INTERACTIONS OF MICROORGANISMS WITH ELECTRICITY

DISSERTATION

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ABSTRACT

Consumers’ demand for minimally processed foods with limited use of chemical additives led to the recent advances in nonthermal food preservation technologies. Pulsed electric field (PEF), high hydrostatic pressure, intense pulsed-light, ultraviolet light, and ultrasound are some of the newly developed technologies. The food industry is also interested in potent antimicrobials (e.g., ozone) as alternatives to conventional sanitizers.

PEF is one of the emerging technologies in the food industry. PEF inactivates microorganisms in liquid foods such as orange juice, milk and liquid egg. The mechanism of microbial inactivation by PEF is not well understood. Further research is needed to confirm the mechanisms of microbial inactivation, identify the pathogens of most resistant to PEF, develop validation methods to ensure microbiological effectiveness, and standardize and develop effective methods for monitoring treatment. The goals of this study were (a) to verify the association between cell injury and death by PEF and the increase in membrane porosity, (b) to investigate the role of the cell envelope in the resistance to PEF, (c) to explore the factors that weaken cell envelope and sensitize cells to PEF, and (d) to investigate effect of sublethal ohmic electrical treatment on the growth beneficial bacteria.

The association between cell injury and death by PEF and the increase in membrane porosity was verified by using a fluorescent nucleotide-binding probe, Propidium Iodide (PI) to quantify the membrane damage to PEF. Cell suspensions of
Lactobacillus leichmannii ATCC 4797 Listeria monocytogenes Scott A and Escherichia coli O157:H7 were subjected to PEF. Cells treated or untreated with PEF were stained with PI, and changes in fluorescence intensities were measured by a spectrofluorometer. Increase in field strength decreased the count of survivors and proportionally increased the fluorescence intensity; this observation indicated that cell inactivation by PEF is caused by membrane damage.

Cell envelope of a gram-negative bacterium (E. coli O157:H7) was modified by EDTA, lysozyme or their combination before applying the PEF treatment. The combination of lysozyme with PEF treatment did not significantly increase the inactivation of E. coli O157:H7 when compared to PEF treated cells (p >0.05). When cells were pre-treated with EDTA, followed by a PEF treatment, significantly higher inactivation (p < 0.05) was observed (2.1 log_{10} CFU/ml) than when PEF treatment was applied alone (1.8 log_{10} CFU/ml). More microbial inactivation (2.5 log_{10} CFU/ml) was obtained when PEF was applied to EDTA plus lysozyme treated E. coli O157:H7 cells (p <0.05). Fluorescence staining technique showed that pre-treatment with lysozyme and EDTA increased the PI uptake by the PEF-treated cells.

Selected physical and chemical factors affecting cytoplasmic membranes (i.e., incubation temperature and ozone) were investigated for possible sensitization of cells to PEF. Ozone attack unsaturated fatty acids in membranes. Incubation temperature may alter cell membrane structure and fluidity. In this study, L. monocytogenes Scott A was grown at 7, 22 and 37°C and treated with PEF at 20 and 25 kV/cm. PEF treatment decreased the population of L. monocytogenes, which was grown at 7°C by 1.4 and 5.4 log_{10} CFU/ml, respectively. Cells grown at 22°C were inactivated 1.2 and 2.0 log_{10}
CFU/ml by 20 and 25 kV/cm electric field strength. The greater inactivation (3.3 and 6.1 log$_{10}$ CFU/ml) was obtained when *L. monocytogenes* Scott A cells were grown at 37°C and treated with 20 and 25 kV/cm electric field intensities, respectively. Relative fluorescence intensities significantly increased by PEF treated (20 kV/cm) cells when incubation temperature was increased. The higher the incubation temperature, the higher the inactivation and relative fluorescence intensity were determined.

*Lb. leichmannii*, *E. coli*, and *L. monocytogenes* were suspended in 0.1% NaCl and treated with ozone, PEF and ozone plus PEF. Cells were treated with 0.25 to 1.00 μg ozone/ml of cell suspension, PEF at field strengths of 10 to 30 kV/cm; and selected combinations of ozone and PEF. Synergy between ozone and PEF varied with the treatment level and the bacterium treated. *Lb. leichmannii*, treated with PEF (20 kV/cm) after exposure to 0.75 and 1.00 μg/ml ozone, was inactivated by 7.1, and 7.2 log$_{10}$ CFU/ml, respectively. However, ozone at 0.75 and 1.00 μg/ml and PEF at 20 kV/cm inactivated 2.2, 3.6 and 1.3 log$_{10}$ CFU/ml, respectively. Ozone at 0.5 and 0.75 μg/ml inactivated 0.5 and 1.8 log$_{10}$ *E. coli* CFU/ml, respectively, and PEF at 15 kV/cm inactivated 1.8 log$_{10}$ CFU/ml. Ozone (0.5 and 0.75 μg/ml) followed by PEF (15 kV/cm) inactivated 2.9 and 3.6 log$_{10}$ CFU/ml, respectively. Population of *L. monocytogenes* decreased 0.1, 0.5, 3.0, and 0.8 log$_{10}$ CFU/ml when treated with 0.25, 0.5, and 0.75 μg ozone/ml, and PEF (15 kV/cm), respectively. However, when the bacterium was treated with 15 kV/cm, after exposure to 0.25, 0.5, and 0.75 μg ozone/ml, 1.7, 2.0, and 3.9 log$_{10}$ CFU/ml were killed, respectively. Exposure of microorganisms to ozone followed by the PEF treatment showed a synergistic bactericidal effect. Synergy was more apparent at mild than severe doses of ozone, and when the combination treatment was applied to *Lb. leichmannii* than to *E. coli* or *L. monocytogenes*.
Lag period of *Lactococcus lactis* subsp. *lactis* ATCC 11454 decreased 2.5, 1.8, and 1.56 hours by low-voltage ohmic heating when compared with conventional heating at 25, 30, and 37°C, respectively. Sublethal ohmic heating resulted in lower nisin activity in the fermented medium than conventional heating. Electrical current increased during the fermentation probably due to the production of lactic acid and other polar components.

In conclusion, a fluorescence staining technique was useful in accessing membrane damage associated with PEF treatments and in determining relative sensitivities of microorganisms to PEF. The efficacy of PEF against *E. coli O157:H7* increased when the cell envelope was altered by the combination of EDTA and lysozyme. *L. monocytogenes* Scott A grown at 37°C were easily inactivated by PEF, when compared to those grown at 7 and 22°C. Exposure of cells to ozone sensitized them to the action of PEF. It might be useful in food fermentations to apply electricity to decrease the lag period of cultures at the initial stage of growth, and then apply conventional heating at the later stages. The measurement of electrical current when ohmic heating is applied at a constant voltage may be used to monitor the growth of culture instead of plate counting or measuring absorbance.
Dedicated to the following individuals for their
unconditional love and support:

My wife, Nurdan Unal
My parents, Ahmet and Atiye Unal
My brothers and sister, Mehmet Ihsan Unal, Salim Nida Unal, and Serife Arik
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INTRODUCTION

Consumers' demand for fresh-like products with minimal losses of vitamins and changes in flavor has led food industry to the study of rapid methods of heating and nonthermal microbial inactivation technologies (Jayaram et al., 1993). This has resulted in important attention towards technologies using electrical energy in food preservation.

Use of electricity for microbial inactivation in foods is not a new approach. There is not sufficient data how the electricity affects microorganisms. Understanding such effects would be useful in developing new methods such as microbial inactivation, and microbial growth enhancement by electricity.

