MRS broth and the other vessel was heated conventionally using a heated water jacket. Fractions of the fermentation over time were collected for microbial enumeration as well pH measurement. Fermentation by *S. thermophilus* at 35°C with 45Hz MEF showed a reduced lag time of 2 hours 6 minutes as compared to the control which had a lag time of 2 hours 45 minutes. Increasing the temperature or frequency resulted in loss of the reduced lag time for *S. thermophilus*. *L. bulgaricus* showed no significant difference in growth or pH between the control and all treatments.

The effect of pulsed electric field on the growth kinetics of various strains of lactic acid bacteria (LAB) was also investigated. Two fermentation vessels (for treatment and control) were filled with MRS broth and held at either 25°C or 35°C. The broth was inoculated with *L. bulgaricus*, *S. thermophilus,* or *Lactococcus lactis* (*L. lactis*) and was exposed to PEF at strengths ranging from 2-10 kV/cm during fermentation. Fractions of the fermentation collected over time were plated for microbial enumeration, tested for pH, and in the case of *L. lactis*, assayed for bacteriocin production. There was no growth stimulation effect observed for any culture at the conditions tested in these experiments. A general trend of reduced growth was seen as electric field was increased for both *S. thermophilus* and *L. bulgaricus*. It was hypothesized that *L. lactis* may produce the

bacteriocin nisin at an increased rate due to cell stress and self induction from pore formation caused by the PEF treatments, but the opposite was found. The control produced nisin slightly faster showing the first clearing zone (200 arbitrary units) at 10 hours with the treated showing the first clearing zone (200 arbitrary units) at 11 hours. Although a stimulation effect was not observed in these experiments, further research utilizing differing treatment parameters would be needed in order to claim that mild PEF does not cause the stimulation of bacterial growth.

Acknowledgments

I would like to offer my deepest gratitude to my advisor, Dr. Ahmed Yousef, for believing in me and taking me on as student in his lab at The Ohio State University. You always offered your knowledge and wisdom not only in the area of academia but also in life. You have taught me how to be a better scientist, teacher and person and for that I will be forever grateful. To my committee members; Dr. Sudhir Sastry and Dr. Luis Rodriguez-Saona, thank you for all your suggestions and help.

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Literature Review

Lactic Acid Bacteria

Lactic acid bacteria (LAB) are a clade of bacterial species that exhibit similar phenotypic properties. The following are typical phenotypic traits expressed amongst LAB: Gram-positive, non-spore-forming, catalase-negative, devoid of cytochromes, fermentative with aerotolerance, fastidious, acid tolerance, and lactic acid is the major end product during sugar fermentation. (Salminen and others 2004). Although these are the typical traits expressed by LAB, there are exceptions to all of these rules, except for Gram reaction. LAB have been thoroughly studied due to their common presence in nutrient rich food like milk and meat as well as their presence in the mouth, intestinal tract, and vagina of mammals (Wood and Holzapfel 1995).

In the food industry, LAB are of particular interest due to their ability to ferment milk into yogurt or cheese, meat into sausage, cabbage into sauerkraut, and others. In the last couple of decades, LAB probiotics have become a point of interest. Some of the species within the LAB clade that are reported to have probiotic effects include *Lactobacillus* spp. and *Lactococcus* spp. Some of the probiotic effects of LAB include the treatment and prevention of diarrhea, irritable bowel syndrome (Halpern and others 1996), inflammatory bowel disease (Saarela and others 2002), as well as the possible

reduction of cancerous colon tumors (Reddy 1999). The fermentation of food with LAB has been done for over 8,000 years (Yildez 2009). LAB are common contaminates of milk due to their ubiquitous nature as well as their ability to ferment lactose (the main sugar in milk) and were unknowingly used to make fermented food products. One of the most common fermented food products around the world is yogurt. While many different kinds of LAB can ferment milk, in the United States and Europe, a fermented milk product can only be called yogurt if it is fermented with *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (US-FDA 2011a).

Streptococcus salivarius* subsp. *thermophilus

Streptococcus salivarius subsp. *thermophilus* (*S. thermophilus*) was not always classified under *salivarius* species. Originally it was only classified as *Streptococcus thermophilus.* With the rise of genotyping in the 1980's, the *Streptococcus* genus was split into three genera, *Streptococcus, Enterococcus,* and *Lactococcus* (Stiles and Holzapfel 1997). *Streptococcus* is farther divided into three general groups: pathogenic, oral, and other. The oral group is then divided into five phylogenetic subgroups: *salivarius, mitis, anginosus, bovis, mutans* (Salminen and others 2004). While currently thermophilus is a sub-species, there are some who believe it deserves to be a species of *Streptococcus* (Stiles and Holzapfel 1997). *S. thermophilus* is a Gram-positive, facultative anaerobic coccus that arranges in chain patterns. This microorganism was first described by Orla-Jensen (Sherman and Stark 1931). It was named thermophilus due to its ability to grow at elevated temperatures, 45ºC to 50ºC, and its dislike for lower temperatures (4ºC).

Although it can grow at higher temperatures, *S. thermophilus'* optimal temperature is 37ºC (Sherman and Stark 1931). Currently *S. thermophilus* is the only *Streptococcus* isolate that is used in food technology. It is used predominantly as a yogurt starter culture and is inoculated along with *Lactobacillus bulgaricus* due to their synergistic effect (Yildez 2009).

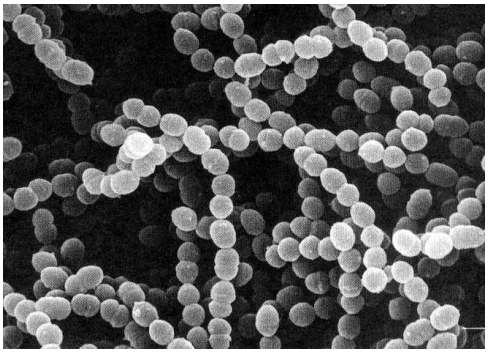

Figure 1.1 *Streptococcus salivarius* subsp. *thermophilus*

(http://www2.unibas.it/parente/Risorsepersonale/Stherm.jpg)

Lactobacillus delbrueckii* subsp. *bulgaricus

Lactobacillus delbrueckii subsp. *bulgaricus* (*L. bulgaricus*) is a Gram-positive, non-motile, anaerobic, non spore-forming rod, that has an optimal growth temperature of 44ºC (Germond and others 2003). It was named bulgaricus after the Bulgarians whose known longevity and healthy lives were attributed to the consumption of large amounts of yogurt (Yildez 2009). Today, *Lactobacillus* is divided into three groups based on the most common fermentation pathway utilized by each isolate; obligate homofermenter, obligate heterofermenter, or facultative heterofermenter. *L. bulgaricus* falls under the category

obligate homofermenter (Yildez 2009). *L. bulgaricus* is one of three subspecies of *L. delbrueckii*. The others are *L. delbrueckii* subsp. *lactis* and *L. delbrueckii* subsp. *delbrueckii*. While the others are used in fermentations, *L. bulgaricus* is one of the two cultures used in the production of yogurt and is considered the flavor producing strain (Tribby 2009). *L. bulgaricus* is inoculated alongside *S. thermophilus* due to their synergistic effects.

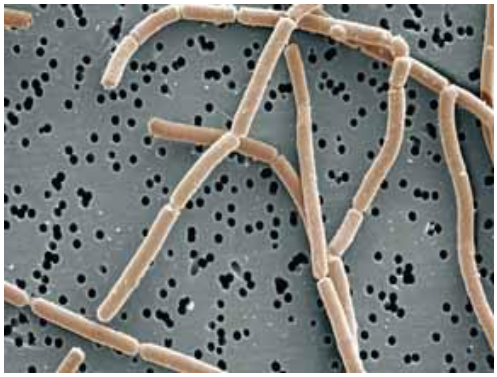

Figure 1.2 *Lactobacillus delbrueckii* subsp. *thermophilus* (Yildez 2009)

Synergy between *L. bulgaricus* and *S. thermophilus*

Synergy in biology is when two or more organisms work together to achieve an enhanced effect that would not be produced singly (Biology-online.org 2011). In the case of synergy between *L. bulgaricus* and *S. thermophilus* in milk, the two grow faster in the presence of each other then they do separately. When fermented together, *S. thermophilus* grows the fastest in the beginning due to its aerotolerance. *S. thermophilus* has greater peptidase activity while *L. bulgaricus* has a greater proteolytic ability

(Chandan 2006). The combination of these enzymes allows for faster acquisition of nutrients that would not be available if milk was fermented with each organism separately. *S. thermophilus* also acidifies the environment which creates better growing conditions for *L. bulgaricus*. Lastly, *S. thermophilus* produces carbon dioxide which has also been found to stimulate *L. bulgaricus* growth (Driessen and others 1982). Today, this synergistic effect is utilized in the lactofermentation of yogurt.

Lactofermentation

Fermentation is an anaerobic process microorganisms utilize to get energy from various carbon sources. In lactofermentation, there are two fermentation pathways: heterofermentation (heterolactic), homofermentation (homolactic). Homolactic bacteria (e.g., *L. bulgaricus*, *S. thermophilus*) use the Embden-Meyerhof (EM) pathway (Mehrotra, R.S., Sumbali, G. 2009).

$$\text{Glucose} + 2\text{ADP} + 2\text{P}_i \rightarrow 2\ \text{Lactic acid} + 2\text{ATP}$$

This is considered a homolactic fermentation due to the fact that there is only one byproduct, lactic acid. Heterolactic bacteria use the pentose phosphate pathway.

$$\text{Glucose} + \text{ADP} + \text{P}_i \rightarrow \text{Lactic acid} + \text{ethanol} + \text{CO}_2 + \text{ATP}$$

This pathway yields less energy per glucose molecule as compared to the EM pathway but includes the production of ethanol and CO_2. In both pathways lactose is the beginning carbon source. It is a disaccharide made of galactose and glucose. The lactose is hydrolyzed by splitting the β-linked glucose/galactose. The galactose is then converted into glucose, the starting sugar of the EM and pentose phosphate pathways (Figure

1.3). Some of the more common food fermentations carried out by LAB today include yogurt (*Streptococcus* spp. and *Lactobacillus* spp.), certain cheeses (e.g., *Lactococcus* spp.), sauerkraut (e.g., *Leuconostoc* spp.), sausage (e.g., *Pediococcus* spp.), and kimchi (variety of LAB).

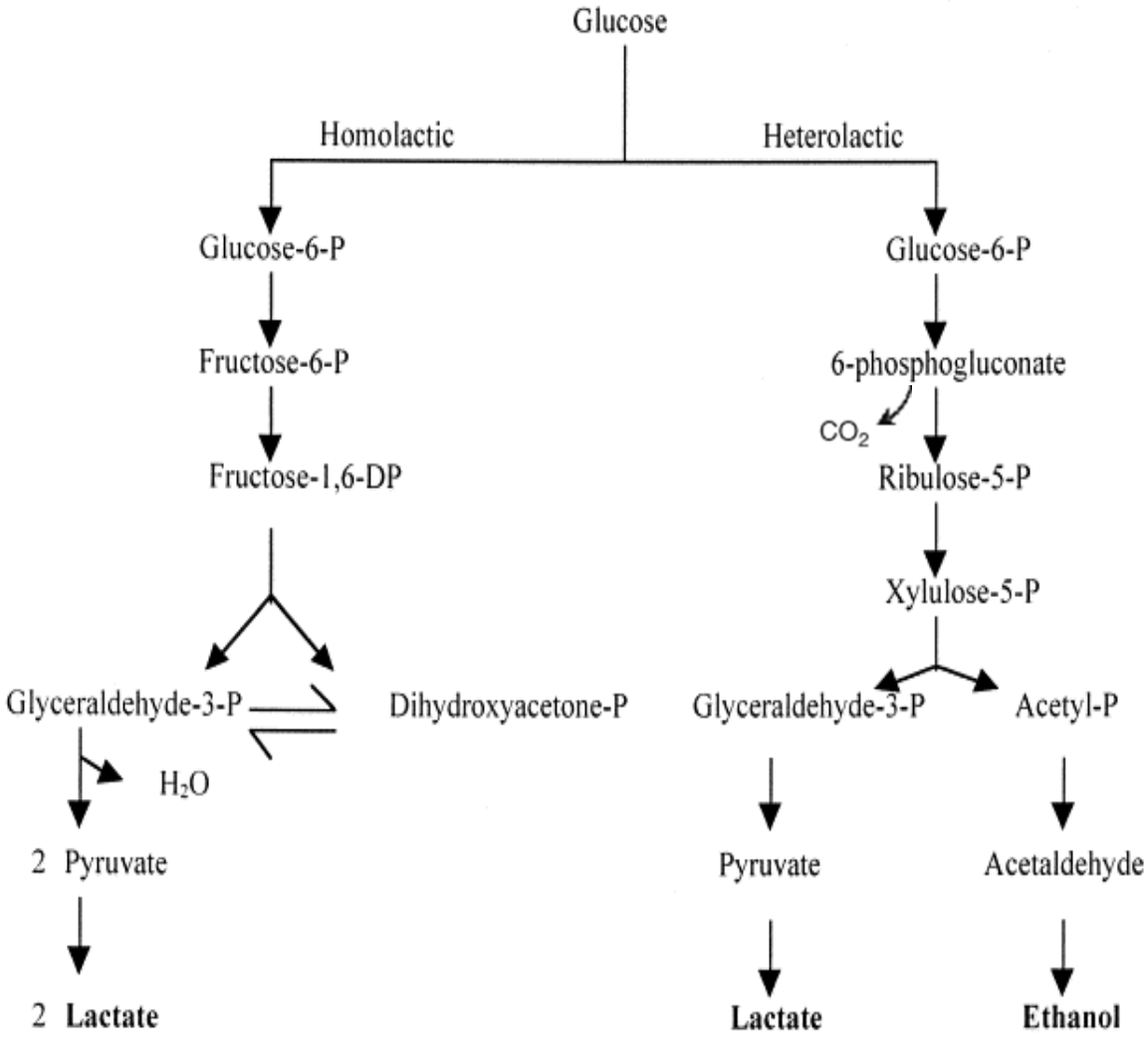

Figure 1.3 Homofermentation and Heterofermentation pathways (Caplice 1999)

Yogurt

Yogurt is produced through the fermentation of milk with *S. thermophilus* and *L. bulgaricus*. The bacteria consume the lactose present in the milk and through fermentative pathways, excrete lactic acid. This lactic acid, along with other excreted acids (i.e., formic acid), reduce the pH of milk (approx. 6.5) to about a pH of 4.5. At this lower pH, milk proteins, especially casein, with the help of proteolytic enzymes, denature and coagulate to form curds (Yildez 2009). The temperature used for fermentation is 43ºC. The optimal growth temperature of *S. thermophilus* is about 39ºC while for *L. bulgaricus* it is 45ºC. Traditionally the ratio of the inocula of *S. thermophilus* to *L. bulgaricus* is 1:1 but inoculation at higher levels of *thermophilus* to *bulgaricus* is also used (Chandan 2006). This is due to the fact that *S. thermophilus* is considered the main pH decreaser while *L. bulgaricus* is considered the main flavor producer. Varying these ratios changes fermentation time as well as flavor and yogurt gel structure.

There are two methods of fermentation that are used in the yogurt industry today; stirred style and set style. Stirred style is a batch fermentation where large fermentors are used to make yogurt in large quantities. The resulting product is then stirred and dispensed into smaller packages. Set style fermentation is where the inoculated milk with all of the added ingredients is put into the final package before the fermentation begins. Set style can be done in batch or can be done continuously. The cups are then incubated for approximately 4-6 hours before being cooled and shipped. In the food industry today, having a continuous process is highly efficient and desired. Considering that stirred style yogurt batch fermentation cannot be a continuous workflow, decreasing the fermentation

time while maintaining yogurt quality has been a point of interest. The fermentation is considered complete when the pH reaches 4.2-4.3 (Yildez 2009). Changing the standard conditions to favor for a faster fermentation normally leads to undesired effects. Soukoulis and others (2007) shortened the incubation period by adding whey powder but found this produced a consistency of yogurt that was undesirable. Aguirre-Ezkauriatza and others (2008) decreased the fermentation period by agitating during fermentation but also reported a decrease in viscosity as well as whey retention. Milk pre-treated with proteolytic enzymes before fermentation showed a reduced fermentation period but the resulting product had poor water retention and different physical properties than standard yogurt (Gassem and Frank 1991).