PEF processing represents a promising, nonthermal method of food preservation. The mechanism contributing to microbial inactivation by PEF is not well known. Zimmermann (1986) considered the cell membranes as capacitors that accumulate externally applied electrical charges, predominantly on their surfaces, causing increased transmembrane potential. Due to the attraction of opposite electrical charges induced on the inner and outer surfaces of the cell membrane, compression pressures occur resulting in a decrease in membrane thickness. These instabilities on the cell membranes due to compression result in decrease in membrane resistance, leading to its irreversible breakdown (Tsong, 1989, 1991; Jayaram, 1992). Other theory is relating the microbial inactivation by pulsed electric field to the formation of pores in cell membranes (Tsong, 1991). Currently, there is no sufficient experimental proof supporting this hypothesis.
Determination of viability of microorganisms is much more complex than it is thought. The state of a microorganism can be termed as dead, moribund, starved, dormant, resting, quiescent, viable but non-culturable, injured, sublethally damaged, inhibited, resuscitable, living, active and vital (Breeuwer and Abee, 2000). The viability of microorganisms is crucial for detection and enumeration of food spoilage and pathogenic microorganisms. Fluorescent probes are very good indicators for detection of microbial cells by flow cytometry and spectrofluorometry (Shapiro, 1995). The bacterial cell membrane is assumed to be the primary target for the inactivation of microorganisms by PEF treatments applied in the food industry. Fluorescent stains commonly used for determination of membrane integrity or damage are ethidium bromide (EB) or propidium iodide (PI). Both dyes are mutagenic, positively charged and almost membrane impermeable. They can enter cell via damaged membranes of stressed, injured or dead cells and intercalate into DNA and RNA, where fluorescence is enhanced. PI uptake has been shown to correlate nicely with membrane damage (Ueckert et al., 1995). Therefore, PI may be conveniently used to determine the correlation between the damage of bacterial cell membrane by pulsed electric field and cell viability.

The loss of membrane integrity by PEF and other non-thermal treatments such as ozone and high hydrostatic pressure depends on the characteristics of the cell membrane and other constituents of cell envelope. Besides, factors that increase the strength of cell envelope and decrease the fluidity of cell membrane are likely to increase the resistance of bacterial cells to pulsed electric field treatments. When the inherent and environmental factors that weaken cell envelope are defined, this may make it easier to develop effective PEF treatments.
Gram-positive and gram-negative bacteria possess cell wall peptidoglycans, which give the characteristic cell shape and provide the cell with mechanical protection. Peptidoglycan layer may protect cell membrane integrity during sublethal pulsed electric field treatments. It is likely that bacterial cells inherently lacking peptidoglycan would be more susceptible to pulsed electric field treatments than those with a thick peptidoglycan wall. Cells grown or treated to contain less peptidoglycan in their envelope may become more susceptible to pulsed electric field treatments than the untreated cells. Therefore, an enzyme, lysozyme, may be used to hydrolyze the β-linkages between muramic acid and glycosamine of the glycopolysaccharides of cell wall. Gram-positive bacteria are lysed in an environment with low osmotic pressure when treated with lysozyme. It is well known that gram-negative bacteria such as Salmonella typhimurium, E. coli 0157:H7, Campylobacter jejuni are all resistant to the antimicrobial activity of lysozyme due to the presence of the outer membrane (Hughey et al., 1987). It is likely that lysozyme treated gram-positive cells (Listeria monocytogenes ScottA) are more susceptible to PEF treatments than the gram-negative cells (Escherichia coli O157:H7).

One of the major components of the outer membrane of gram-negative bacteria is lipopolysaccharide (LPS), a complex molecule consisting of a lipid A anchor, a polysaccharide core, and chains of carbohydrates. LPS acts as a hydrophobic barrier and carries important antigenic determinants of the cell. Holes created in the outer membrane of a gram-negative bacterium as a result of pulsed electric fields treatment may be sublethal to the bacterial cells unless similar holes are generated in the inner membranes. In the past, EDTA has been used to destabilize the outer membrane of gram-negatives. Various chemicals such as EDTA, and lysozyme can alter the cell envelope of gram-negative and gram-positive bacteria. This may increase the efficacy of pulsed electric field treatment against gram-negative and gram-positive bacteria.
Today, the food industry is making use of a combination of factors to achieve food preservation. The simultaneous combination of preservative factors becomes a hurdle to the microorganism, producing a shelf stable and safe food products. The use of selected hurdles may provide an additive effect on microbial inactivation, and a synergistic effect can be observed when the hurdles take up different targets of microbial cell. In practical application of ozone in the food industry, safety-of-use is an important issue. Alterations in the sensory attributes depend on the chemical composition of food, ozone dose, and treatment condition. Therefore, low ozone doses can be chosen, and combined with low level of pulsed electric field strength (5-20 kV/cm). Ozone may sensitize the microbial cells and make them more susceptible to PEF treatment.

In addition to lethal effects of electricity against microorganisms, electricity can also enhance the microbial growth. The effects and interactions of low voltage ohmic heating on the growth and metabolic activity of microorganisms have not been studied very well. Rowley (1972) reported that electrical current can be used either enhance or inhibit the growth of microorganisms depending on the experimental conditions. Low voltage ohmic heating enhanced the growth of *Lactobacillus acidophilus* (Cho et al., 1996). Therefore, *Lactococcus lactis* ssp. *lactis* ATCC 11454 was used to study the effect of low-voltage electric treatment on the growth and metabolic activity of this culture in broth medium.
CHAPTER 1

LITERATURE REVIEW

EMERGING FOOD PROCESSING TECHNOLOGIES

Consumer's demand for foods with particular attributes or functions is constantly changing. Today, consumers want to purchase more fresh and natural products. Thus, new food-processing applications should be designed to keep the natural quality of foods. Thermal processing such as pasteurization and sterilization not only kills microorganisms but also modifies the natural taste, color, and nutritional quality of foods. Recently, non-thermal processing technologies are heavily investigated as alternatives to conventional thermal processing methods. These novel technologies may help the processors produce safe foods with fresh-like taste and texture. One of these technologies is the pulsed electric field (PEF). Combination of PEF and other processing treatments such as pH, ionic strength, temperature, and high hydrostatic pressure, are promising.

Pulsed electric field is used in the areas of genetic engineering and biotechnology. Electric pulses are applied to microbial cells to cause electroporation of cell membrane. Foreign materials such as DNA, RNA, are added to infuse into the cell. When the electric pulses are stopped, microbial cells repair their membranes, sealing the electropores. In genetic engineering and biotechnological applications, the process has to be controlled in order to maintain the viability of microorganisms during the application of pulsed electric
field. Technically, application of PEF in food applications uses the same principle. However, the treatment time or intensity of pulsed electric fields is kept higher than in genetic engineering and biotechnological applications in order to maintain an irreversible breakdown (inactivation) of the microbial cell membrane.

**Pulsed Electric Field**

PEF processing involves the application of pulses of high voltage (typically 20 - 80 kV/cm) to foods placed between two electrodes. When high electric voltage is applied, electrical current flows through liquid food materials. Liquid foods are electrical conductors due to the presence of electrical charge carriers such as metal ions (Zhang et al., 1995a). Because of a very short period of discharge time (i.e. microseconds and/or nanoseconds), heating of foods is minimized. PEF treatment is applied at ambient, sub-ambient, slightly above ambient temperature for less than 1 s, and energy loss due to heating of foods is minimized. For food quality attributes, PEF technology is considered superior to traditional heat treatment of foods because it greatly reduces the detrimental changes of flavor, color, taste, nutrients, and textural attributes (Dunn and Pearlman, 1987; Jia et al., 1999; Knorr et al., 1994; Mertens and Knorr, 1992; Qiu et al., 1998). Even though some researchers made some conclusions that PEF keeps the nutritional value of foods, effect of PEF on nutritional and chemical impacts of foods should be better understood before it is used in food preservation (Qin et al., 1995). Sale et al. (1967, 1968) was the first to conduct systematic studies on the effect of pulsed electric fields on the inactivation of microorganisms. They showed that the electric field strength and treatment time (which is the product of the number of pulse and pulse width) were
the two most important factors involved in microbial inactivation. By suspending microorganisms in a gel that was impermeable to products of electrolysis, they proved that inactivation was not the products of electrolysis. In their experiments, the temperature increase was less than 10°C, and therefore they concluded that inactivation was not due to thermal effects. Sale and Hamilton (1967) proposed that the electric field caused an irreversible loss of membrane’s function, as a semipermeable barrier between the bacterial cell and its environment and that this was the cause of cell death. They obtained an approximately 2-log reduction of various microorganisms. They reported that the pulse electric field treatment caused the loss of motility and inhibited the synthesis of enzymes in microorganisms but that it had no effect on those enzymes already present.

Mechanism of microbial inactivation by pulsed electric field

The mechanism of microbial inactivation by high voltage pulsed electric fields was not fully investigated. Hamilton and Sale (1967, 1968) suggested that intense electric pulses might cause permanent or temporary loss of the integrity of the microbial cell membranes. They found that when the applied field reached a potential difference of 1 V across the microorganism’s membrane, the cell membrane began to lose its function as the semipermeable barrier between the cell and its environment (Sale and Hamilton, 1967; 1968). The electron micrographs showed that the flattened appearance and irregular outline of the treated cells were resulted from the leakage of intracellular contents (Hamilton and Sale, 1967).

The cell membrane can be considered as a capacitor filled with dielectric constant of 2, compared to the dielectric constant of 80 for water (Zimmermann, 1976; 1986,
Castro et al., 1993). The cell membrane is filled with dielectric substances made of cellular components. Therefore, free charges can be accumulated at both sides of the membrane. The application of electric field pulse causes an increase in the transmembrane potential and the charges at the two membrane surfaces are opposite and attract each other. The attractive force compresses the membrane and reduces the thickness of membrane. Due to the decrease of membrane thickness, the electric compressive forces increase more quickly until a local breakdown of membrane occurs (Zimmermann, 1986). The dielectric breakdown of a lipid bilayer cell membrane is explained by Zimmermann (1986) and showed in Fig. 1.1. The membrane is regarded as a capacitor and the normal resting potential difference across the membrane is 10 mV. When the membrane is exposed to an external electric field, the membrane potential difference increases due to charge separation across the membrane. The breakdown of the membrane occurs when a critical breakdown voltage is reached with the increase of the external electric field intensity. This breakdown leads to the formation of transmembrane pores. The breakdown is reversible if the size of the pores is relatively small compared to the total membrane surface. Irreversible breakdown occurs at the supercritical field strengths and causes inactivation of the cell.

Tsong (1989; 1991) suggested the electroporation of cell membrane as a mechanism of the effect of PEF on cells. Electroporation process is shown schematically in Fig. 1.2. When an external electric field is applied, electroporation occurs at protein channels due to protein denaturation caused by heating or electric modification of their functional groups. Electroporation leads to an osmotic imbalance of the cell membrane. The osmotic pressure of cellular macromolecules inside the cell would drive the influx of water and small solutes. The cell swells and later ruptures, leading to the destruction of
the cell. The mechanisms of pore formation and destabilization of bacterial cell membranes by high voltage pulsed electric fields are not completely understood. Even though several studies have demonstrated the effect of pulsed electric field on the death of microorganisms, the underlying mechanisms involved in the breakdown of cell membrane leading to cell death are not clear, especially in view of the conflicting results of electric field strength and temperature effects.

Susceptibility of membrane to damage by PEF is a likely to depend on the composition of cell envelope and strength. A typical bacterial cell envelope consists mainly of a phospholipid membrane, a peptidoglycan (murine) layer, and in case of Gram-negatives, a carbohydrate-rich outer membrane, known as the lipopolysaccharide layer (LPS). Even though its structure varies, the cell envelope of all bacteria has a considerable tensile strength (Neidhardt et al., 1990). Cell membrane damage/integrity and or membrane potential can be determined and it is closely associated with cell viability (Shapiro, 1995). Our preliminary studies with propidium iodide proved that vital staining is a powerful method for rapid measurements of cell membrane integrity and viability.

**Factors affecting the inactivation of microorganisms by PEF**

Pulsed electric field strength and treatment times are the two major factors influencing the inactivation of ratio in PEF processing (Hulsheger et al., 1981; Sale and Hamilton, 1967; Sale and Hamilton, 1968; Hamilton and Sale, 1967; Matsumoto et al., 1991; and Jayaram et al., 1992). Based on the literature, the other factors influencing the inactivation ratio are discussed below.
Pulse shape, pulse polarization and pulse frequency

Qin et al. (1994) compared three different pulse waveforms (square, exponential, and oscillatory) for the inactivation of microorganisms. They reported that square wave pulses were more efficient than exponential pulses. Oscillatory decay pulses were the least efficient since they prevented the cells being continuously exposed to a high-intensity electric field for an extended period of time, thereby preventing the cell membrane from irreversible breakdown.

Ho et al. (1995) and Qin et al. (1994) reported that bipolar pulses were more efficient than are mono pulses. A rapid reversal of the applied electric-field orientation changes the movement direction of the charged groups on the cell membrane. The alternating stress produced by bipolar pulses results in a structural damage of the membrane and in the enhanced susceptibility of the cell membrane to electrical breakdown.

Hulsheger et al. (1981) did not find any significant effect of pulse frequency on inactivation ratio. Increasing pulse frequency increases the capacity of a continuous PEF processing unit. However, as the pulse frequency increases, the command charging power supply and the high-speed electrical switch become more expensive (Zhang et al., 1995a). This also increases the energy added to the medium. Therefore, a cooling system must be built to maintain the temperature below the normal conditions in order to reduce thermal degradation.

Characteristics of medium:

Hulsheger et al. (1981) reported that there was a synergistic effect of the temperature of the treated medium with PEF treatment on the inactivation ratio.
Increasing the medium temperature decreases the breakdown of transmembrane potential of the cell membrane in addition to causing thermal-injury effects, thus resulting in a higher inactivation ratio (Pothakamury et al., 1996).

Jayaram et al. (1993) studied the effects of liquid-medium conductivity on the inactivation ratio of *Lactobacillus brevis* during PEF treatment. As the conductivity of the fluid was increased, the resistance of the treatment chamber was reduced. This reduced the pulse width, which consequently reduced the inactivation ratio. In order to study the effects of conductivity, the inactivation ratio was determined while keeping the pulse width constant by connecting external resistors in parallel with the treatment chamber. They observed higher inactivation in lower conductivity medium. Lowering conductivity of medium increased the difference in ionic concentration between the cytoplasm and the medium, thus facilitating an increased flow of ionic substances across the membrane. Jayaram et al. (1993) believed that this, in turn, caused a drain on cell energy reserves and eventually weakened the membrane structure, thereby making the membrane susceptible to pulse application.

Vega-Mercado et al. (1996) studied the effects of pH and ionic strength of medium on inactivation of microorganisms during PEF treatments. The inactivation ratio was reduced in higher ionic strength solutions. As the pH was reduced from neutral, the inactivation ratio increased. The PEF treatment and ionic strength were responsible for pore formation and compression of the cell membrane, whereas the pH of the medium affected the cytoplasm when the pore formation was completed. Vega-Mercado et al. (1996) believed that these factors disturbed the homeostasis of the microorganisms, thereby leading to an increased inactivation ratio.
Characteristics of microorganisms

Sale and Hamilton (1967) reported that microorganisms differed in their sensitivity to PEF exposure. Yeasts were more sensitive than vegetative bacteria. Sale and Hamilton (1968) reported experimental values of breakdown transmembrane potential for various microorganisms and they ranged from 0.7 to 1.15 V. Hulsheger et al. (1983) conducted comparative studies on the lethal effects of PEFs on gram-negative and gram-positive bacteria and on yeast cells. They reported that gram-positive bacteria and yeasts were less sensitive to PEF treatment than were gram-negative bacteria. Zhang et al. (1994) studied the inactivation of E. coli and Saccharomyces cerevisiae by PEF treatment. Their results showed that yeast was more sensitive than was gram-negative bacteria. These results were in agreement with the results of Sale and Hamilton (1967), but contrary to the result of Hulsheger et al. (1983). Yeast cells are larger than bacterial cells and thus yeast cells show a lower breakdown transmembrane potential.