Lactococcus lactis* subsp. *lactis

Lactococcus lactis subsp. *lactis* (*L. lactis*) is a LAB of great importance to dairy fermentation. This species is very common in the food industry today and is typically a cheese starter culture. It is a Gram-positive, nonpathogenic, which is facultatively anaerobic, with a main fermantative end product of L-lactic acid (Teuber M. 2009). This microorganism used to be categorized under *Streptococcus lactis* but further investigation of DNA showed that a group of the previous *Streptococcus* genus showed significant similarities that were different from the rest of the genus, thus warranting a new genus (Schleifer and others 1985). *L. lactis* is a natural contaminant of milk and today the culture is used in the production of cheeses that are cooked around the temperature of 40ºC (Fox and McSweeney 2004). *L. lactis* is also used in the food industry for its

production of a class I bacteriocin, nisin. This bacteriocin is known to have antimicrobial properties against a wide range of Gram-positive bacteria (Teuber M. 2009; Ramasamy and others 2006). *L. lactis* is not only used in the food industry but also in the medical sciences and is considered to be the most fully sequenced lactic acid bacterium (Bolotin and others 2001).

Current medical research regarding the use of *L. lactis* has been in the area of using the bacterium in vaccination. The bacteria are genetically engineered to produce proteins of a specific pathogen and inoculated in the nasal mucosa (Norton and others 1997). An immunological response is elicited and the appropriate antibodies are created without there ever being a threat of infection. This has been shown to be effective in mice for various pathogens including *Helicobacter pylori* (Lee and others 2001), *Brucella abortus* (Ribeiro and others 2002), *Streptococcus pneumoniae* (Hanniffy and others 2007), as well as non-bacterial diseases such as HIV (Xin and others 2003), malaria (Ramasamy and others 2006), and human papillomavirus (Bermudez-Humaran and others 2005).

Bacteriocin

Bacteriocins are antimicrobial peptides that express activity against other Gram-positive bacteria with a ranging effectiveness. They are categorized into the following groups; lantibiotics (Class I); small non-lantibiotics (class II); large non-lantibiotics (class III) (Cintas and others 2001). The larger (>30 kDa) non-lantibiotics are heat sensitive while the smaller (<10 kDa) are heat stable (Cintas and others 2001). The bacteriocins

mode of inactivation vary depending on the specific bacteriocin but the most common modes of inactivation are increased permeability of the membrane through pore formation and dissipation of the proton motive force (PMF) (Héchard and Sahl 2002).

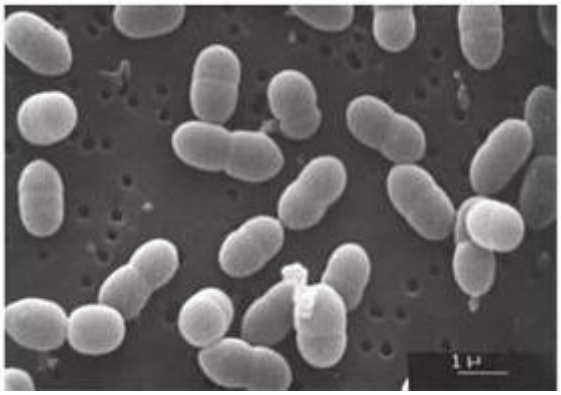

Figure 1.4 Scanning electron microscope picture of *Lactococcus lactis* subsp. *lactis* (Teuber M. 2009).

While some bacteriocins act slowly through inhibiting biosynthesis of the cell wall, others, like nisin, rapidly kill cells by pore formation and the leaking of intracellular materials (Héchard and Sahl 2002). The producing strains themselves are protected from their own bacteriocin through the expression of immunity proteins (Cintas and others 2001). Although there are similarities between bacteriocins and antibiotics, they are considered different for several reasons including toxicity and activity (Table 1.2).

The most studied and commercially used bacteriocin is nisin (class I) (Cintas and others 2001). Nisin is produced by the species *Lactococcus lactis* and is known for having very strong activity against a wide range of Gram-positive bacteria including

Staphylococcus aureus, *Listeria monocytogenes* and *Clostridium botulinum* (Hurst 1981; Cintas and others 1998). Nisin is also unique in that it is stable at low pH. This makes it a great bacteriocin to be used as a food additive and is currently the only bacteriocin approved by FDA (US-FDA 2011b).

Due to bacteriocins antibacterial activity and the wide range of food products they can be used in, many studies have been completed in the area of increasing the production of bacteriocins (Hirsch 1951; Jozala and others 2005; De Vuyst and others 1996) including the use of electric fields (Loghavi and others 2007)

Table 1.1 Differences between bacteriocins and antibiotics (Cleveland and others 2001)

Characteristics	Bacteriocins	Antibiotics
Application	Food	Clinical
Synthesis	Ribosomal	Secondary metabolite
Activity	Narrow spectrum	Varying spectrum
Host cell immunity	Yes	No
Mechanism of target cell resistance or tolerance	Usually adaptation affecting cell membrane composition	Usually a genetically transferable determinant affecting different sites depending the mode of action
Interaction requirements	Sometimes docking molecules	Specific target
Mode of action	Mostly pore formation, but in a few cases possibly cell wall biosynthesis	Cell membrane or intracellular targets
Toxicity/side effects	None known	Yes

Electric Field

Electric fields were first defined by physicist Michael Faraday. Electric fields are maps of the force that is exerted on electrically charged objects (test charge) in the vicinity of an electrically charged object (Figure 1.5). This force is described in Newtons per coulomb (N C^{-1}) or volts per centimeter (V/cm). The relationship of the object's charge (test charge) and its position within the electric field in relation to force is described as follows: F = q* E (Pender 1910); where "F" is the force experienced by the particle, "q" is the charge of the particle, and "E" is the position of the charge within the electric field. Electric field strength can be defined as the force exerted on a positive test charge of 1 coulomb. In the case of using two electrodes, electric field strength can be defined as the voltage divided by distance.

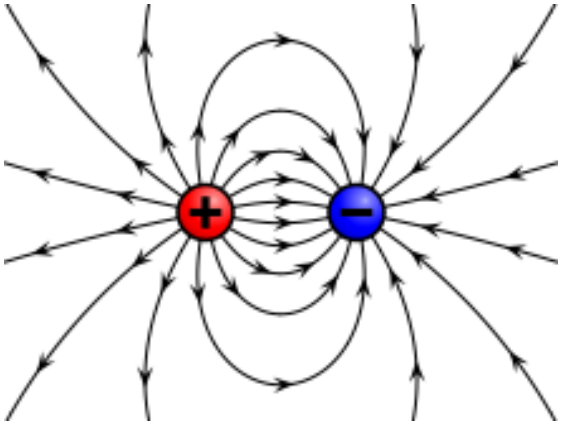

Figure 1.5 Electric field (arrows) surrounding a positive and negative charge

Electricity

Electricity is a phenomenon where charged particles (e.g., electrons) interact with the atoms of a substance. For simple physics, electricity can be defined by these three parameters: resistance, current, and voltage. Resistance is a property of a substance which resists the flow of an electrical charge. Insulators have very high resistance while conductors have low resistance. This property is measured in Ohms (Ω). Current is the measurement of the flow of the electrical charge and is measured in amps (A). One ampere is the flow of 6×10^{18} electrons per second through a substance (Anderson 2008). Voltage (V) is the measure of the potential or the ability to move an electrical charge through a resistance. These three parameters of electricity are related by the following equation: V = I*R (voltage = current * resistance).

Current can be further defined as alternating current (AC) or direct current (DC). Alternating current periodically reverses the flow of electrons. This change in current typically takes the form of a sine wave (Figure 1.6). One period is the time it takes for the signal of the sine wave to complete one cycle (the distance it takes before the sine wave repeats itself). The number of cycles completed in 1 second is called a Hertz (Hz). The electricity most common in households today is alternating current at 50 or 60 Hz depending on the country. The wave form of alternating current does not necessarily have to be a sine wave. With the proper equipment (i.e. wave generator) you can generate waves in the form of pulses, saw-tooth, triangle, etc. (Figure 1.7).

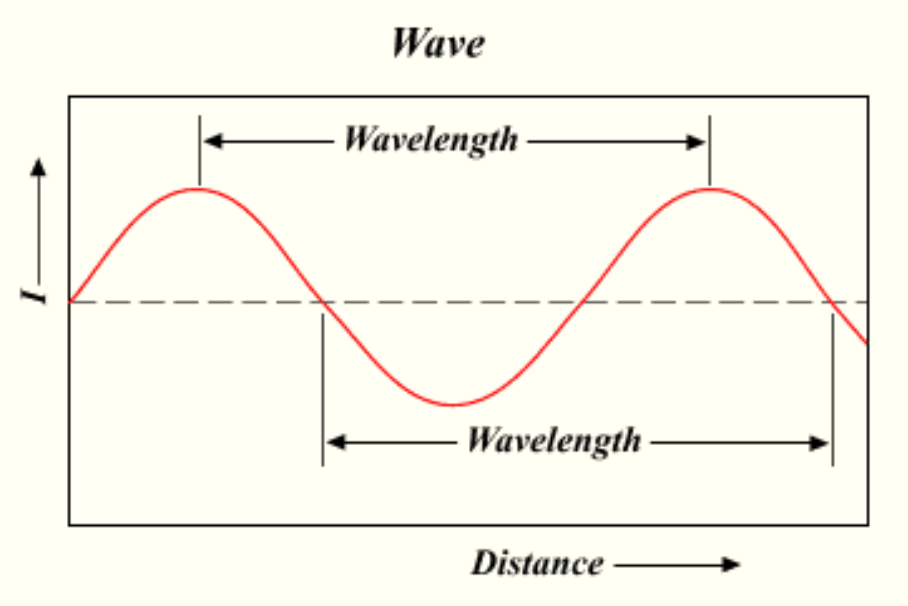

Figure 1.6 Sine wave with parameters

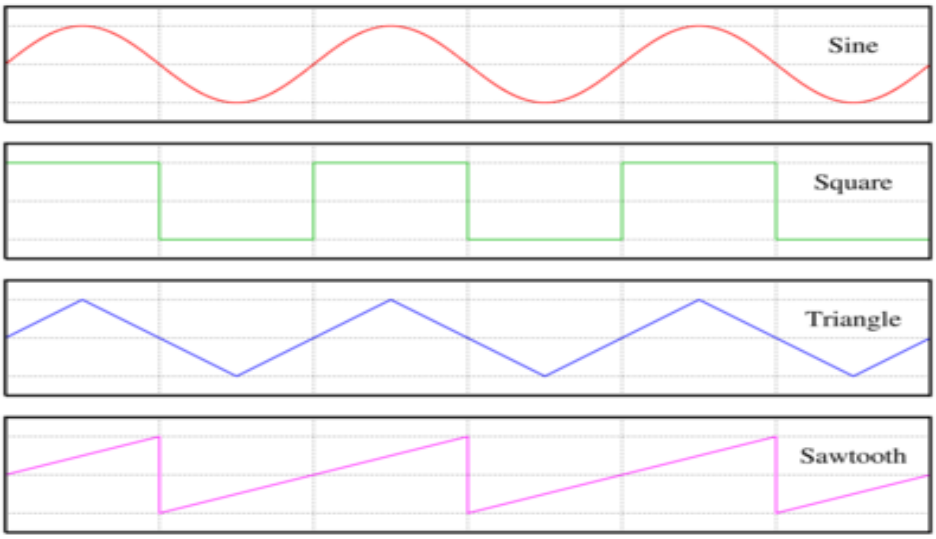

Figure 1.7 Various wave shape possibilities

Electroporation

The use of electric field to create pores in membranes of prokaryotic and eukaryotic cells has been studied since the 1970's. Electroporation is the formation of a pore across the membrane due to electromechanical compression and electric field-induced tension (Wouters and others 2001b). Electropermeabilization is the term used to describe the phenomenon in which there is a significant increase in membrane permeability due to electric fields (Ohshima and others 1995; Wouters and others 2001a; Wouters and others 2001b). The degree of electric field required in order for the breakdown of the membrane is in the range of 200-1,000 mV across the membrane (Sale and Hamilton 1967; Chassy 1988). This critical membrane potential varies due to different membrane composition, cell diameter, and cell-wall characteristics (Chassy 1988). Two theories have been proposed as the cause of death from high electric fields. The first is that pores are formed across the membrane and that the uncontrolled flow of water in, and intracellular fluids out due to osmotic pressure, causes the cell to swell and eventually rupture (Figure 1.8) (Vega-Mercado and others 1996). The second theory suggests cell death from chemical stress associated with molecular transport (Wouters and others 2001a; Wouters and others 2001a). Today, electroporation is primarily used in transformation of microorganisms. Strong electric fields are generated and pulsed across a suspension of target cells and DNA. DNA, which is normally unable to cross the cell membrane, is now able to do so due to pore formation.

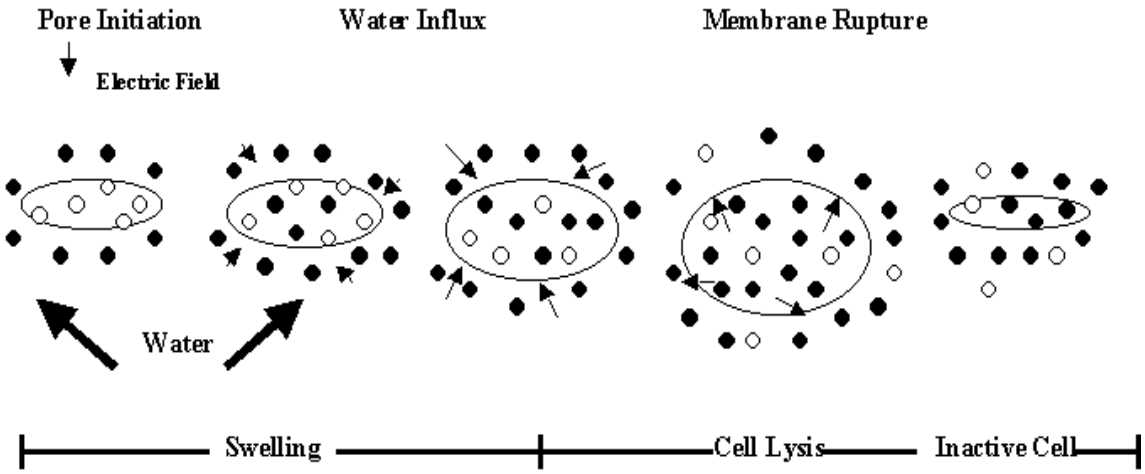

Figure 1.8 Theorized method of inactivation of vegetative cells via Pulsed Electric Field (Vega-Mercado and others 1996)

Pulsed Electric Field

Pulsed electric field (PEF) is an emerging non-thermal processing technology. It uses electric fields, in the 20-80 kV range, that are pulsed to inactivate microorganisms without the application of heat. This can allow for a better retention of the food product structure/flavor and overall "freshness" as compared to thermal processing (Evrendilek and others 2000; Rivas and others 2006). While heat is not the mode of inactivation, there is some heat generated due to Joule's Law: $Q = I^2 * R * t$. Where Q = heat expressed (in Joules), I = current (in amperes), R = resistance (in ohms), and t = time (in seconds). The increase in heat of the food product is minimal due to the short time exposed to electric fields (in the range of microseconds) and varies depending on the conductivity of

the food sample, electric field strength, and pulse duration. PEF is suitable for the pasteurization of liquid or semi-liquid food including milk, juices, yogurt, soups, and liquid eggs (Raghupathy and others 2005). Currently, Genesis Juice Corp. is the only known company in America approved by the FDA to processes juice via PEF.