As PEF treatment inactivates microorganisms based on the electromechanical instability of microorganisms, lethal effects vary not only for different species but also for different growth phases of each species. Hulsheger et al. (1983) reported that cells harvested from the exponential growth phase were more sensitive to PEF treatment than were those from stationary phase. Pothakamury et al. (1996) also reported that E. coli cells in exponential phase were more sensitive to PEF treatment when compared with cells in stationary and lag phases.

Characteristics of treatment chamber

Treatment chambers usually consist of two electrodes held in position by insulating material. The insulating material forms an enclosure that contains the medium or the food to be treated. Parallel plates, wire-cylinder, rod-rod, rod-plate, and coaxial
(concentric) cylinders are all potential electrode configurations (Zhang et al., 1995a). Coaxial electrode and parallel plates configurations were used in most of the studies reported. Parallel plates produce uniform distribution of electric field strength and are simple in design. Coaxial electrodes, on the other hand, provide smooth and uniform product flow and are attractive for industrial applications (Zhang et al., 1995a).

Matsumoto et al. (1991) studied three different electrode configurations, namely, a wire-cylinder electrode system, a "converged electric field" electrode system, and a rod-rod electrode system. In the wire-cylinder electrode system, a high-voltage wire positioned at the center of a grounded cylindrical electrode, and the liquid medium was placed in the annular space. As expected, the electric field was not uniform, thus rendering the treatment less effective than that in other designs. In the rod-rod electrode system, two-rod electrodes were placed in the treatment chamber, and a high-voltage electric field was created between the two points of the electrode rods. This yielded effects similar to those associated with the electrohydraulic treatment; therefore, the formation of arcs makes it unacceptable for use in the treatment of food. The converged electric field-type electrode system (Fig. 1.3) is suitable for continuous treatment of food. In this system, liquid food was continuously fed through holes in the disc electrode. An insulating plate (Teflon, 1 cm thickness) with small holes was placed between parallel disc electrodes. The size and number of holes in the Teflon plate varied. Since the current was converged into the small holes of the Teflon plate, a converged high-voltage electric field was formed on these holes. The advantage of this electrode system is that the overall resistance of the load in the treatment chamber was increased, thus reducing the cost for production of pulses.
Yin et al. (1997) used a continuous type co-field flow PEF treatment chamber (Fig. 1.4) to process the fluid foods. In co-field flow PEF treatment chamber, the fluid flows through a series of treatment zones with a high voltage electrode on one side of each zone and a low voltage electrode on the other. The PEF process is defined by the electric field strength, or the voltage applied per distance between electrodes, and the treatment time. Martin-Belloso et al. (1997) compared the inactivation ratio between continuous circulation treatment and a stepwise treatment and reported that there was no significant difference between the two treatment methods.

Promising results have been obtained with PEF treatment of foods at the laboratory level, there were some inconsistencies. The high initial cost of setting up the PEF processing system is the major obstacle confronting the concept of industrial application of this method. However, PEF systems are attractive because of their low operating costs and because they yield high quality, minimally processed products. A continuous PEF processing system suitable for industrial application has yet to be developed. It is a challenge for food and electrical engineers to design a continuous treatment chamber and a pulse generator that are capable of satisfying industrial requirements. Inactivation tests in viscous and particulate foods need to be conducted as well. Further experiments have to be conducted to optimize process conditions (e.g., validation of different combinations of electric field strength and treatment time required to pasteurize or sterilize various foods). Treatment chambers need to be modified for certain foods. Innovative developments in high-voltage pulse technology are also required to reduce the cost of the pulse generator and make PEF processing competitive with thermal processing methods.
Low Voltage Ohmic Heating

Recent interest of industry has resulted in renewed attention towards technologies utilizing electricity in foods. Most of the research using electrical energy hold promise of microbial inactivation and provide high quality and shelf-stable. One of these technologies is the ohmic heating. The ohmic heating effect is produced by passing an electrical current at an appropriate voltage throughout the liquid or liquid particulated food products such as milk, fruit juices, and tomato juices (Fig. 1.5).

Ohmic heating is an emerging food processing technique suitable for liquid food pasteurization (Fryer et al., 1993). Low voltage ohmic heating was recently tested in lactic acid fermentation (Cho et al., 1996). Growth kinetics of Lactobacillus acidophilus and associated production of lactic acid and bacteriocin were studied when the fermentation was carried out under conventional and non-lethal electrical heating (sublethal ohmic heating). The lag period for L. acidophilus was affected by the method of heating depending on the fermentation temperature. There was a 94% decrease in lag period under non-lethal electrical heating (30°C, 15V) when compared with conventional heating method. The electrical current enhanced the early stages of growth, but inhibited the late stages of growth. Ohmic heating resulted in higher pH and lower bacteriocin activity in the fermented medium. The authors suggested that the lag period decreased by non-lethal electrical heating at 30°C could be beneficially used in food industry to shorten the time for processing cheese and yogurt. However, electrical current decreased and showed some fluctuations during the fermentation due to the noise from the electric source, the high ionic strength of medium, and lack of accurate and sensitive measuring devices at the level of current in their study.
The effects and interactions of sublethal ohmic heating on the growth and metabolic activity of microorganisms have not been studied in details. Firstenberg et al. (1984) used impedance measurement techniques to monitor the microbial growth and metabolic activities. Parrot (1992), and Wolcott et al. (1969) reported that the changes in the composition of growth medium following growth of microorganisms are associated with the changes of impedance. Rowley (1972) reported that electrical current generated by electrochemical reactions enhanced the growth of bacteria. Results showed that bacterial counts were 100 fold higher than controls. Rowley (1972) concluded that electrical current can be used either enhance or inhibit the growth of microorganisms depending on the experimental conditions.

**PHYSICAL PROPERTIES OF BACTERIAL CELL ENVELOPE**

Most bacterial cells are surrounded by rigid cell envelopes, which protect the cytoplasm from influences of the outer environment. The envelope of gram-positive bacteria consists of the following components (from inside to outside): (a) cytoplasmic membrane (proteins & phospholipids), (b) cell wall (peptidoglycan & teichoic acid), (c) capsule (usually polysaccharides). Gram-negative cell envelope contains the following layers: (a) cytoplasmic membrane (proteins & phospholipids), (b) cell wall (peptidoglycan), (c) periplasmic space (membrane-derived oligosaccharides, transport proteins & degradative enzymes), (d) outer membrane (lipopolysaccharide, proteins, & phospholipids), (e) capsule (usually polysaccharide). These envelopes also maintain the characteristic shape of the cells and give protection against osmotic lysis. The bacterial cell envelope unusually varies in structure and chemical composition. This variation reflects the enormous number of environmental challenges encountered by microorganisms (Neidhardt et al., 1990).
Susceptibility and resistance of microorganisms to PEF depends on cell envelope composition and strength. Bacterial cell envelope consists mainly of a phospholipid membrane, a peptidoglycan layer (murein), and a carbohydrate-rich outer membrane, known as lipopolysaccharide (LPS). Even though its structure varies, cell envelope of all bacteria has tensile strength (Neidhardt et al., 1990). Variations in the structure of bacterial cell envelope are caused by the groups of factors listed below:

a. Thickness and structure of peptidoglycans: Gram-negative bacteria are deficient in peptidoglycans while gram-positive cell wall is rich in this substance.

b. Presence of LPS: LPS are mainly present only in gram-negative cell envelope.

c. Fatty acids chain length and the degree of saturation.

d. Presence of flagella and pili.