Although PEF is a promising new technology, there are limitations for its application in food. Products with high conductivity or bubbles can cause arcing which will drastically increase local temperature and cause the formation of unwanted radicals. It has also been found that when food products of lower conductivity are exposed to PEF, there is greater inactivation as compared to those of higher conductivity (Wouters and others 2001b). The effect of product pH on inactivation varies dependent on the microorganism (Wouters and others 2001a). Lower water activity has been shown to reduce inactivation of target microorganisms (Mi and others 2002). Another limitation is that PEF is only effective against vegetative cells and not spores (Grahl and Märkl 1996). The effectiveness against vegetative cells varies depending on species and strain (Figure 1.9). It has been suggested that efficiency of PEF treatment depends on cell size and shape (Kekez and others 1996; Wouters and others 2001b). Lastly, there is a limitation in how research completed in this area can be compared. All of the variables in Table 1.1 need to be taken into consideration when comparing results of various PEF research designs. While some processing variables can be accounted for easily (e.g., pulse length and electric field strength), others, including gap, volume and chamber configuration are difficult to assess their contribution to process lethality. It is also important to note what growth phase the microorganisms are in when subjected to PEF. It has been shown that

cells in exponential phase are more sensitive to PEF treatment then lag and stationary phases (Pothakamury and others 1996). While PEF is intentionally used in the processing of food samples there are also lower electric fields that occur unintentionally as a result of electrically heating (ohmic heating) food.

Table 1.2 Various Pulsed Electric Field parameters (Wouters and others 2001b)

Process parameters	**Microbial characteristics**	**Product parameters**
Electric field strength	**Growth conditions:** Initial inoculum	Composition
Pulse length	Medium composition	Conductivity
Number of pulses	Temperature	Ionic strength
Start temperature	Oxygen concentration	pH
End temperature	**Growth phase:** Time of incubation	A_w
Pulse shape	**Recovery conditions:** Medium composition	
Treatment chamber: Configuration	Temperature	
Volume	Recovery time	
Gap	Oxygen concentration	
Flow rate[a]		
Frequency		
Specific energy		
Residence time[a]		

[a] For continuous process

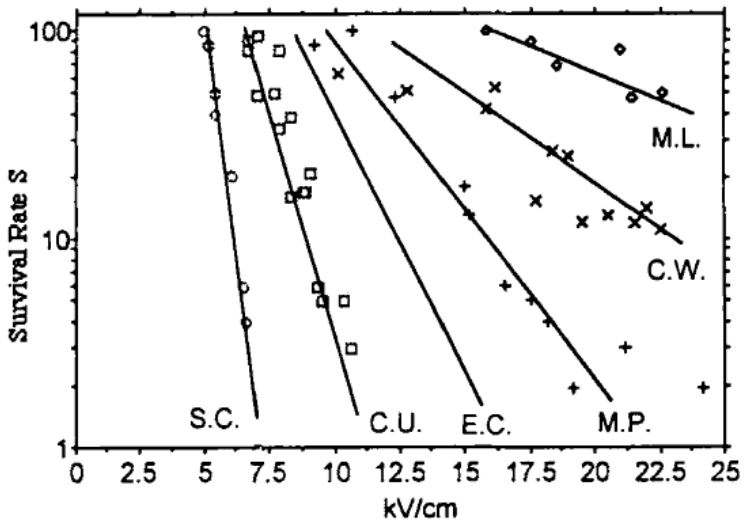

Figure 1.9 Relationship between field strength and microbial inactivation. Ten pulses of 20 μs; S.C., *Saccharomyces cerevisiae;* C.U., *Candida ufilis;* E.C., *Escherichia coli;* M.P., motile pseudomonad; C.W., *Closfridium welchii;* M.L., *Micrococcus lysodeikficus* (Sale and Hamilton 1967).

Moderate Electric Field

Moderate electric field is a relative term used to describe the electric field strengths that occur during ohmic heating. Sastry describes it as "electric fields less than or equal to 1000 V/cm" (Sastry 2008). While ohmic heating is used primarily for inactivation of microorganisms through thermal processing, recent studies have shown an interaction between MEF and membranes of prokaryotic and eukaryotic cells. When MEF was used as a pretreatment, it has been shown to improve the drying rate of vegetable tissue (Wang and Sastry 2000). Extraction of juices from apples and potatoes increases with MEF treatment (Praporscic and others 2006). During blanching, mushrooms showed a greater shrinkage under ohmic heating as compared to conventional heating (Sensoy and Sastry

2004). Fermentation by *L. acidophilus* exhibits a reduced lag time as well as increased metabolite production under MEF conditions, as compared to conventional heating (Cho and others 1996). Further tests were conducted and demonstrated an increased uptake of a fluorescent dye by *L. acidophilus* from media when under MEF conditions (Loghavi and others 2009). All of these tests show that interactions of electric fields and membranes can and do occur at moderate electric field conditions.

Ohmic heating

Ohmic heating has gone by many different names throughout the years including joule heating, electrical resistance heating, direct electrical resistance heating, electro-heating and electroconductive heating (Mitelut and others 2011). Ohmic heating is the process of applying alternating current (ac) to an electrically conductive liquid/semi-liquid that has a resistance. When an electric current is applied to anything with resistance, energy is given off in the form of heat. This conversion of energy to heat is very efficient and can be as high as 90% (Ghnimi and others 2007). The uniqueness of ohmic heating comes from the ability to heat from within the food system instead of heating from the outside in, which is considered the conventional method (Richardson 2004). This is especially important for liquids with small particulates. With ohmic heating, it is possible to heat the inside of particulates faster than the surrounding fluid, which is impossible with conventional methods. This allows for a faster come up time, which in turn reduces the amount of energy needed to achieve uniform heating. This reduced time of processing also allows for greater retention of the original structure/flavor of the food system as well

as a greater retention of vitamins and nutrients when compared to conventional methods (Raghupathy and others 2005).

Ohmic heating was first developed and implemented in the processing of milk around 1919 (Arthur K. Anderson, Rubin Finkelstein 1919). The process was called the Electropure Process. The electrodes were set up so that the milk would have to flow continuously in order to complete the electrical circuit. If the milk stopped flowing, the circuit would be broken and no electric current would be applied to the milk. Many researchers of the time claimed that there was a lethal, non-thermal effect on microorganisms caused by the electricity. An in-depth review of the effects of electric field on microorganisms by Palaniappan et al. concluded just the opposite. They found that none of the early studies were able to appropriately separate the electrical and thermal effects on bacteria thus making the claims of non-thermal lethal electrical effects unfounded. Another issue of that time was the problems with using electrodes that would corrode. The rate of this corrosion is dependent upon the type of material that makes up the electrode. Ohmic heating fell out of favor as a method of thermal processing around the 1950's due to the lack of technology to provide inert electrode materials (Richardson 2004).

In the food industry today, ohmic heating is used as a unique way of thermally processing liquids/semi-liquids. Due to consumer demands for minimally processed, safe food, a revived interest has occurred starting in the 1990's. New advancement in ohmic systems allows for particulate foods to be processed at the rate of a high temperature short time (HTST) processes without the problems of uneven heat transfer (Richardson

2004). The price and availability of industrial grade inert electrodes was also a factor in the revisiting of ohmic technology. Currently products such as fruit in sauces and syrups, liquid egg, and low-acid particulate products in a can have been processed using ohmic technology (Raghupathy and others 2005) . While bacterial inactivation is normally the focus of ohmic heating, there are other applications including blanching, starch gelatinization, thawing, peeling of fruits, dehydration, extraction, fermentation and inactivation of proteins (Anderson 2008).

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Chapter 2
Growth Kinetics of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* Under Moderate electric field

Abstract

Common yogurt starter cultures, *Lactobacillus bulgaricus* and *Streptococcus thermophilus,* were subjected to moderate electric field (MEF) during fermentation. The electric field strength remained constant throughout all experiments at 1 V/cm. A fermentation vessel that was heated conventionally (control) and another that was heated ohmically (treatment) via platinized titanium electrodes, were filled with 1.5 L of MRS broth and inoculated side by side. Treatment variables were frequency (45 Hz or 60 Hz), temperature (35°C or 44°C), and microorganism (*L. bulgaricus* or *S. thermophilus*). Fractions of the fermentation over time where collected and plated (for microbial enumeration), pH was determined in each fraction. Fermentation by *S. thermophilus* at 35°C with 45 Hz MEF showed a reduced lag time ($p < .05$) as compared to the control. Increasing the temperature or frequency resulted in the loss of significant difference between the lag times of the control and the treated. *L. bulgaricus* showed no significant difference in growth or fermentate pH between the control and all treatments. Results indicate that reaction of microorganisms to MEF may be species specific as well as temperature and frequency specific.

Introduction

Recent technological strides in electric field setups combined with consumer demands for high quality, minimally processed food products has increased interest in electrical processing such as ohmic heating and pulsed electric field (PEF). The effect of electric field on prokaryotic cells has been an area of research since 1909 with George E. Stone looking at the effects of electric field on *Pseudomonas* spp. and *Bacillus* spp. in water and milk (Stone 1909). The majority of the studies following Stone's work looked at the lethal effect electric fields against microorganisms and the feasibility of implementing this technology in the industry. A review of these studies concluded that no non-thermal lethality occurs at low electric fields commonly produced during ohmic heating and while there are benefits to ohmic heating, microbial lethality was not one of them (Palaniappan and others 1990). While lethality does not occur at moderate electric fields, (field strengths lower than 1,000 V/cm) recent research has shown a relationship between MEF and increased diffusion across cell membranes of eukaryotes (Wang and Sastry 2000; Wang and Sastry 2002; Sensoy and Sastry 2004; Praporscic and others 2006) as well as prokaryotes (Cho and others 1996; Loghavi and others 2007; Loghavi and others 2008; Loghavi and others 2009). MEF has been shown to increase inactivation of spores as compared to conventional heating under similar heating profiles (Cho and others 1999; Somavat and others 2012). Sub-lethal pretreatment of *Escherichia coli* with MEF led to increased inactivation when followed with conventional heating (Palaniappan and others 1992).

MEF has been shown to reduce lag time as well as increase the production of the bacteriocin lacidin A in *Lactobacillus acidophilus* in MRS at an electric field of 1 V/cm under sub-optimal temperatures (Cho and others 1996; Loghavi and others 2008). The effects of electric fields and the amount required to achieve various effects differs between species (Chassy 1988). Decreasing the fermentation time of food products without sacrificing quality has been a point of interest in the food industry. Yogurt fermentation takes about 4-6 hours and in most cases any attempt to reduce the fermentation time results in unwanted changes in traditional yogurt properties.

In the present study, our objective was to investigate the effects of MEF at various frequencies on lag time and acid production of common yogurt starters, *S. thermophilus* and *L. bulgaricus*. Further, we investigate the effect of increasing the fermentation temperature above the optimum for *Streptococcus thermophilus*.

Materials and Methods

Bacterial Strains

Lactobacillus bulgaricus subsp. *delbruekii* ATCC 11842 and *Streptococcus salivarius* subsp. *thermophilus* ATCC 19258 were acquired from the Food Safety Laboratory culture collection at The Ohio State University (Columbus) and were tested in this study. Stock cultures were suspended in De Man, Rogosa and Sharp broth (MRS; Difco, Franklin Lakes, NJ) + 40% (vol/vol) glycerol, and kept at -80%C. Twenty four hours before experiments, a loop-full of the appropriate frozen culture was transferred to MRS

broth and incubated at 35ºC. The identity of the stock culture of *Lactobacillus bulgaricus* was confirmed using 16s rRNA sequencing as indicated in Figure 2.1.

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HM058989.1	Lactobacillus delbrueckii subsp. bulgaricus culture-collection IMAU:8	1554	1554	70%	0.0	95%
HM058987.1	Lactobacillus delbrueckii subsp. bulgaricus culture-collection IMAU:8	1554	1554	70%	0.0	95%
HM058963.1	Lactobacillus delbrueckii subsp. bulgaricus culture-collection IMAU:8	1554	1554	70%	0.0	95%
HM058962.1	Lactobacillus delbrueckii subsp. bulgaricus culture-collection IMAU:8	1554	1554	70%	0.0	95%
HM058940.1	Lactobacillus delbrueckii subsp. bulgaricus culture-collection IMAU:8	1554	1554	70%	0.0	95%

Figure 2.1 Confirmed *Lactobacillus bulgaricus* from blast results of 16s sequence.

MEF Setup

A lab-scale MEF fermentor was constructed from a 5L glass sterilizable flanged vessel with a lid with an exterior water jacket. The lid was fashioned out of polypropylene and was drilled with several holes that allowed an inoculation port, two thermocouples (T-type copper/constantan), one in the center of the vessel the other close to the positive electrode, a sample collection port, and electrodes (Figure 2.2). Each electrode was made of platinized titanium to prevent chemical reactions from occurring with the electrodes. Each electrode had a surface area of 73.5 cm^2. There was a distance of 14 cm between electrodes. A second vessel was created to be used as a control with exactly the same dimensions as the MEF vessel except for the holes for electrodes. Both vessels were stirred (Corning PC 351,353 stir plates) throughout the experiment but at very low rpm. The purpose of stirring was to maintain a homogenous distribution of microorganisms for

sampling as well as to avoid any hot spots that could be created due to the nature of electrically heating the media. Temperature was maintained for each vessel by two separate water baths (Haake G, Fisher Scientific 133, Waltham, MA) and temperature controllers (Haake DC 30, Fisher scientific isotemp 2100, Waltham, MA) and pumping water through the water jackets (Figure 2.3). For the treatment vessel, the temperature of the water was approximately 3ºC less than the desired temperature of the MEF vessel. The water jacket was used to cool the slight heating of the media due to heat being produced from the passing of current through a resistance (the media). The opposite was true of the conventional vessel; water bath temperature was approximately 2ºC higher than the desired temperature.

Wave form

Alternating current at 60 Hz or 45 Hz pure sine wave was applied to the treated vessel at 14 volts via AC power source (ELGAR, San Diego, CA) and a function generator (GW instek, Chino, CA) (Figure 2.3). The amperage remained constant at 1 amp, approximately, throughout all experiments. The electric field strength was 1 V/cm due to the distance of the electrodes.

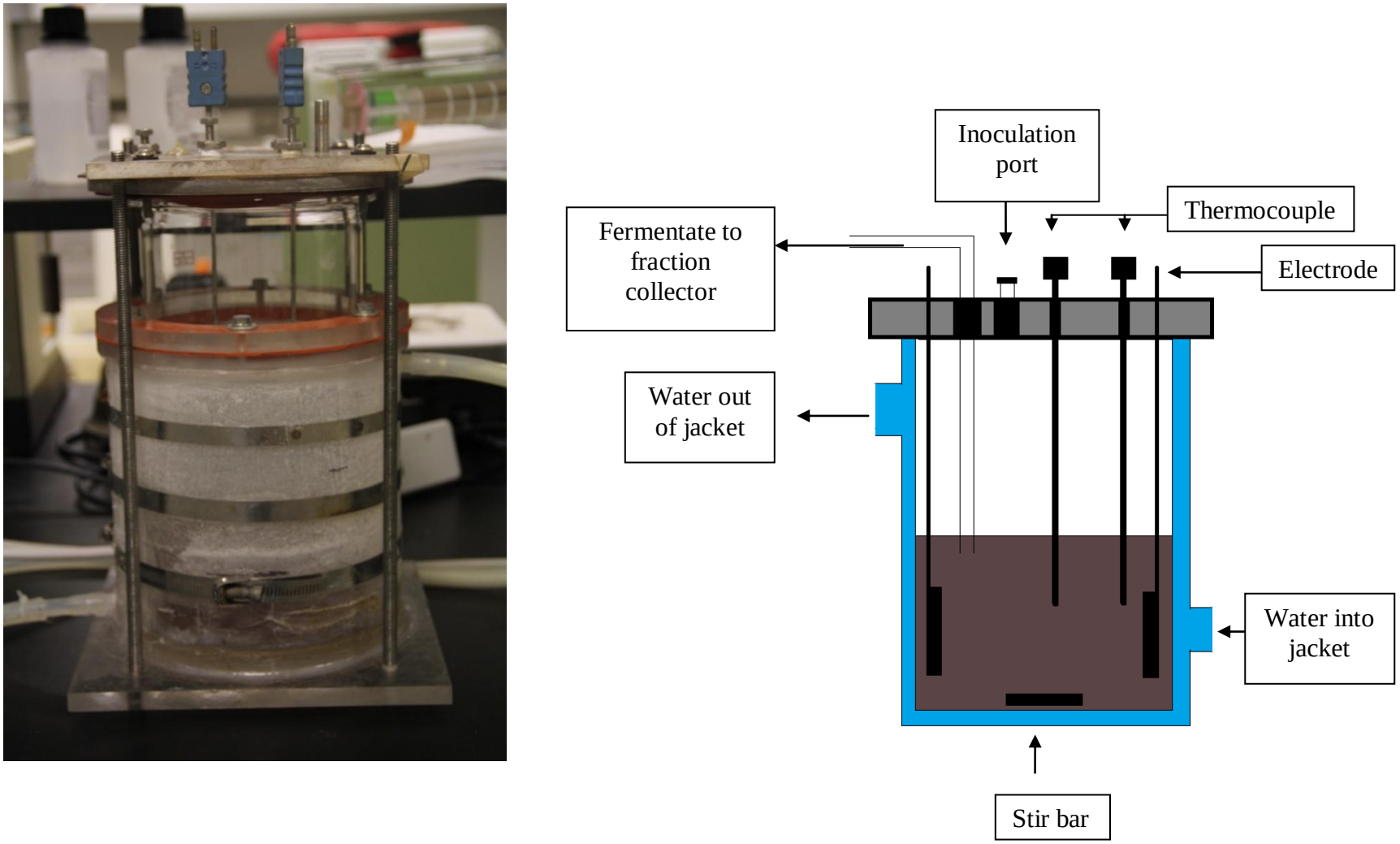

Figure 2.2 Real picture of fermentation vessel (Left) Schematic of fermentation vessel (not drawn to scale; Right)