Following section addresses the cellular membrane structures of gram-negative and gram-positive bacteria. It is useful to know the physical properties of bacterial cell envelope in order to study the resistance and susceptibility of gram-negative and gram-positive bacteria to pulsed electric field.

**Bacterial cell envelope**

Structural and functional properties of the bacterial cell envelope are directly related to adaptive strategies of bacteria. The components of the bacterial cell envelope are adopted to take up nutrients, often from highly dilute solutions, and exclude certain toxic compounds. Surface components participate directly in the adherence of bacteria to surfaces and interfaces and in the transfer of genetic information. Many of the properties used to distinguish bacteria taxonomically based on differences of surface components (Neidhardt et al., 1990).
Cell membrane

All bacteria have a cell membrane of a lipid bilayer. Approximately 70% of the mass of the membrane is attributable to proteins. Basically, the cell membrane is an osmotic barrier modified by the presence of specific transport systems. Since most of the macromolecular syntheses and metabolic reactions occur in the cytoplasm, the cell membrane is the true boundary between cell interior and the outside. The high protein content of the bacterial cell membrane suggests that this structure is involved in multiple and unique functions. Some of the known functions of bacterial cell membrane are: 1) osmotic barrier, 2) transport of specific solutes (nutrients and ions), 3) synthesis of membrane lipids (including lipopolysaccharide in gram-negative cells), 4) synthesis of wall murein, 5) assembly and secretion of extracytoplasmic proteins (membrane periplasmic, outer membrane, extracellular), 6) respiratory electron transport, 7) chromosome segregation (probably), 8) chemotaxis (both motility and sensing function) (Neidhardt et al., 1990).

Cell wall

Bacterial cells are particularly tough, and difficult to break by mechanical forces. They retain their shape even under extreme environmental conditions. The toughness of bacteria is related to their cell wall structure, which provides rigid mechanical support and prevents pressure from bursting the cell (osmotic lysis) (Neidhardt et al., 1990). Besides, the cell wall represents a chemical and physical defense against noxious chemicals that may harm the cell membrane. Gram-positive and gram-negative bacteria differ considerably in the structure of their cell walls (Table 1.1, and Fig 1.6). Cell wall structure copes with environmental changes. Any portion of the wall of gram-positive
cells looks like a thick blanket while that of most gram-negative cells like a flimsy sheet. In reality, even the thin gram-negative wall has considerable tensile strength (Rogers, 1983; Ghuysen and Hakenbeck, 1994; Neidhardt et al., 1990). Table 1.1 summarizes the major classes of chemical constituents in the walls and envelopes of gram-positive and gram-negative bacteria.

**Gram-positive cell wall**

The gram-positive cell wall consists of a thick coat of murein (peptidoglycan). Peptidoglycan layer is a glycan backbone of repeating groups of β-1,4-linked disaccharides of β-1,4-N-acetylmuramyl-N-acetylglucosamine. Tetrapeptides of L-alanine-D-isoglutamic acid-L-lysine (or diaminopimelic acid)-n-alanine are linked through the carboxyl group by amide linkage of muramic acid residues of the glycan chains; the D-alanine residues are directly cross-linked to the ε-amino group of lysine or diaminopimelic acid on a neighboring tetrapeptide, or they are linked by a peptide bridge. In *Staphylococcus aureus* peptidoglycan, a glycine pentapeptide bridge links the two adjacent peptide structures. The extent of direct or peptide-bridge cross-linking varies from one peptidoglycan to another. The staphylococcal peptidoglycan is highly cross-linked, whereas that of *E. coli* is much less so (Ghuysen and Hakenbeck, 1994).

The structure of the peptidoglycan is illustrated in Fig 1.7. The β-1,4 glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine is specifically cleaved by lysozyme. Widely distributed in nature, this enzyme is present in human tissues and secretions and can cause complete digestion of the peptidoglycan walls of sensitive organisms. When lysozyme is allowed to digest the cell wall of gram-positive bacteria
suspended in an osmotic stabilizer (such as sucrose), protoplasts are formed. These protoplasts are able to survive and continue to grow on suitable media in the wall-less state. Gram-negative bacteria treated similarly produce spheroplasts, which retain much of the outer membrane structure. The dependence of bacterial shape on the peptidoglycan is shown by the transformation of rod-shaped bacteria to spherical protoplasts (spheroplasts) after enzymatic breakdown of the peptidoglycan. The mechanical protection afforded by the wall peptidoglycan layer is evident in the osmotic fragility of both protoplasts and spheroplasts (Ghuysen and Hakenbeck, 1994).

The other major components of the gram-positive wall are teichoic acids and teichuronic acids, which may account for up to half the wall mass in some species (Neithardt et al., 1990). These compounds are found only in certain gram-positive bacteria (such as Staphylococcus, Streptococcus, Lactobacillus, and Bacillus spp). So far, they have not been found in gram-negative organisms. Teichoic acids are polyolphosphate polymers, with either ribitol or glycerol linked covalently by phosphodiester bonds to the peptidoglycan (Ghuysen and Hakenbeck, 1994). The structures of teichoic acids are illustrated in Fig 1.8. Substituent groups on the polyol chains can include D-alanine, N-acetylglucosamine, N-acetylgalactosamine, and glucose; the substituent is characteristic for the teichoic acid from a particular bacterial species and can act as a specific antigenic determinant.

**Gram-negative cell wall and the outer-membrane**

Gram-negative bacteria have evolved a radically different solution to the problem of protecting the cytoplasmic membrane. Their murein layer is much thinner than that of gram-positive bacteria, and they make a completely different structure called outer membrane (Table 1.2). The outer membrane is built up outside a thin murein layer. The
outer membrane is chemically distinct from the usual biological membranes and has the ability to resist damaging chemicals. It is a bilayered structure, and its inner leaflet resembles in composition that of the cytoplasmic membrane. Its outer leaflet, on the other hand, has a unique constituent in the place of phospholipids. This component is the bacterial lipopolysaccharide, or LPS (Neidhardt et al., 1990; Rogers, 1983). LPS consist of three portions: The lipid A, core, and O antigen. The lipid A of LPS is inserted with phospholipids to create the outer leaflet of the bilayer structure. Lipid A is an unusual glycolipid, because the fatty acids attached to its disaccharides are shorter than usual (14 carbons long instead of the more common 16 or 18); phosphate groups are also attached to the disaccharides. All of these fatty acids are saturated and have hydroxyl group constituents (Neidhardt et al., 1990).

A short series of sugar constitutes the core. Its structure is relatively constant among gram-negative bacteria and includes two characteristic sugars, ketodeoxyoctonoic acid and heptose.

A long carbohydrate chain, up to 40 sugars in length, makes up the O antigen. The hydrophilic carbohydrate chains of the O antigen cover the bacterial surface. Although this is a loose structure, at least compared with the LPS layer of gram-positive bacteria, it is highly effective in excluding hydrophobic compounds. Mutants that make no O antigen become sensitive to compounds like bile salts and certain antibiotics to which the wild type is resistant. In addition to these components, the outer membrane possesses several major outer membrane proteins; the most abundant is called porin. The assembled subunits of porin form a channel that limits the passage of hydrophilic molecules across the outer membrane barrier. Evidence also suggests that hydrophobic
pathways exist across the outer membrane and are partly responsible for the differential penetration and effectiveness of certain β-lactam antibiotics (ampicillin, cephalosporins) that are active against various gram-negative bacteria (Neidhardt et al., 1990). Several outer membrane proteins are involved in the specific uptake of metabolites (maltose, vitamin B₁₂, nucleosides) and iron from the medium. Thus, outer membranes of the gram-negative bacteria provide a selective barrier to external molecules and thereby prevent the loss of metabolite-binding proteins and hydrolytic enzymes (nucleases, alkaline phosphatase) found in the periplasmic space.