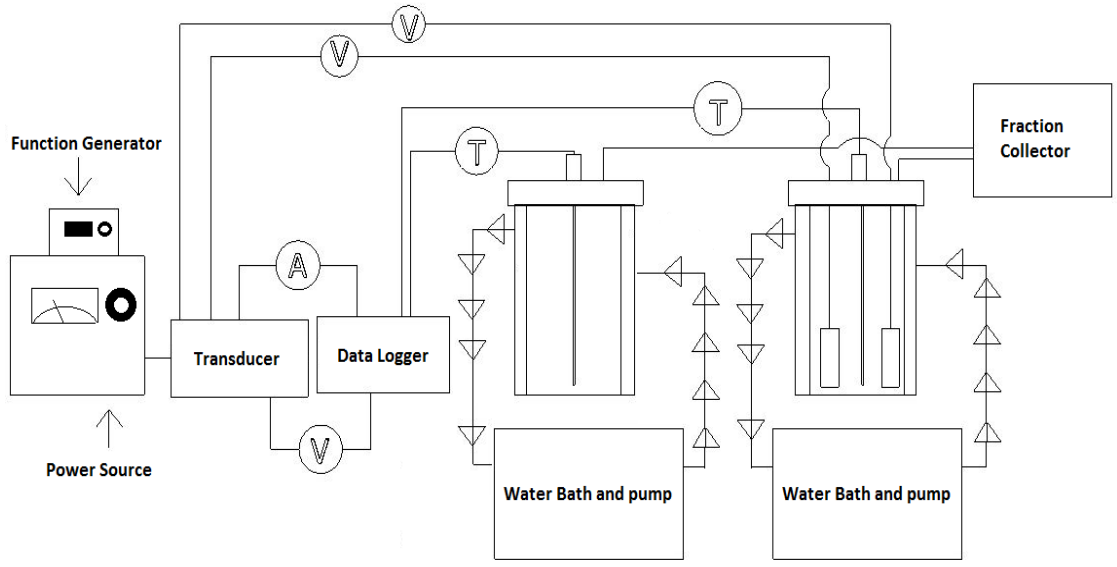

Figure 2.3 Schematic of MEF treated and control fermentation vessels. Data was collected via data logger. T= Temperature, V= Volts, A = Amps. Arrows denote flow of water.

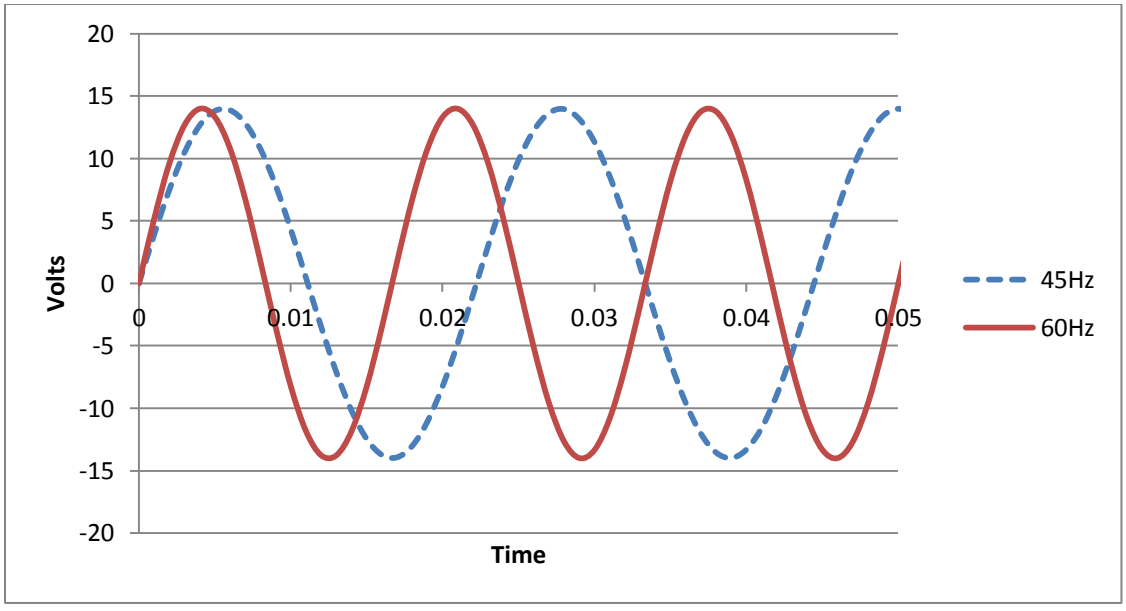

Figure 2.4 Sine wave of voltage at 45 Hz and 60 Hz

Experimental Procedure

A culture of either *S. thermophilus* or *L. bulgaricus* was prepared 24 hours in advance of the experiment. Both fermentor vessels were filled with 1.5 L (just enough to submerge both electrodes) of MRS broth and autoclaved for 20 minutes at 121ºC. The vessels were then placed into the respective water jacket and cooled down to running temperature (35ºC or 44 ºC ± .5ºC). The MRS broth was then inoculated with the appropriate culture at 0.1% (v/v). Temperature, voltage, and amperage were all recorded via a data logger at one minute intervals over the entire course of the fermentation to verify consistent temperature and application of electric field. Samples were treated at frequencies of 45 Hz or 60 Hz at 35ºC as well as 45 Hz at 44ºC (Table 2.1). Samples were pumped out of the vessels via a peristaltic pump (Masterflex, Cole-Parmer, Vernon Hills, Il) to a fraction collector that was refrigerated at 4ºC. Fractions were approximately 4 mL and were taken every 30 minutes. Fractions were then plated on M 17 +10% Lactose agar (Oxiod, Hampshire, UK) and incubated aerobically for *S. thermophilus* or on MRS agar incubated in anaerobic jars with three oxygen consuming, CO_2 producing sachets (GasPak EZ Anaerobe Container System; BD, Franklin Lakes, NJ) for *L. bulgaricus* at 35ºC for 48 hours. Colony forming units were counted and used to determine the amount of bacteria present at a given time. Overall metabolic activity was measured by the changes in pH over time.

Table 2.1 Treatment conditions

Treatment	Culture	Electric Field (V/cm)	Frequency (Hz)	Temperature (ºC)	Duration (h)
Conventional					
	L. bulgaricus	E=0	N/A	35	20
	S. thermophilus	E=0	N/A	35 or 44	12
MEF					
	L. bulgaricus	E=1	45 or 60	35	20
	S. thermophilus	E=1	45 or 60	35 or 44	12

Data analysis

All treated fermentations were done in duplicate and averages of growth parametres and pH values are reported. Bacterial growth was determined through making appropriate dilutions of the collected fractions and plating on suitable microbiological media. Colony forming units (CFU) were counted and the values changed into log scale. The collected data was then fitted to the Gompertz model ((Zwietering and others 1990; Cho and others 1996) as follows:

$$y(t) = A + C \exp\{ -\exp[-B(t-M)]\}$$

where $y(t) = \log_{10}$ CFU at time t. Model parameters can be interpreted as follows: A is the initial population, C is equal to the maximum population minus the initial population (total log increase), B is the maximum specific growth rate (slope at the inflection point), and M is the time the culture reaches the inflection point of the curve which represents the maximum growth rate the culture attains. The lag time, as defined by Zwietering, is where the slope of the inflection point crosses the x-axis (Zwietering and others 1990).

In order to determine the inflection point without bias, the growth data were fitted to the nonlinear Gompertz 4P model in the statistical program JMP version 10.0 (SAS Institute, Cary, NC). The modeled parameters were then used to calculate lag period, final log count, and minimum generation time. Lag period (hours) = M - (1/B); final log count = A+C ; minimum generation time (hours) = $(\log_{10}2)*(e/(B*C))$.

The change in pH was recorded (accumet model 15, Waltham, MA) with a pH probe (Inlab Semi Micro, Columbus, OH) by measuring the fractions at one hour intervals. The curve of pH is different than that of bacterial growth and is best fitted to the logisitc model (Cho and others 1996):

$$Y_h = A_1 + \frac{C_1}{(1 + \exp(-B_1 (X - M_1))}$$

where Y_h = 14-pH, X = time (in hours), A_1, B_1, C_1, and M_1 are model parameters. M_1 is the time (in hours) it takes the pH to reach the inflection point. To eliminate bias, data were fitted to the Logistics model 4P of the statistical program JMP. All collected data regarding growth curve or pH were analyzed in SAS version 9.2 (SAS Institute, Cary, NC).

Results

Streptococcus thermophilus

The differences in growth parameters of *Streptococcus thermophilus*, in response to the electric treatment, can be seen in Table 2.2. There was a significant ($P < 0.05$) difference in lag time between the treated and control of the 45 Hz treatment at 35°C but not at

44°C. The lag time was reduced, on average, by 39 minutes when *S. thermophilus* was treated with an electric field of 1 V/cm at 45 Hz, as compared to the control. There was no difference in lag time between cells treated with 60 Hz and control. The pH inflection point of the 45 Hz, 35°C treatment was significantly different than the control. The maximum growth at 45 Hz, 44°C treatment was significantly less than that at 45 Hz 35°C.

Lactobacillus Bulgaricus

The changes in growth parameters of *Lactobacillus bulgaricus*, due to the electric treatment, can be observed in Table 2.3. There was no significant difference in the growth parameters of the treatments and their controls. There was also no significant difference between treatments. The pH changes were not included in Table 2.3.

Table 2.2 Growth parameters and pH change for different treatments of *Streptococcus thermophilus*

Treatment	Frequency	Lag time (min)	Maximum specific growth	Minimum generation time (h)	maximum growth	Total pH decline	pH inflection point (h)
Con 35°C	0	137.9[a]	0.41	0.42	9.26[a]	1.74	7.69[a]
MEF 35°C	60	138.5[a]	0.43	0.42	9.16[a]	1.64	7.72[a]
Con 35°C	0	165.1[b]	0.47	0.37	9.18[a]	1.76	8.12[b]
MEF 35°C	45	126.1[c]	0.45	0.38	9.36[a]	1.88	7.35[c]
Con 44ºC	0	146.9[a]	0.51	0.44	8.24[b]	1.63	8.50[d]
MEF 44ºC	45	150.8[a]	0.49	0.45	8.31[b]	1.67	8.24[d]

[abcd]Denotes significant difference ($P<0.05$) within column

Table 2.3 Growth parameters of *Lactobacillus bulgaricus* under different treatments conditions

Treatment	Frequency	Lag time (min)	Maximum specific growth	Minimum generation time (h)	maximum growth
Con 35°C	0	5.533	0.225	0.969	7.607
MEF 35°C	60	5.550	0.226	1.011	7.445
Con 35°C	0	4.315	0.199	1.078	7.548
MEF 35°C	45	4.245	0.219	1.042	7.343

Discussion

The first documented use of moderate electric field (MEF) in order to stimulate growth of microorganisms was reported by George Stone (Stone 1909). He studied the growth of microorganisms in milk and water under MEF using copper and zinc electrodes and found a 1 to 2 log increase in bacteria of the treated compared to the control. Since then, the little research that has been conducted in this area has shown varying results. Rowley concluded that alternating current in the range of 1-60 Hz did not affect microorganisms but that direct current increased generation time (Rowley 1972). Shimada and Shimarahara (1997) showed that alternating current of 50 Hz influenced the growth of *Escherichia coli* but results were dependent on various variables including medium, inoculum size, and shaking during incubation. A review of these and other research relating to MEF stated that conclusions about MEF could not be drawn due to the lack of experimental details (Palaniappan and others 1990).

In recent years, MEF research has been conducted on *Lactobacillus acidophilus* at the Ohio State University (Cho and others 1996; Loghavi and others 2007; Loghavi

and others 2008; Loghavi and others 2009). Researchers concluded that MEF reduced the lag time and increased the production of lacidin A, a bacteriocin of *L. acidophilus*. These effects were found to differ depending on the frequency and the temperature.

Our results show that MEF at 45 Hz significantly reduces the lag time of *S. thermophilus* when incubated at 35°C (Figure 2.6). Increased permeabilization of the cell membrane leads to the uptake of the surrounding nutrients without the expenditure of energy leading to the shortened lag time. The exact means of increased permeabilization are unknown but theorized to be either caused through the electrical formation of pores (electropermeabilization) in the cell membrane or through the alteration of cell membrane protein conformation (Loghavi and others 2007; Loghavi and others 2009).

The reduction of lag time was lost when the fermentation temperature was increased to 44°C (typical yogurt fermentation temperature). This shows that temperature affects whether or not the reduced lag time caused by MEF can be seen. These findings are in agreement with the findings of Cho et al. (Cho and others 1996). They showed that the reduction in lag time of *Lactobacillus acidophilus* observed during MEF treatment was only apparent at sub-optimal temperatures (30°C). Once temperatures were increased in the range of 35-40°C the differences in lag time were not significant. Loghavi tested at the increased uptake of propidium iodide (PI), a florescent molecule to which cell membranes are impermeable, in varying growth stages of *L. acidophilus* under MEF (Loghavi and others 2009). She concluded that cells in the lag phase had the greatest uptake of PI and therefore were the most permeable of all the cells. Considering lag phase cells are the most permeable to MEF treatment, it would seem

reasonable to say that the longer the lag phase occurred the greater the stimulation effect of MEF would be observed. While this explanation seems to fit, the lag time at 44°C was similar to that of the control lag time at 35°C, suggesting that other interactions between MEF and temperature must be occurring. The critical membrane potential was found to be dependent on temperature (Coster and Zimmermann 1975) and could be a factor in the permeability of cell membranes.

The reduction in lag time of *S. thermophilus* at 45 Hz was significantly greater than that at 60 Hz. This is consistent with the findings of Loghavi where lower frequency electric fields generated the greatest reduction in lag time for *L. acidophilus* (Loghavi and others 2007; Loghavi and others 2009). Loghavi and co-workers proposed a theory as to why lower frequencies generated greater permeabilization, stating that at lower frequencies the charges building up around the cell membrane remain there longer as compared to higher frequencies due to the nature of alternating current. The increase in time may allow enough charges to build up to cause increased membrane permeability (Loghavi and others 2007).

The changes in metabolic activity of the LAB during MEF fermentation were deduced by monitoring lactic acid production via a pH probe. The decrease in pH was most likely related to the biomass and not an increase in acid production. Considering that in industry, yogurt fermentations are stopped once the pH reaches approx. 4.2-4.3 (Yildez 2009), decreasing the time it takes to reach the final pH could be advantageous.

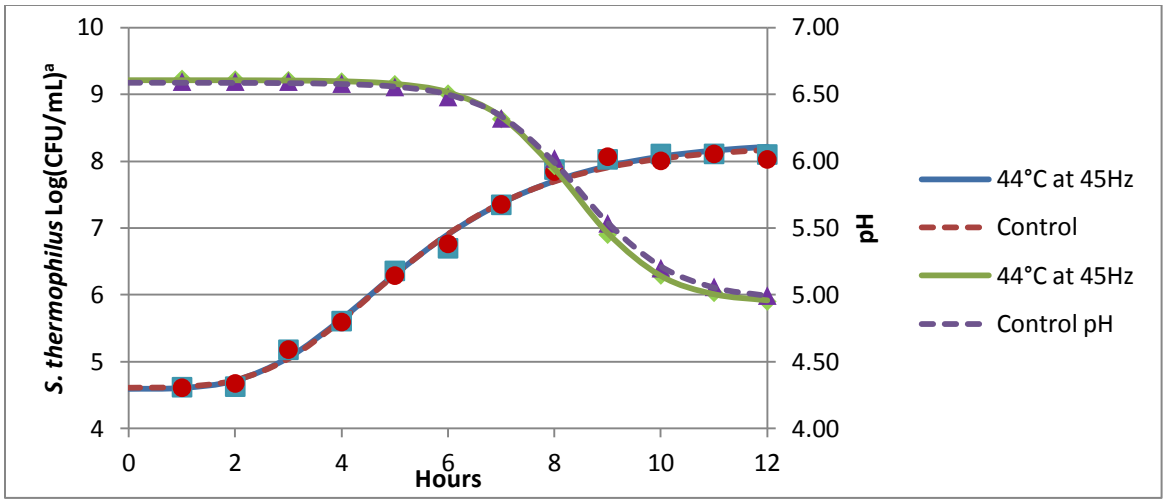

Figure 2.5 Changes in log CFU[b] and pH over time of *S. thermophilus* treated with 45 Hz MEF at 44°C

[a]Fitted with the Gompertz model

[b]Colony forming unit

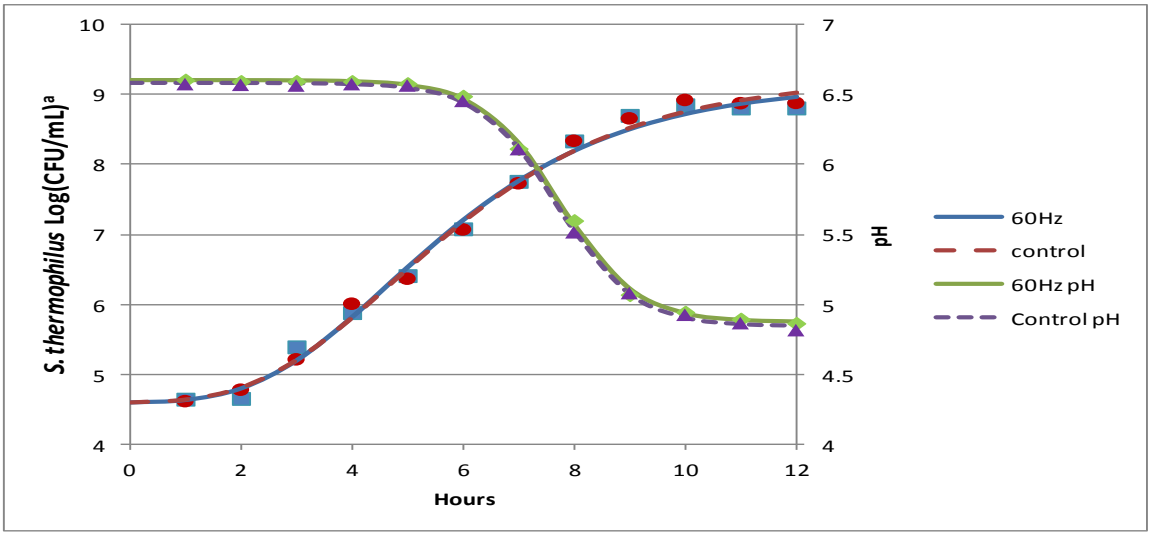

Figure 2.6 Changes in log CFU[b] and pH over time of *S. thermophilus* treated with 60 Hz MEF at 35°C

[a]Fitted with the gompertz model

[b]Colony forming unit

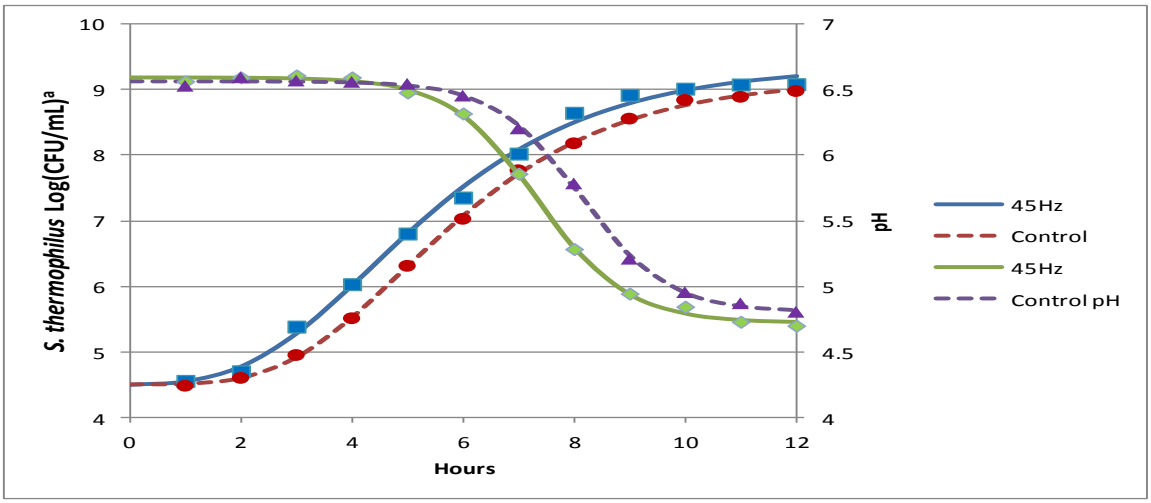

Figure 2.7 Changes in log CFU and pH over time of *S. thermophilus* treated with 45 Hz MEF at 35°C

[a]Fitted with the Gompertz model

[b]Colony forming unit

There was no significant difference in any of the growth parameters of *L. bulgaricus* for all treatment conditions (Table 2.3). The cause for the difference in reaction to the 45 Hz MEF between *S. thermophilus* and *L. bulgaricus* is uncertain. While it is known that *S. thermophilus* is a Gram-positive coccus and *L. bulgaricus* is a Gram-positive rod, these differences alone do not explain why their reactions to MEF were so different. Loghavi et al. treated *L. acidophilus*, which is a Gram-positive rod, with MEF and saw significant reduction in lag time of the treated vs. the control (Loghavi and others 2007). It is known that some strains of *L. bulgaricus* are extremely sensitive to electric fields as compared to *S. thermophilus* (Chassy 1988) but as to why and how this plays a role in membrane response to MEF is still an area that needs further research.

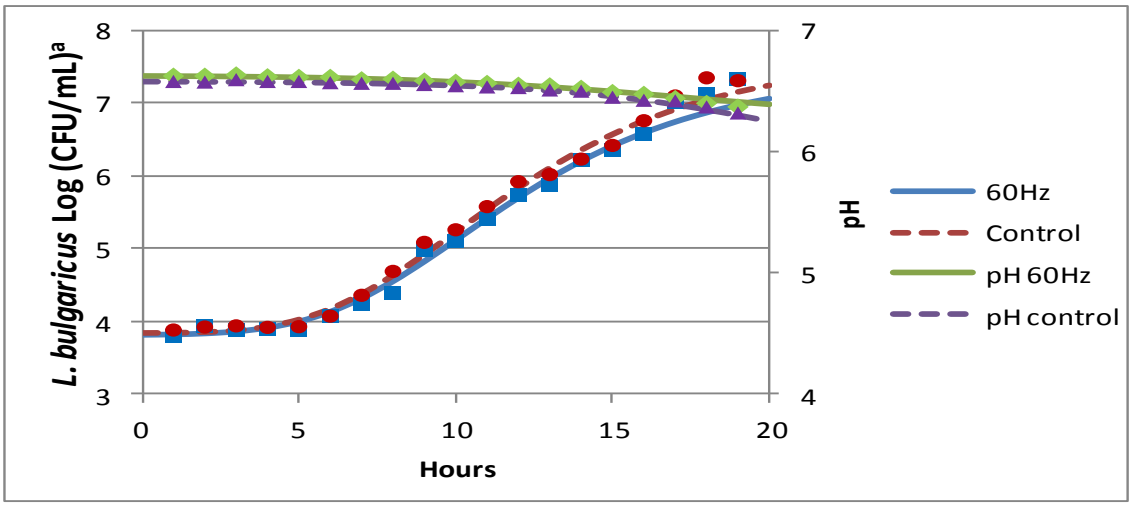

Figure 2.8 Changes in log CFU[b] and pH over time of *L. bulgaricus* treated with 60 Hz MEF at 35°C

[a]Fitted with the Gompertz model

[b]Colony forming unit

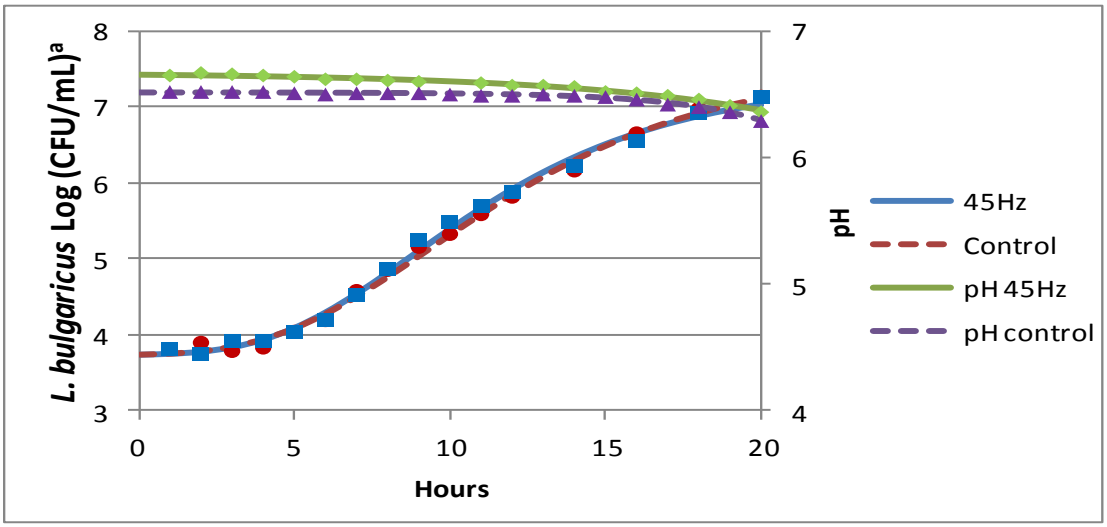

Figure 2.9 Changes in log CFU[b] and pH over time of *L. bulgaricus* treated with 60 Hz MEF at 35°C

[a]Fitted with the Gompertz model

[b]Colony forming unit

Conclusion

This study has shown that the application of moderate electric field (1 V/cm) on *S. thermophilus* during fermentation at near optimum temperature (35°C) can reduce the lag time. Reducing the lag time could decrease the overall fermentation time which could be very useful in the production of yogurt or other fermented foods utilizing *S. thermophilus* as a starter culture. However, increasing the temperature as well as the frequency of the alternating current has shown to reduce this effect. *L. bulgaricus* did not respond to the electric fields the same way as *S. thermophilus* suggesting that the stimulation of growth under moderate electric field could be strain dependent.

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Chapter 3
Growth Kinetics of Lactobacillus bulgaricus, Streptococcus thermophilus, and Lactococcus lactis Treated With Pulsed Electric Field

Abstract

Various strains of lactic acid bacteria (LAB) were subjected to continuous pulsed electric field (PEF) during fermentation. Two fermentation vessels (one for PEF-treated, and the other for the control) were filled with MRS broth and held at either 25°C or 35°C. The MRS was then inoculated with *Lactobacillus bulgaricus, Streptococcus thermophilus,* or *Lactococcus lactis* and fermentor contents were exposed to PEF at strengths ranging from 2-10 kV/cm. During fermentation, fractions where collected over time and microbial population was determined by plating on microbiological media. The fractions also were tested for pH, and in the case of *L. lactis*, assayed for bacteriocin production. Under these experimental conditions, exposure to PEF did not stimulate the growth of any culture or bacteriocin production. A general trend of reduced growth was seen as electric field was increased for both *S. thermophilus* and *L. bulgaricus*. It was hypothesized that *L. lactis* may produce the bacteriocin, nisin, at an increased rate due to cell stress and self induction from pore formation caused by the PEF treatments, but the opposite was found. The control produced nisin slightly faster showing the first clearing zone (200 arbitrary units) at 10 hours with the treated showing the first clearing zone (200 arbitrary units) at 11 hours. Although a stimulation effect was not observed in these

experiments, further research utilizing differing treatment parameters would be needed in order to claim that mild PEF does not cause the stimulation of bacterial growth.

Introduction

Pulsed electric field (PEF) is a non-thermal treatment of food products where high voltage is discharged across electrodes while the food product passes through leading to inactivation of various microorganisms. Microbial inactivation via PEF was first demonstrated by Sale and Hamilton in 1967. They reported electric fields in the range of 25 kV/cm having lethal effects on *Escherichia coli* (Sale and Hamilton 1967). They concluded that irreversible structural changes of the membrane led to the loss of selective permeability due to the formation of pores. Studies have shown that permeability increases with increased field strength and time exposed to the electric fields (Castro and others 1993; Zhang and others 1995; Wouters and others 2001a). Permeability of cell membranes were found to be reversible depending on pulse strength and duration (Coster and Zimmermann 1975). When electric field is applied, charged particles accumulate on both sides of the cell membrane. When a critical membrane potential is reached, approximately 1 V for bimolecular lipid membranes, dielectric breakdown occurs (Zimmermann and others 1974). This critical membrane potential was found to be inversely dependent on temperature (Coster and Zimmermann 1975).

Although the majority of research done in this area has addressed the lethality of PEF and the mode of inactivation (Wouters and others 2001b), some research was done in the area of sub-lethal PEF and membrane permeability. Wouters investigated

membrane permeabilization of *Lactobacillus* spp. subjected to PEF (Wouters and others 2001a). The researcher measured the uptake of a fluorescent dye by *L. plantarum* treated with 10-12 kV/cm PEF and found very little uptake of the dye as well as very little death. Once increased to 15-25 kV/cm, a linear relationship of dye uptake and cell death was found. Ohshima et al. applied sub-lethal electric field to *Saccharomyces cerevisiae* in the range of 0-10 kV/cm and found the release of intracellular proteins without cell death (Ohshima and others 1995).

Moderate electric fields in the form of sinusoidal waves of approximately 1 V/cm have been shown to increase membrane permeability and enhance cell growth during fermentation (Cho and others 1996; Loghavi and others 2007; Loghavi and others 2008; Loghavi and others 2009). It was also found that frequency and wave form affected permeability (Loghavi and others 2008). Loghavi et al. hypothesize that at lower frequencies, there is longer time for charges to build up on the cell membrane leading to an increase in electroporation.

To our knowledge, there has been no research done with continuous sub-lethal PEF treatment during a fermentation. Our goal is to investigate the effects of continuous sub-lethal PEF treatment on growth kinetics of various lactic acid bacteria as well as bacteriocin production by *Lactococcus lactis.*

Bacterial Strains

Lactobacillus bulgaricus subsp. *delbruekii* (ATCC 11842), *Streptococcus salivarius* subsp. *thermophilus* (ATCC 19258), *Lactococcus lactis* subsp. *lactis* (ATCC 11454), and

Lactobacillus cellobiosus (OSU 919) were acquired from the Food Safety Laboratory culture collection at The Ohio State University (Columbus) and were tested in this study. Stock cultures were suspended in De Man, Rogosa and Sharp broth (MRS; Difco, Franklin Lakes, NJ) + 40% (vol/vol) glycerol, and kept at -80%C. A working liquid stock of *L. bulgaricus* and *S. thermophilus* was prepared by transferring frozen culture to MRS broth and incubating at 35ºC for twenty four hours, then transferring and incubating at same conditions. Twenty four hours before experiments, 10 µL of liquid stock of the appropriate culture was inoculated into 10 mL of MRS broth and incubated at 35ºC. A loop-full of frozen culture of *L. lactis* was transferred to MRS broth and incubated for 24 hours at 35ºC. Two more successive overnight transfers were done before each experiment. *L. cellobiosis* was used as an indicator for nisin activity and was grown overnight at 35ºC before testing.