The complex lipopolysaccharide is also called endotoxin, thereby distinguishing these cell-bound, heat-stable toxins from heat-labile, protein exotoxins secreted into culture media (Rogers, 1983; Wright and Tipper, 1979; Ghuysen and Hakenbeck, 1994). Endotoxins possess powerful biologic activities and play an important role in the pathogenesis of Gram-negative bacterial infections. Endotoxic properties of LPS reside largely in the components of lipid A (Neidhardt et al., 1990).

LPS is held in the outer membrane by relatively weak cohesive forces (ionic and hydrophobic interactions) and can be dissociated from the cell surface with surface-active agents. LPS molecules are assembled at the plasma or inner membrane. These newly formed molecules are initially inserted into the outer-inner membrane adhesion sites (Neidhardt, 1990).

The connection between the outer membrane and the cytoplasmic membrane is not well understood. These two structures can be mechanically separated by plasmolysis (placing bacteria in a hypertonic medium). Gram-negative cell's LPS is protected by the outer membrane, which is impermeable to lysozyme. Two general techniques can be used to remove the LPS layer of these microorganisms and, in an isoosmotic medium, generate
osmotically sensitive protoplast-like forms. The outer membrane can be made permeable to lysozyme by the use of the bivalent ion chelator EDTA, which loosens the structure of LPS. Freezing certain strains of *E. coli* also achieves the same thing. In the presence of penicillin cell growth is not inhibited, bacteria continue to synthesize their constituents, which, when accumulated in sufficient amounts, make a bulge through the murein layer. Eventually, the whole cell is extruded and becomes spherical.

**Periplasm**

The dual membrane system of gram-negative bacteria creates a complex compartment called periplasmic space, or periplasm. The periplasm is the region between the outer surface of the inner (plasma) membrane and the inner surface of the outer membrane (Neidhardt et al., 1990, Smit, 1987). In addition to the hydrolytic enzymes, the periplasm holds binding proteins (proteins that specifically bind sugars, amino acids, and inorganic ions) involved in membrane transport and chemotactic receptor activities. Plasmid-encoded β-lactamases and aminoglycoside-modifying enzymes in periplasm produce antibiotic resistance by degrading/modifying an antibiotic in transit to its target sites on the membrane (penicillin-binding proteins) or on the ribosomes (aminoglycosides). These periplasmic proteins can be released by subjecting the cells to osmotic shock and after treatment with EDTA (Neidhardt et al., 1990).

**Acid-fast cell wall**

In addition to the major cell wall polymers, the walls of some gram-positive bacteria possess polysaccharide molecules linked to the peptidoglycan. Acidic polysaccharides attached to the peptidoglycan are called teichuronic acids. *Mycobacteria*
have peptidoglycolipids, glycolipids, and waxes associated with the cell wall (Ghuysen and Hakenbeck, 1994). Having such a protective cover makes these organisms nearly impermeable to many toxic chemicals, including acids. If a dye is introduced into these cells, for example by brief heating, it cannot be removed by dilute HCl, as would be the case in all other bacteria. These microorganisms are therefore called acid-fast or acid-resistant (Neidhardt et al., 1990).

**Effects of Temperature, Salt and Pressure on Bacterial Cell Membrane**

All microbial cells must have an intact cell membrane for their survival and growth. The composition of cell membrane changes in response to changes in the external medium in order to maintain its functions. The most important environmental factors in the context of food spoilage and poisoning are the lowering of water activity by preservative solutes (e.g. salts), pH and temperature. All of these processing parameters affect the structure and function of membranes. Some novel processing techniques such as the use of ultra-high pressure also targets cell membranes for their biological effects.

The physiology of bacteria depends on the adaptation of their membrane composition to external stimuli. Most changes in lipids are necessary to maintain a particular state of fluidity in the membrane bilayer. The lipids are directly responsible for such properties as passive permeability and indirectly influence others through interaction with membrane proteins (Russell, 1989). A better understanding of the mechanisms and regulation of membrane lipid changes in response to preservation methods may help in designing novel methods of protecting foods with limited amount of additives or processing (Russell and Gould, 1991).
Cell membrane response to temperature

In order for growth to occur at low temperatures, the cytoplasmic membrane must retain sufficient fluidity to ensure the necessary physical state, required for membrane structure and function (Sinesky, 1974). The major response of membrane composition to a change in temperature is an alteration in fatty acid component of lipids (Russell, 1992). Changes in the head-group composition are generally minor. In most bacteria, the proportion of unsaturated fatty acids in phospholipids increases as the temperature decreases. This change maintains the fluidity of cell membrane despite decreasing temperature because the melting point of lipids decreases as their proportion of unsaturated fatty acids increases (Neidhardt et al., 1990).

The fatty acid composition of *L. monocytogenes* is dominated to an unusual extend by branched-chain fatty acids, and at low temperatures *anteiso*-C<sub>15:0</sub> becomes the major fatty acid (Edgcomb et al. 2000). Jones et al. (1997) reported that *L. monocytogenes* had a lower proportion of *anteiso*-C<sub>17:0</sub> and a higher proportion of *anteiso*-C<sub>15:0</sub> and short chain fatty acids when grown in continuous culture at 10°C compared to 30°C. According to Mastronicolis et al. (1998), *anteiso*-C<sub>15:0</sub> increased in all lipid classes in *L. monocytogenes* grown at 6°C. Horward and Russell (1984) reported that the primary response in *Lactobacillus plantarum* to a decrease in growth temperature is a marked increase in the level of unsaturation of the membrane lipids. The changes in *L. plantarum* are characteristic of an organism which uses an anaerobic pathway of fatty acid biosynthesis in that most of the increase in unsaturated fatty acids is in 18:1. *Clostridium botulinum* uses the anaerobic pathway for the synthesis of fatty acids. The major fatty acids in *C. botulinum* are 14:0, 15:1, 16:0, 16:1, and 18:1. When the growth
temperature is lowered from 37°C to 8°C, the main change in composition is also an increase in the level of unsaturation from 27% to 40%, together with a small decrease in the mean acyl chain length from 15.9 to 15.3 (Russell et al., 1995). Therefore, all of these three psychrotolerant organisms respond to changes in temperature by modulating their fatty acid composition to maintain membrane fluidity at lower temperatures.

**Cell membrane response to salt**

In contrast to the response of membrane composition to temperature, the major change in response to salt is in the head-group of lipids (Russell et al., 1995). When water activity is lowered by means of preservative solutes, the common alteration is the increase in the proportion of anionic phospholipid and glycolipid. Such changes occur in response to charged or uncharged solutes and are a means of preserving the membrane lipids in the proper bilayer phase (Sutton et al., 1990, Sutton et al., 1991). They occur in a wide range of bacteria which can grow at low water activity, including those causing food-spoilage (Russell, 1993). Russell et al. (1995) reported that *L. monocytogenes* responded to the addition of 2% (w/v) NaCl to the growth medium by increasing the ratio of diphosphoglycolipid (DPG)/phosphatidylglycerol (PG) in the presence of salt compared with control cultures. Therefore, they studied the effects of solutes on the lipid composition of *L. plantarum*. Salt did not stimulate a rise in lysyl-phosphatidylglycerol (LPG) content. They reported a small decrease in LPG which may have resulted from the slower growth rate. It was also observed that as the batch cultures went into the stationary phase of growth, the portion of LPG halved with a corresponding increase in PG content. Besides the change in membrane lipid composition, the other major change in cellular
composition, which is triggered by growth at low water activity, is the accumulation of compatible solutes. In many bacteria, the major compatible solute is glycinebetaine, particularly when they are grown in rich media, which would mimic the situation in proteinaceous food materials. For example, *L. plantarum* accumulates glycinebetaine when it is available exogenously, but glutamate, alanine and proline if it is not. *L. monocytogenes* accumulates, besides glycinebetaine, the novel compatible solute, carnitine (Kets et al., 1994; Verheul et al., 1995). Compatible solutes stabilize intracellular enzymes and other proteins and enable them to continue functioning when the water activity decreases. They are present in many foods and can be taken up from the external medium. When glycine-betaine was added to the growth medium, *L. plantarum* responded by synthesizing membranes with a much higher proportion of LPG at the expense of both PG and DPG. *L. plantarum* contains, besides PG and DPG, the lysyl derivative of PG. In *S. aureus*, the proportion of this lipid has long been known to increase when the pH of the growth medium falls, and this is believed to help prevent the influx of protons into the cell (Haest et al., 1972). Whether this is related to changes in the proton permeability of the membranes under these growth conditions is not known. However, it would be interesting to determine how this property of membranes is influenced by compatible solutes such as glycine-betaine, which are believed to stabilize membranes against the effects of low water activity.