PEF Setup

Fermentor

The PEF fermentor was constructed from a 5-L glass sterilizable flanged vessel with a lid that was sealed by a rubber gasket and C-clamps. The lid was fashioned out of polypropylene and was drilled with several holes that allowed an inoculation port, one thermocouple (T-type copper/constantan), two tubes for the drawing and return of media to and from the treatment chamber and one tube to collect fractions. A second vessel was assembled with exactly the same dimensions except for the drawing and returning tubes and was run side by side as a control.

PEF System

Inoculated MRS was pumped from the vessel via a digital gear pump (Cole Parmer, Vernon Hills, Il), with a p23 fitting, to the PEF system (OSU-4ERRC) at 60 mL/min. The PEF system was designed for The Ohio State University by Dr. Howard Q. Zhang, Andrew Bigley, and Timothy Schurmann at the Eastern Regional Research Center (Wyndmoor, PA) (Figure 3.1). The PEF system had a voltage output range of .1 - 16 kV and a frequency maximum of 500 Hz.

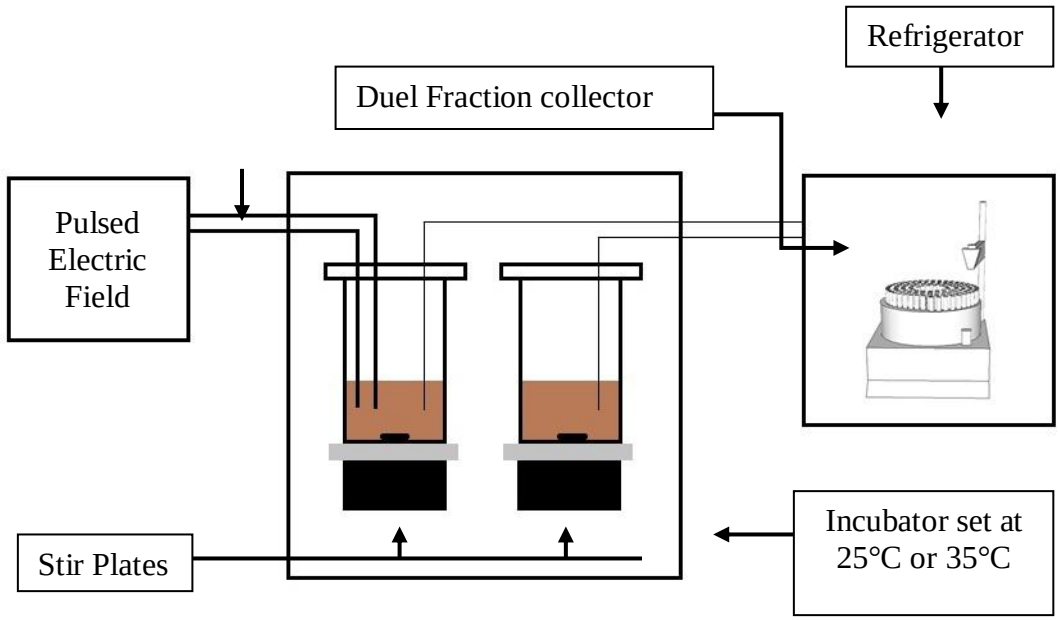

Figure 3.1 Schematic of Pulsed Electric field experimental setup

A cooling water bath (Isotemp 10135, Fisher Scientific, Waltham, MA), was used to chill the electrical parts used to generate the high voltages. The treatment chamber consisted of two stainless steel electrodes with a Teflon insulator (rated approx.

140ºC). Eight holes were drilled in the Teflon (Figure 3.2) allowing for multiple electrode gaps to be created. Four of the eight holes were used in order to make four treatment chambers. The electrode gap was 0.5 cm and the diameter of the hole was 0.29 cm (Figure 3.4). The flowing media first entered a cooling water bath then was PEF-treated twice before returning to the water bath and being treated two more times, returning one last time to the water bath before going back to the vessel (Figure 3.5).

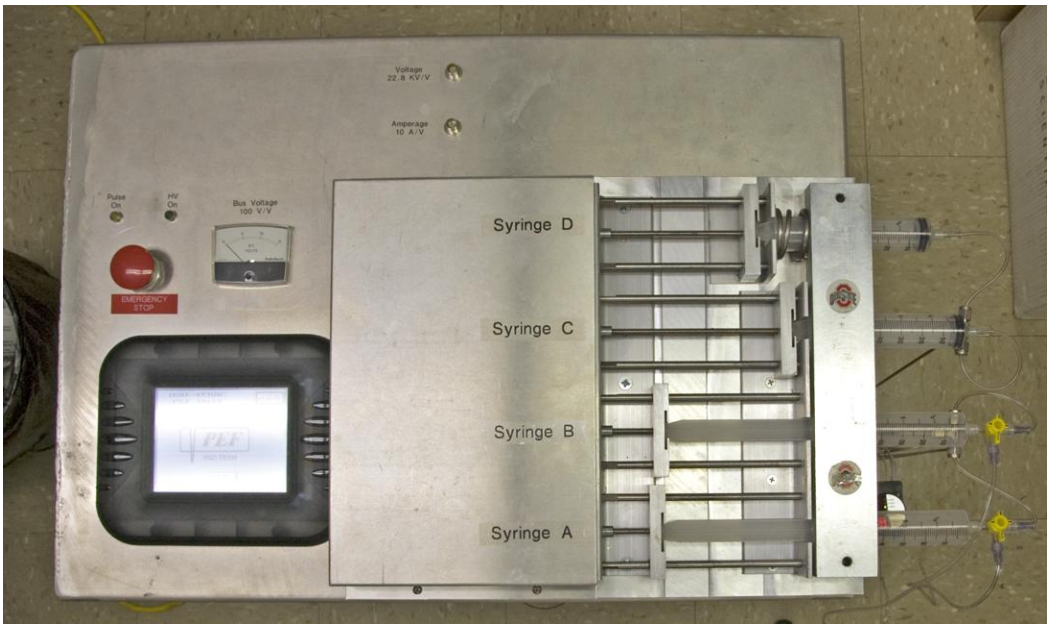

Figure 3.2 Top view of pulsed electric field system. Programmable logic controller touch screen on the left. Syringe pumping was bypassed by digital gear pump (not shown in photo)

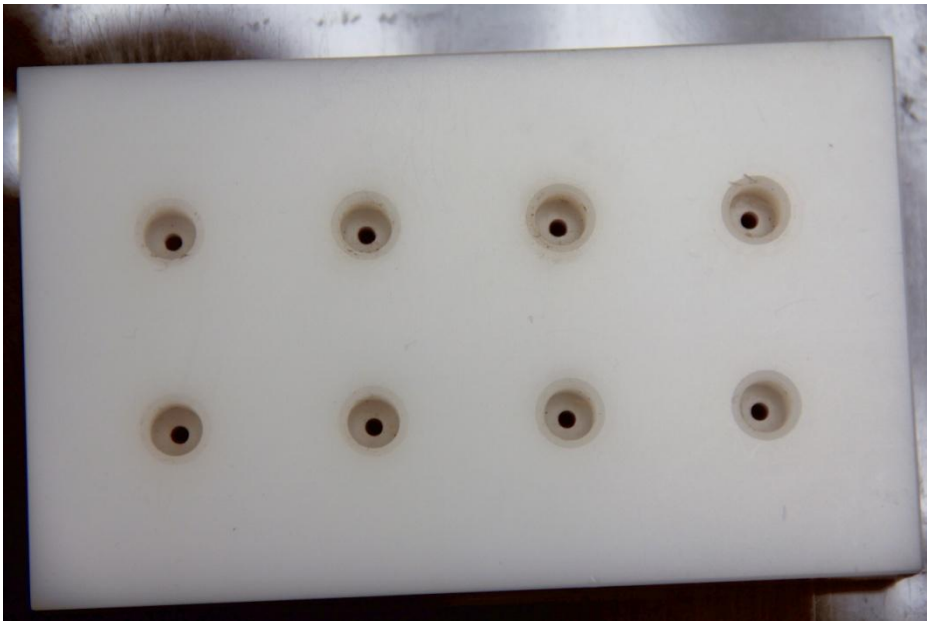

Figure 3.3 Treatment chamber Teflon insulation with drilled holes to create isolated treatment chambers (only four chambers were used out of the eight)

This water bath insured that the desired fermentation temperature remained constant as well as negating any increase in temperature from PEF treatment (1ºC increase at the highest voltage and conductivity).

Wave Form

The wave form used in these experiments was a bi-polar square wave with a square pulse (Figure 3.5). Each positive and negative pulse duration was 3 µs long with a 4 µs gap between them. The distance from the beginning of the first pulse to the end of the second pulse was one period. The frequency (periods/second) used in all experiments was 30 Hz. The amplitude of the pulse relates to the voltage across the electrode gap by a form

factor of 1V = 22.8 kV for voltage and 1V = 10 A for amperage. The voltages used ranged from 2 kV-10 kV

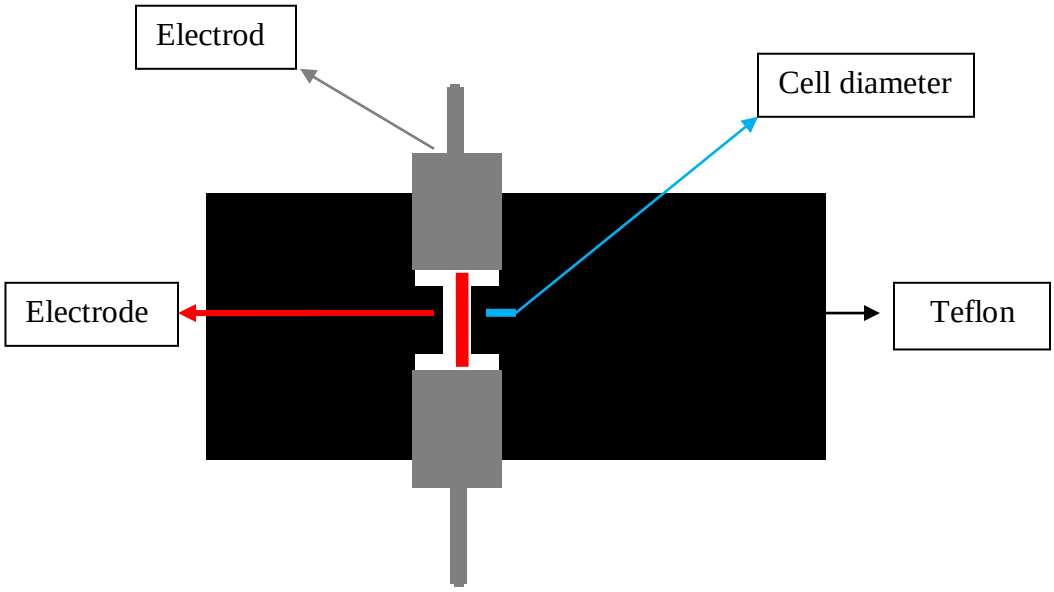

Figure 3.4 Vertical cross section of the Teflon insulation.

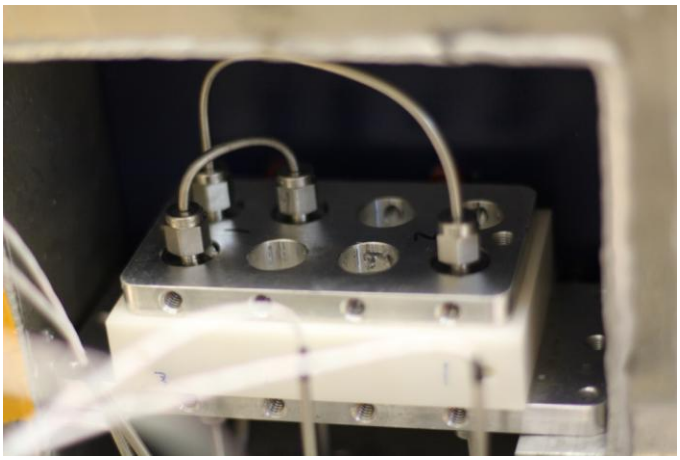

Figure 3.5 PEF treatment area with four treatment chambers in use.

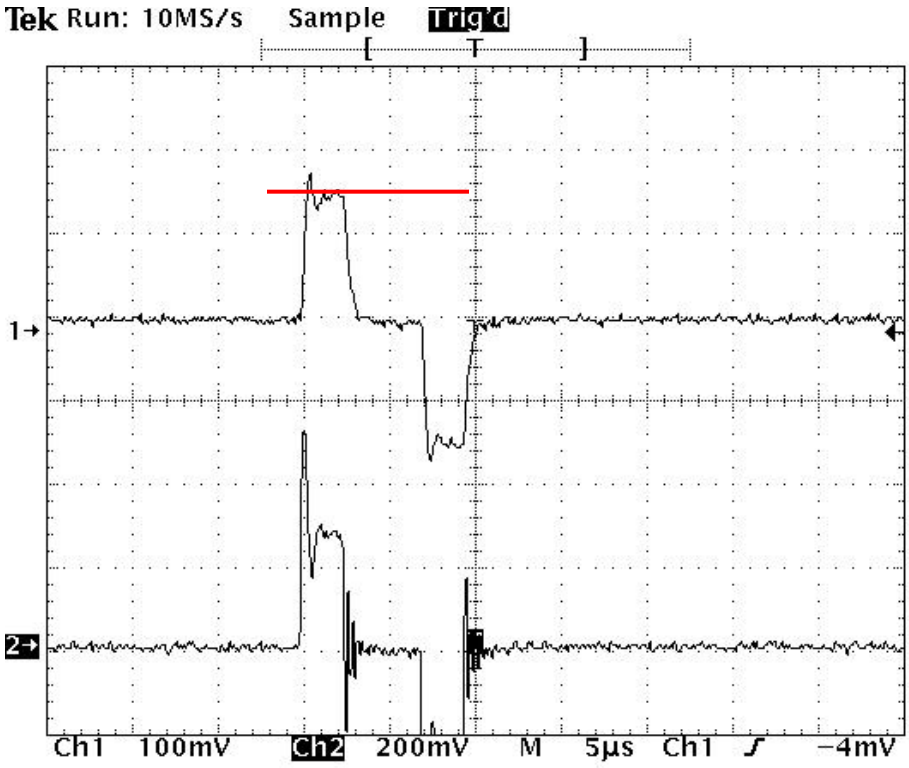

Figure 3.6 Bi-polar square wave. Channel 1 = voltage. Channel 2 = amperage. Y-axis is 5 µS per square. X-axis per square is 100 mV for voltage and 200 mV for amperage. The amplitude was determined by averaging the peak (as indicated by red line)

PEF Treatment Exposure

The total amount of exposure to electric fields is calculated through the following equation.

$$\frac{pulse\ duration * frequency * \#\ of\ chambers * electrode\ gap * cross\ sectional\ area}{product\ flow\ rate}$$

The same treatment exposure was used in all experiments. Pulse duration (both positive and negative pulse) was 6 µS. Frequency: 30 Hz. Number of chambers: 4. Electrode gap: 0.5cm. Cross sectional area: 0.066 cm^2. Product flow rate: 60 mL per min. The resulting treatment exposure to PEF was 23.8 µS. It is important to note that this treatment time is per time running through the PEF. PEF was run continually throughout fermentation and theoretically the entire fermentation medium (1 L of MRS broth) has passed through the PEF chamber once every 16.7 minutes. The total amount of treatment time per cell is difficult to calculate due to the fact the cells are growing and multiplying but the entirety of the MRS broth was treated for a total of 1028 µs.