Salt and other solutes may also influence the fatty acid composition of the membrane lipids (Russel, 1989, 1993). However, 2% (w/v) NaCl did not significantly affect the fatty acid composition of the lipids of *L. monocytogenes*. *L. monocytogenes* is known to grow in the presence of 10% NaCl in a rich medium like brain heart infusion
(BHI) broth. The lack of change in the fatty acid composition upon imposition of a salt stress might be related to the low salt concentration used, which is probably not very stressful for this bacterium. The osmotic tolerance of *L. monocytogenes* in defined medium is much lower than in BHI, due to the presence of osmoprotectants such as betaine, carnitine and peptides in BHI (Beumer et al., 1994, Amezaga et al., 1995; Verheul et al., 1995). Compatible solutes are believed to interact with membranes, so the fatty acid composition of *L. monocytogenes* grown in BHI in the presence of salt reflects not only the influence of salinity but also the effect of compatible solutes.

**Cell membrane response to ultra-high pressure**

Because of its inactivating effect on microorganisms, the potential use of pressure technology as a novel food preservation method is currently being investigated. Growth of microorganisms is generally inhibited at pressures in the range of 20 to 130 MPa while higher pressures of between 130 and 800 MPa may result in cell death; the maximum pressure allowing for growth or survival depends on the species and medium composition (Abee et al., 1999). Since membranes have been implicated as targets for pressure damage, the fatty acid composition of the mutants was compared to that of the parent strain, but no significance differences could be found (Hauben et al., 1997). MacDonald (1992) reported that less fluid bacterial cell membranes are more sensitive to ultra-high pressure (UHP). Russell et al. (1995) studied the susceptibility of *L. plantarum* to UHP in cultures grown under a variety of conditions temperature, salinity and pH, because the known changes in membrane lipid composition, which occur under such stress conditions, could alter susceptibility and thus the efficacy of UHP as a food-processing technique. According to Russell et al. (1995), cell with a high DPG content (which would
stiffen the membrane if there was calcium in the external environment) are more susceptible to UHP and that generally an increase in membrane fluidity has the opposite effect, i.e. decreases the sensitivity to UHP.

Fatty acid chain length and degree of saturation affect greatly the fluidity of the lipid bilayer comprising cell membranes. When cell membrane predominantly contains long and saturated fatty acids (i.e., of high melting point), the membrane fluidity decreases and susceptibility of cells to mechanical damage decreases appreciably. Since fatty acid structure of cell membrane is an intrinsic cell property that might be altered experimentally through variation in culture's environment. The fluidity and fatty acid composition of cell membrane of psychrotrophic, mesophilic and thermophilic bacteria vary. Therefore, it might be possible to make a correlation between the relative membrane fluidity and susceptibility of microorganisms to novel food processing methods such as pulsed electric field, high hydrostatic pressure and other food preservation technologies. It may be possible that psychrotrophic bacteria should be more responsive, and the thermophilic bacteria should be the least responsive to the increase in treatment temperature.

RAPID ASSESSMENTS OF CELL VIABILITY

Cell viability is conventionally defined as the reproductive capability of the cell. Several characteristics of viable cells could be measured and used to estimate the reproductive ability (Amsterdam, 1991; Chen and Smiley, 1993; Shapiro, 1995). For example, the determinations of membrane integrity and/or membrane potential can be associated with viability (Shapiro, 1995). In a broad sense, the alternative approaches to
viability assays include growth-based viability assays, measurements of metabolic activities, redox potential, electrical conductivity, dye-based viability assays, microcalorimetry, radiometry and bioluminescence (Piddock, 1990).

The conventional method for determining cell viability is to measure ability of these cells in forming colonies on nutritionally rich media. The culture viability is then the ratio of these cells that formed colonies to the total cell count in the original sample, which is determined microscopically. However, there are several problems associated with this technique, not the least of which is the length of time required to obtain the results. For some slowly growing microorganisms, it may take several weeks to determine how many cells were viable in the original sample. Even when the sample contains fast-growing organisms and the plates are incubated under optimal growth conditions, a minimum of overnight growth is usually required before the resulting colonies can be counted. For economical reasons, such a delay is often unacceptable. Therefore, many rapid methods have been developed to allow a rapid assessment of the viable microbial load in a sample (e.g., Adams and Hope, 1989; Fung, 1994; Harris and Kell, 1985; Jones, 1987).

These alternative rapid viability measurements include a variety of stain-based methods. The so-called vital stains that have been used in attempts to estimate microbial viability fall into three broad categories.

(1) The intact membranes of viable cells exclude some dyes, such as propidium iodide (PI). Therefore, the presence of the dye within the cell indicates disruption of the cell membrane and may be expected to be correlated with cell death.
(2) Viable cells actively accumulate other dyes, such as rhodamine 123; thus, the number of brightly stained cells reflects the viability of the sample. However, in some cases more active cells can actually pump such dyes out (Jernaes and Steen, 1994). Additionally, energized membranes less tightly bind some dyes, so that the more active cells are less brightly stained.

(3) In the case of dyes such as fluorescein diacetate (FDA), a membrane-permeant nonfluorescent precursor is converted to a membrane-impermeant fluorescent molecule by the activity of intracellular enzymes, and thus is an indication of metabolically active cells.

A plate count only indicates how many of the cells can replicate under the conditions provided for growth. In the case of environmental samples, laboratory media, the temperature, and the other factors may differ substantially from those in the original sample (Roszak and Colwell, 1987). Thus, the proportion of cells that can divide and form colonies may be much lower than the number of cells that would score as viable using the dye-based rapid methods (Amann et al., 1995). Microscopic examination is largely a qualitative technique, wherein a judgment of alive or dead is not possible.

Flow cytometry offers an alternative method of determining the amount of fluorescent dye taken up by each cell in a population (Davey and kell, 1996; Kell et al., 1991; Lloyd, 1993; Shapiro, 1995). Since quantitative measurements can be made very rapidly on a large number of individual cells, an accurate picture of the distribution of dye uptake by many thousands of cells is possible within a few minutes.
**Growth-based viability assays**

The classical approach requires a suitable growth media and conditions for the organism or organisms present in the sample, as well as the use of a suitable method of growth detection for the organisms. In practice, because of limited time, materials, and prior knowledge, the most convenient method is all too often chosen. Nutrient-rich media such as Trypticase soy broth (TSB) is often used for these procedures to ensure growth. Growth detection is usually measured either as colony-forming units (CFU) on a solid agar plate or as turbidity in liquid media.

Many microorganisms have growth requirements that are very different from the standard conditions applied. Microorganisms with known growth requirements may reside in a physiological state in which the standard culture conditions do not support growth, or do so only for a small fraction of the population, or only after long lag phases. Physiological states that can be difficult or impossible to detect include injury (stress), starvation (stationary phase), and dormancy (Kell et al., 1998).