Experimental Procedure

A culture of either *S. thermophilus, L. bulgaricus,* or *L. lactis* was prepared as described in bacterial strains section. Both fermentor vessels were filled with 1 L of MRS broth and autoclaved for 20 minutes at 121ºC. The vessels were then placed into a large incubator set at 35°C. The MRS broth was then inoculated with the appropriate culture at 0.1% (v/v) for *S. thermophilus* and *L. lactis* and 1% (v/v) for *L. bulgaricus*. Temperature and electric field strength was monitored. Samples were treated with electric fields ranging from 2-10 kV/cm at 30 Hz. Samples were pumped out of the vessels via a peristaltic pump (Masterflex, Cole-Parmer, Vernon Hills, Il) to a fraction collector that was refrigerated at 4ºC. Fractions were approximately 4 mL and were taken every 30 minutes. Fractions were then plated on M 17 +10% Lactose agar (Oxiod, Hampshire, UK) and incubated aerobically for *S. thermophilus* or on MRS agar incubated in

anaerobic jars with three oxygen consuming, CO_2 producing sachets (GasPak EZ Anaerobe Container System; BD, Franklin Lakes, NJ) for *L. bulgaricus* at 35ºC for 48 hours. *L. lactis* was also plated on MRS but was incubated aerobically at 35°C for 48 hours. Colony forming units were counted and used to determine the amount of bacteria present at a given time. Overall metabolic activity was measured by the changes in pH over time.

The amount nisin produced during fermentation was measured using the critical dilution assay (Yousef and Carlstrom 2002). Dilutions of the supernatant from the collected fractions were made and 5 µL of each dilution were plated on soft agar seeded with *Lactobacillus cellobiosis* (indicator microorganism). Clearings were observed and the highest dilution that still produced a clear inhibition zone was used in the following equation to calculate the arbitrary units (AU). AU/mL culture = $(1/DF_i)(1000/$ volume spotted in µL). DF_i is the inverse of the highest dilution factor that produced a clear zone of inhibition.

Data Analysis

All treated fermentations were done in duplicate and averages of growth parameters and pH values were reported. Bacterial growth was determined through making appropriate dilutions and plating the collected fractions. Colony forming units (CFU) were counted and the values were transformed into a log scale. The collected data were fitted to the Gompertz model (Zwietering and others 1990; Cho and others 1996).

$$y(t) = A + C \exp\{ -\exp[-B(t-M)]\}$$

where y(t) = $\log_{10}$ CFU at time t, A is the initial population, C is equal to the maximum population minus the initial population (total log increase), B is the maximum specific growth rate (slope at the inflection point), and M is the time the culture reaches the inflection point of the curve and is the maximum growth rate the culture attains. The lag time, as defined by Zwietering, is where the slope of the inflection point crosses the x-axis (Zwietering and others 1990). In order to determine the inflection point without bias, the growth data were fitted to the nonlinear Gompertz 4P model in the statistical program JMP (SAS Institute (version 10.0) Cary, NC). The modeled parameters were then used to calculate lag period, final log count, and minimum generation time. Lag period (hours) = M - (1/B); final log count = A+C ; minimum generation time (hours) = ($\log_{10}$2)*(e/(B*C)).

A pH meter (Fisher Scientific, accumet model 15) equipped with a pH probe (Mettler Toledo, Inlab Semi Micro) was used to measure pH of the fermentation fractions, at 1 hour intervals. The curve of pH is different than that of bacterial growth and is best fitted to the logisitc model (Cho and others 1996)

$$Y_h = A_1 + \frac{C_1}{(1 + \exp(-B_1 (X - M_1))}$$

where Y_h = 14-pH, X = time (in hours), A_1, B_1, C_1, and M_1 are model parameters. M_1 is the time (in hours) it takes the pH to reach the inflection point. To minimize bias, the data were fitted to the Logistics model 4P of the statistical program JMP. All collected data regarding growth curve or pH was analyzed in SAS (SAS Institute (version 9.2) Cary, NC).

Using the critical dilution assay, arbitrary units of nisin were calculated using the following equation: AU/mL culture = $(1/DF_i)(1000/$ volume spotted in µL). The collected data was then analyzed in SAS.

Table 3.1 Various electric field strength treatments applied to various LAB during fermentation

Strain	Electric Field (kV/cm)	Frequency (Hz)	Temperature (ºC)	Duration (h)
S. thermophilus	5	30	35	12
S. thermophilus	7	30	35	12
S. thermophilus	10	30	35	12
L. bulgaricus	2	30	35	12
L. bulgaricus	5	30	35	12
L. bulgaricus	10	30	35	12
L. lactis	7	30	25	12

Results

Streptococcus thermophilus

The growth parameters of *S. thermophilus* are presented in Table 3.2. While there was no significant difference in lag time for the 10 kV/cm treatment of *S. thermophilus* as compared to the control, the inflection point for the pH (an indicator of the rate of fermentation) was significantly different. The pH inflection point of *S. thermophilus* treated at 10 kV/cm coupled with Figure 3.7 seem to indicate a trending of reduced

growth as compared to the control. All the other growth parameters from Table 3.2 were not significantly different from each other.

Lactobacillus bulgaricus

The growth parameters of *L. bulgaricus* are shown in Table 3.3. PEF at 5 and 10 kV/cm significantly increased the lag time of *L. bulgaricus* as compared to the controls. At 10 kV/cm, growth parameters of *L. bulgaricus* were not modeled due to a growth curve not being formed because a death curve was observed. (Figure 3.11). The treatment of *L. bulgaricus* with 2 kV/cm did not significantly change any of the growth parameters (Table 3.3). All treatments of *L. bulgaricus* were different from each other with a trend of increased lag time as kV/cm increased.

Lactococcus lactis

The growth parameters of *L. lactis* are presented in Table 3.4. There were no significant differences in growth parameters of *L. lactis* between the control and the 7 kV/cm PEF treatment. The production of the bacteriocin nisin was delayed when treated with PEF. The control showed the first clearing at 10 hours (200 AU) while the treated showed the first clearing at 11 hours (200 AU). Both the treated and the control finished with the same maximum activity at 12 hours (400 AU).

Table 3.2 Growth parameters and pH change for different PEF treatments of *Streptococcus thermophilus* at 35°C

Treatment	Frequency	Lag time (min)	Maximum specific growth	Minimum generation time (h)	maximum growth	Total pH decline	pH inflection point (h)
Con[a]	30	100.84	0.43	0.39	9.33	1.71	8.20
5 kV/cm	30	102.54	0.43	0.40	9.36	1.75	7.88
Con	30	92.04	0.43	0.43	9.08	1.75	7.36
7 kV/cm	30	118.67	0.52	0.38	9.01	1.75	7.00
Con	30	106.73	0.46	0.40	9.04	1.73	7.57
8 kV/cm	30	111.38	0.49	0.39	8.90	1.74	7.32
Con	30	108.10	0.38	0.48	9.49	1.49	6.81
10 kV/cm	30	117.61	0.56	0.36	9.11	1.80	8.12

[a]Controls were done for each experiment individually

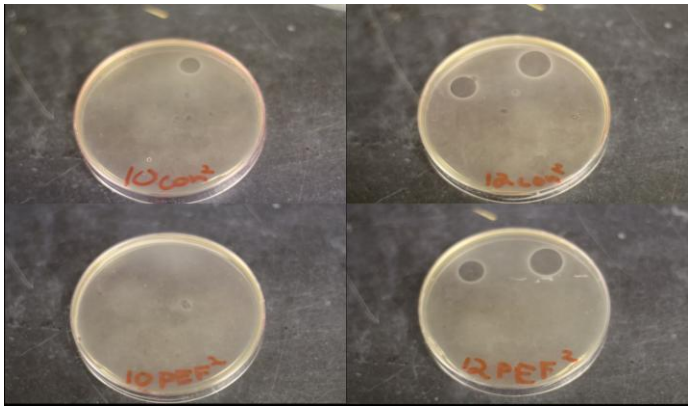

Figure 3.7 Activity of nisin on tryptic soy agar seeded with *L. cellobiosis* at 10 hours (left) and 12 hours (right). Fractions taken from the control (top 2) and from the PEF-treated at 7 kV/cm (bottom 2).

Table 3.3 Growth parameters and pH change for different PEF treatments of *Lactobacillus bulgaricus* at 35°C

Treatment	Frequency	Lag time (min)	Maximum specific growth	Minimum generation time (h)	maximum growth	Total pH decline	pH inflection point (h)
Con	30	205.46[a]	0.55	0.55	8.13	2.33	11.06
2 kV/cm	30	209.01[a]	0.51	0.57	8.25	2.28	11.33
Con	30	246.95[b]	0.47	0.61	8.39	2.92	12.17
5 kV/cm	30	305.60[c]	0.45	0.62	8.44	6.62	40.96
Con	30	220.24[a]	0.48	0.65	8.12	2.89	12.15
10 kV/cm	30	N/A[d]					

[a]Controls were done for each experiment individually
[abc]Denotes significant difference ($P<0.05$) within column
[d]N/A: Growth was inhibited at 10 kV (figure 3.11) therefore no growth parameters were generated

Table 3.4 Growth parameters and pH change for different PEF treatments of *Lactococcus lactis* at 25°C

Treatment	Frequency	Lag time (min)	Maximum specific growth	Minimum generation time (h)	maximum growth	Total pH decline	pH inflection point (h)	Nisin (Arbitrary Units[a]
Con[b]	30	95.96	0.42	0.52	9.46	2.14	4.40	400[c]
7 kV/cm	30	100.51	0.40	0.53	9.42	2.43	3.80	400[c]

[a]Arbitrary units values are unique to the test conditions and the indicator microorganism (*L. cellobiosis*)
[b]Controls were done for each experiment individually
[c]Values represent arbritray units of nisin after 12 hours of fermentation.

Discussion

Traditionally, pulsed electric field is used in one of two ways. The first way is for the inactivation of microorganisms through pulsing of high electric fields in the range around 20-80 kV/cm (Raghupathy and others 2005). The second way PEF is used is for extraction of intracellular material from fruits and vegetables (Lebovka and others 2003). Both of these uses rely on PEF's unique ability to destabilize cell membranes and cause the formation of irreversible pores which leads to microbial inactivation as well as enhanced extraction. To our current knowledge, there have been no attempts to carry out fermentation under the presence of continual pulsed electric field.

Previous research by done by Cho (Cho and others 1996) and Loghavi (Loghavi and others 2007; Loghavi and others 2008; Loghavi and others 2009) on *Lactobacillus acidophilus* and research done by Costello (Chapter 2) on *Streptococcus thermophilus* suggested that growth of these microorganisms may be stimulated using moderate electric field (MEF). The suggested hypothesis for stimulated growth was the formation of small reversible pores which allowed the unmediated passing of nutrients across the cell membrane (Loghavi and others 2007). While the effects of changing temperature and frequency were investigated, changing of the wave form was not. Pulsed electric field can be found in many different wave forms but for the experiments that we conducted, a bi-polar square wave was chosen to mimic that of the alternating current that was used in the previously mentioned research.

Since PEF is known to have lethal effects if the electric field is high enough, and our intent was to create reversible pores, a range of electric fields (2-10 kV/cm) were

tested in order to find the highest electric field each strain could be subjected to without delaying growth. Subjectively, this electric field strength was around 7 kV/cm for *S. thermophilus* and around 2 kV/cm for *L. bulgaricus*. At these field strengths there were no significant differences in any of the growth parameters between the control and the treated cells. While this is helpful in understanding the tolerance of these microorganisms to PEF, the hypothesized stimulation effect was not observed under experimental conditions. It is possible that the square wave form or the duration of the pulses were not conducive to eliciting the desired effects. In comparison, the growth increase of *S. thermophilus* in response to MEF treatment resulted from continual treatment with electric field for 12 hours as compared to PEF which was treated for a total of 1028.16 μs. This could have been a factor in not seeing growth stimulation.

L. bulgaricus was more sensitive to electric fields as compared to *S. thermophilus* due to size and shape differences (Chassy 1988). A general trend of reduced growth as electric field increased was observed as expected.

Since the parameters tested for PEF did lead to growth stimulation of *S. thermophilus* or *L. bulgaricus*, *Lactococcus lactis* (a nisin producing strain) was tested in order to look at the production of bacteriocin during fermentation under continuous PEF. It was hypothesized that since the production of nisin is a self-inducing mechanism, the creation of temporary pores could enhance nisin production by allowing earlier release of the bacteriocin (de Ruyter and others 1996). It was also hypothesized that stress caused by the pulsed electric field could increase the production of defensive molecules such as bacteriocins (Verluyten and others 2003).

We chose to treat *L. lactis* with 7 kV/cm PEF due to the fact that it was a Gram-positive coccus which we believed would respond similarly to PEF treatment as *S. thermophilus* did, thus making 7 kV/cm the highest electric field that could be endured without reduced growth. The maximum amount of nisin produced was observed at 12 hours and was the same for both the treated and the control. The rate of production was slightly faster with the control as compared to the treated and was probably due to slight reduced growth due to PEF. A potential reason for the lack of difference between the treated and control could be due to the fact that nisin production was not found in detectable levels until 10 hours of fermentation (near stationary phase). It is known that bacterial cells are more resistant to PEF treatment when in stationary phase (Pothakamury and others 1996) and this could have been a factor in not seeing increased nisin production.

Conclusion

Sublethal PEF treatments (2-10 kV/cm, and approximately 1028 μs total treatment time) did not stimulate the growth of *S. thermophilus, L. bulgaricus,* or *L. lactis*. As electric field was increased past a specific point (2 kV/cm for *L. bulgaricus,* and 8 kV/cm for *S. thermophilus*) growth inhibition was observed. Although no stimulatory effects were observed under the experimental conditions tested, more research in the area of sub-lethal pulsed electric field is required before any definitive conclusions can be made.

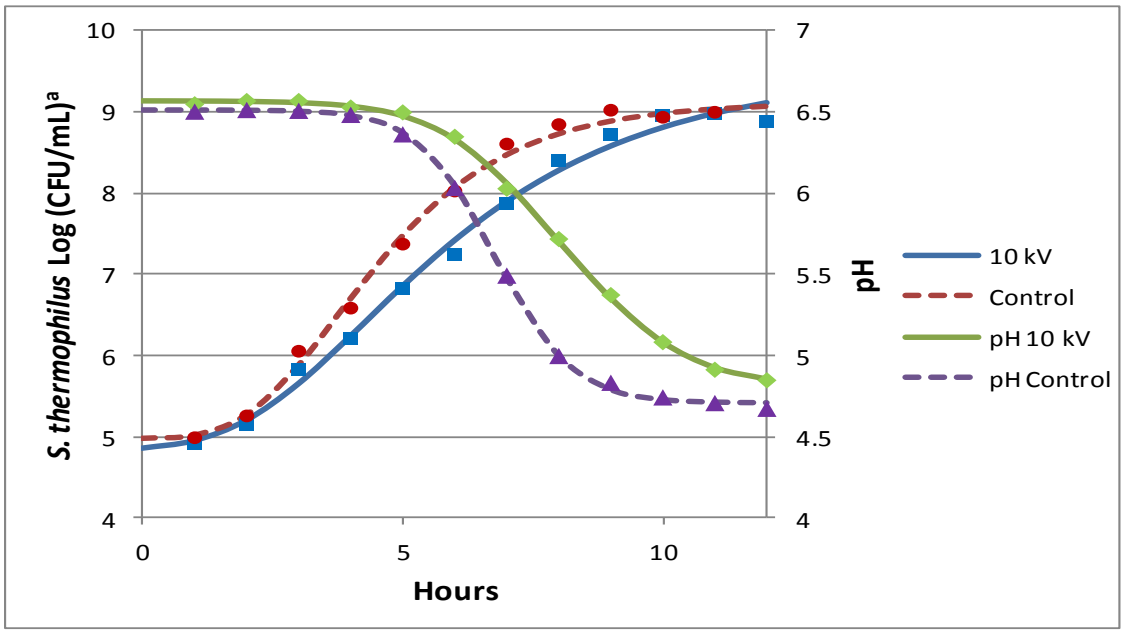

Figure 3.8 Log CFU and pH over time of *S. thermophilus* treated with 10 kV PEF at 35°C

[a]Fitted with the gompertz model

[b]Colony forming unit

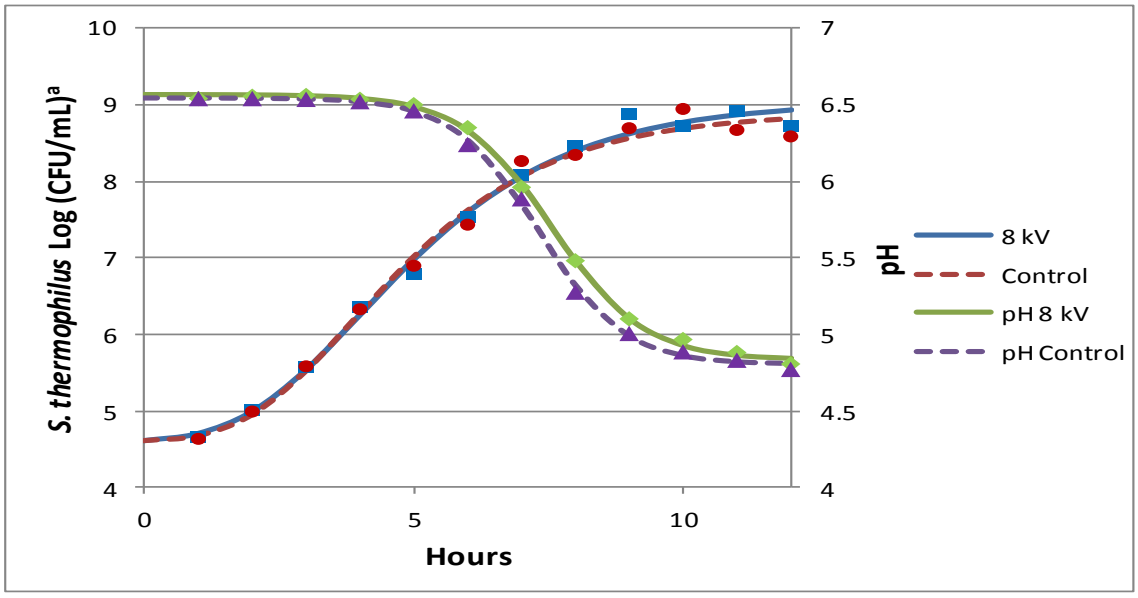

Figure 3.9 Changes in log CFU and pH over time of *S. thermophilus* treated with 8 kV PEF at 35°C

[a]Fitted with the Gompertz model

[b]Colony forming unit

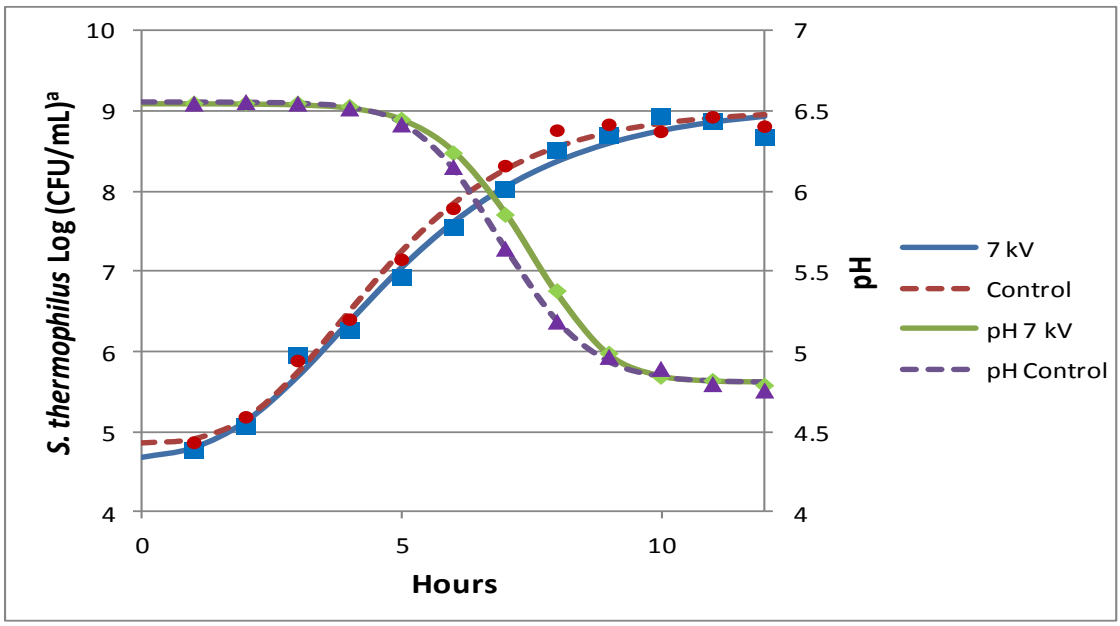

Figure 3.10 Changes in log CFU and pH over time of *S. thermophilus* treated with 7 kV PEF at 35°C

[a]Fitted with the Gompertz model

[b]Colony forming unit

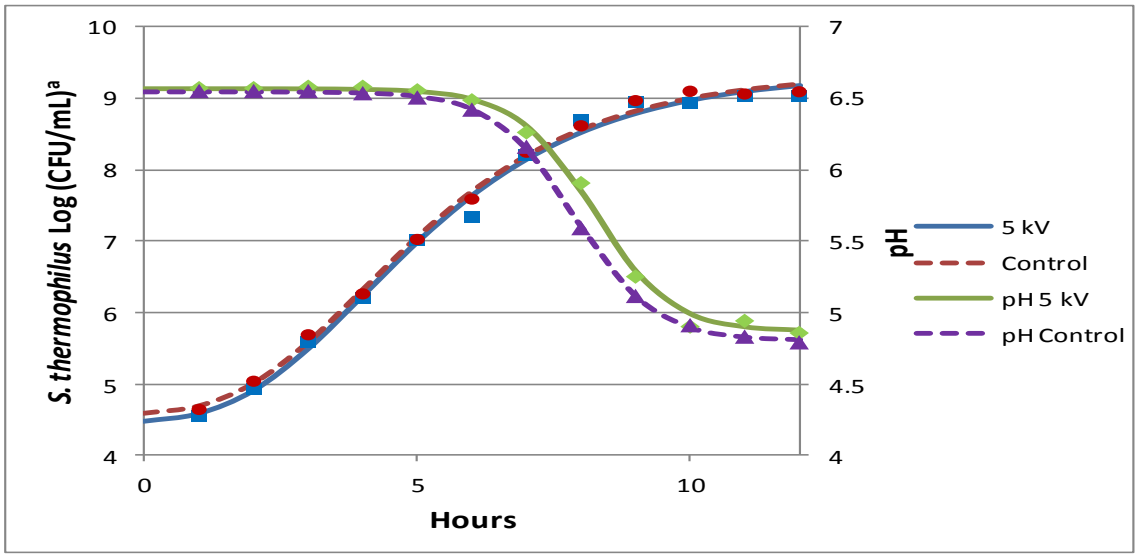

Figure 3.11 Changes in log CFU and pH over time of *S. thermophilus* treated with 5 kV PEF at 35°C

[a]Fitted with the Gompertz model

[b]Colony forming unit

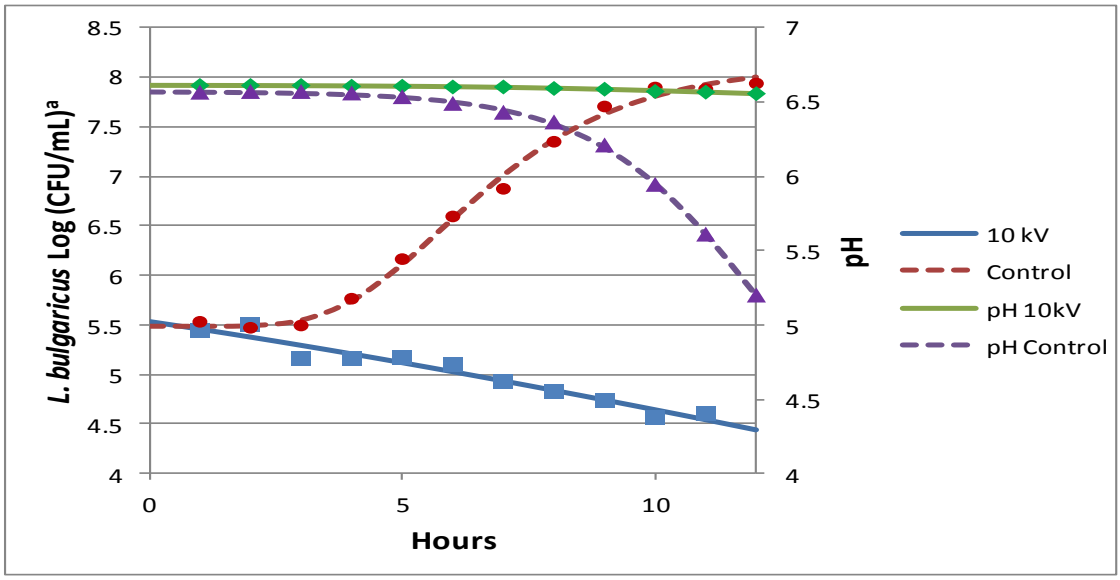

Figure 3.12 Changes in log CFU[b] and pH over time of *L. bulgaricus* treated with 10 kV PEF at 35°C

[a]Fitted with the Gompertz model

[b]Colony forming unit

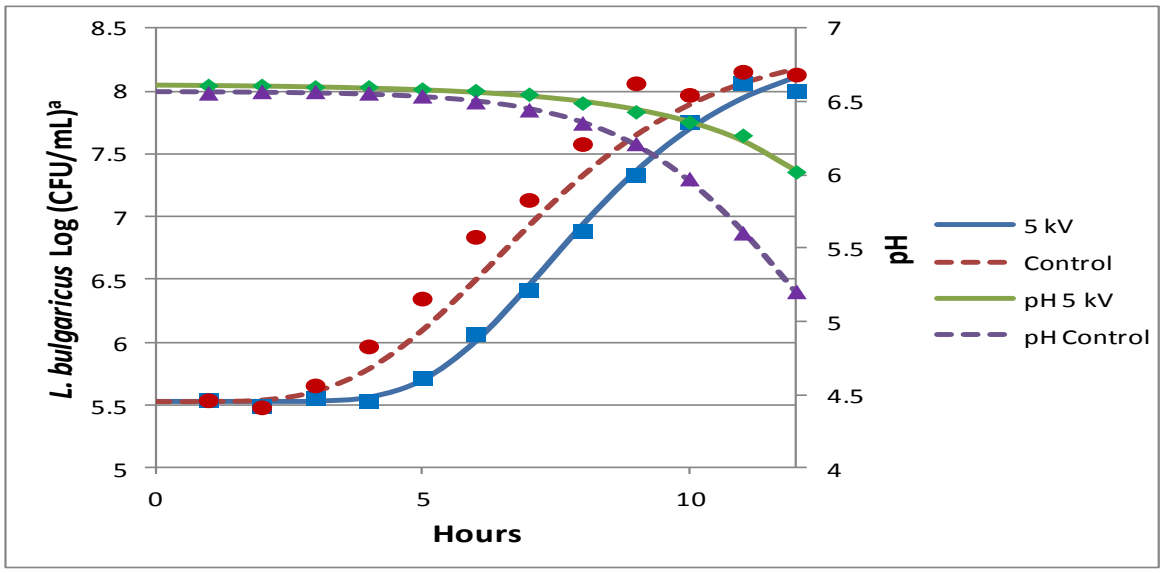

Figure 3.13 Changes in log CFU[b] and pH over time of *L. bulgaricus* treated with 5 kV PEF at 35°C

[a]Fitted with the Gompertz model

[b]Colony forming unit

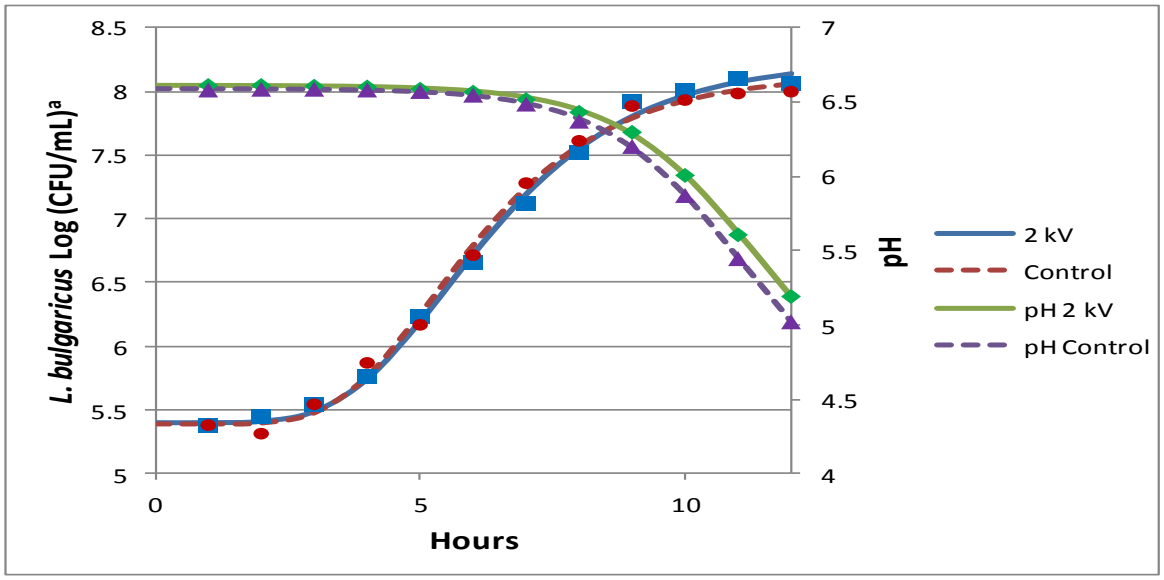

Figure 3.14 Changes in log CFU[b] and pH over time of *L. bulgaricus* treated with 2 kV PEF at 35°C

[a]Fitted with the gompertz model

[b]Colony forming unit

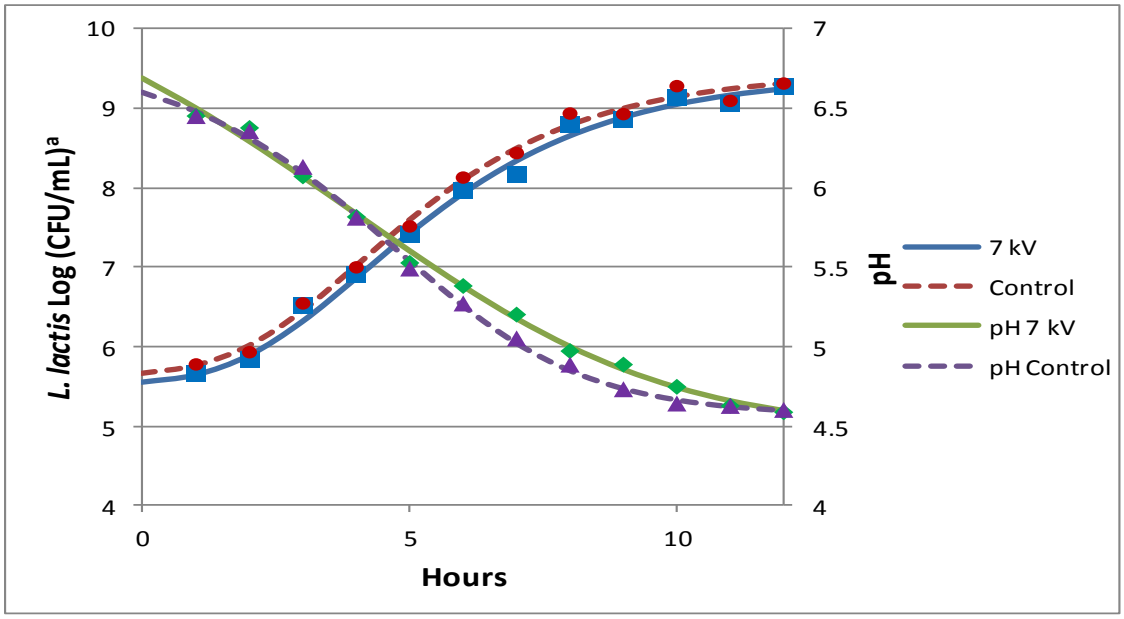

Figure 3.15 Changes in log CFU and pH over time of *L. lactis* treated with 7 kV PEF at 35°C

[a]Fitted with the Gompertz model

[b]Colony forming unit

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