In some cases, growth of viable cells can remain undetected due to the constraints of the growth determination method employed. Organisms showing slow growth rates or long lag phases may not be capable of producing enough biomass to form visible colonies or detectable turbidity during the period of incubation allowed. In some cases, growth may stop after a limited number of divisions (Kell et al., 1998), or the organism may be unable to form colonies on solid media. These factors, alone or in combination, may lead to false-negative results.

Therefore, the main drawback of classical, growth-based viability assays is the possibility of false-negative results; false positives can be excluded by correct sterile
technique. However, if the experimental design equates viability with culturability, only growth-based viability assays make any true sense. Thus, it may be considered to be both necessary and sufficient criteria for cellular viability.

**Methods associated with metabolic activity**

Changes in pH values in a culture can be used to determine the viability. However, acid production depends on the culture’s acid-producing ability. In the presence of an antimicrobial agent, the amount of acid produced in a given time period is correlated with the concentration of antimicrobial agent. However, these methods have some drawbacks to determine the endpoints quantitatively. Therefore, they have not been developed further (Piddock, 1990).

For monitoring the growth or inhibition of a culture, a labeled substrate such as 
$^{14}$C-glucose may be supplemented into the growth media and the production of labeled products such as CO$_2$ is measured. The concentration of antibiotic required to produce 50% less CO$_2$ than that of control correlated well with the result from MIC test in broth (Piddock, 1990).

**Methods based on assessment of membrane integrity and potential**

The ability of the intact cell membrane to exclude acid dyes such as trypan blue, eosin, and nigrosin can determine membrane integrity, a measure of cell viability. Cell, which do no let in the dye, however, are not necessarily viable (Shapiro, 1995). Membrane potential is also related to membrane integrity since only intact membrane retains the achieved potential. Therefore, measurement of membrane potential can be used to indicate the viability of cultures (Petit, 1992). Rhodamine 123 can be used for
measuring membrane potential (Petit, 1992; Skowronek et al., 1990; Vannini et al., 1988), cyanine dyes (Shapiro, 1995) and resazurin (Piddock, 1990). Retention of the positively charged dye is dependent on the presence of a certain membrane potential (Shapiro, 1990). Cells with a lower membrane potential retain less amount of stain. Redox potential determination refers to the reaction of enzymes involved in energy (oxidative) metabolism. For this purpose, tetrazolium salts and methylene blue are used in viability determinations (Amsterdam, 1991; Shapiro, 1995).

**ATP-dependent bioluminescence assays**

ATP-dependent bioluminescence assay is based on the presence of ATP, which is the chemical form of energy in living organisms (Griffiths, 1996). With the reaction of luciferase enzyme from firefly, luciferin is produced, and ATP and Mg$^{2+}$ form a luciferin-ATP complex. When the complex dissociates, light is released and measured by a photometer. The more the ATP is present, the more the light is detected. The level of ATP is assumed to be remaining relatively constant. Therefore, the light produced during reaction is directly related to the number of metabolically active cells (Griffiths, 1996). By comparison of luminescence tubes with and without antibiotics, a ratio can be obtained. Since the measurement is an enzyme-related test, factors affecting the reaction need to be standardized (Griffiths, 1996).

**Methods based on measurement of electrical conductivity**

Progress of a microbial growth in a broth can be monitored and predicted from the changes in the electrical resistance to an alternating current due to the production of
metabolic products (acids, alcohol etc). Electrodes can be incorporated into tubes containing sensitive culture, and the electrical resistance can be continuously monitored (Firstenberg-Eden and Eden, 1984). The main disadvantage of this method is that bacterial population needs to be greater than $10^7$ CFU/ml to be reliable (Piddock, 1990).

The dye-based viability assays

There are occasions in which it is the metabolic activity of cells rather than their viability is the matter of concern. A cell with DNA damage at the origin of replication could not multiply, but the rest of its activities would probably be unaffected.

The dye-based assays have several advantages over growth-based assays. They are generally less time consuming, in some cases providing instantaneous results. They facilitate a method of measuring something that might be correlated with other measures of viability in microorganisms for which suitable growth conditions have not established. For organisms that display extremely slow growth rates, long lag phases, or low growth yields, growth-based methods are often impossible or impractical, and thus the dye-based approach offers an attractive alternative. In some cases (e.g., flow cytometry), the dye-based approach allows simultaneous analysis of taxonomic traits by using specific antibodies or ribosomal RNA probes (Amann et al., 1995; Wallner et al., 1993).

However, these rapid assays also often have their own drawbacks that can make them difficult to interpret. So far, no viability assay has been developed that selectively and reliably detects viable cells without, under any circumstances, giving a signal with dead cells (Kaprelyants et al., 1993). Dead cells are defined as cells unable to form a colony on a plate under any condition tested (Barer et al., 1998; Kell et al., 1998). This is
due to the fact that assays are normally based on single parameters such as membrane energization (often referred to as membrane potential, despite the absence of any direct evidence for it in bacteria; Kell 1988, 1992), enzyme activity, or uptake of a substrate. Some of these criteria might be considered necessary to define viability in most cases, but none of them is sufficient to exclude nonviable cells. For example, a cell could show some enzyme activity but may have lost its ability to divide by lethal lesions in the chromosome. Thus, these assays can give rise to false-positive results.

Commercial viability assays may produce false-negative results. In general, the test populations employed to demonstrate detection of viable microorganisms are either growing cells or cells subjected to rather short periods of stress (e.g., heat or cold). In natural environments, starvation and/or stress may be long-term, and the activity of cells may be reduced to extremely low levels (especially in the case of dormant cells), such that positive results might be below the detection limits. Injured cells may have damaged membranes and score as nonviable in these assay kits, whereas repair of the damage during cultivation on a rich medium would allow subsequent growth (i.e., viability).

The dye-based viability assays might be less time-consuming and more convenient in many cases. However, they do not necessarily provide reliable data because the principles on which they are based are not sufficient criteria for viability. Thus, before use, each method for assessing viability must be validated for each microorganism and for each type of sample in order to avoid false-positive or false-negative results.
**Dye exclusion assays**

The exclusion of dye by an intact membrane is probably the most straightforward viability test to understand and perform. Fluorescent stains normally excluded by living cells are used to assess viability on the grounds that dead cells have leaky membranes that are permeable to the stains. Nucleic acid stains such as propidium iodide (Fig 1.10.) and ethidium bromide are generally excluded by intact plasma membranes and their uptake is often used to indicate cell death (Aeschbacher et al., 1986; Bohmer, 1985; Green et al., 1994; Grogan and Collins, 1990; Jones 1987; Lapinsky et al., 1991; Lopez-Amoros et al., 1995; Schmid et al., 1992). PI is often the dye of choice for viability determinations in animal cells, whether the assay is done using fluorometry, flow cytometry or fluorescence microscopy (e.g., Garner et al., 1997; Maxwell and Johnson, 1997; Ronot et al., 1996). The applicability of excluded dyes for viability determinations needs to be carefully considered for each type of microorganism (Jernaes and Steen, 1994; Lewis, 1994).

**Dye uptake**

Mitochondria of eukaryotic cells have the ability to accumulate lipophilic cations such as rhodamine 123 (Chen, 1988; Chen et al., 1982; Grogan and Collins, 1990; Johnson et al., 1980, 1981). The staining of mitochondria with rhodamine 123 has been used in conjunction with flow cytometry to study their activity (Darzynkiewicz et al., 1981; Iwagaki et al., 1990; Lizard et al., 1990). Viable bacteria accumulate rhodamine 123, while non-viables do not (Diaper et al., 1992). Under certain conditions, individual bacteria take up rhodamine 123 quantitatively which reflects the extent of their viability.
whether they are immediately culturable, nonculturable, or dormant (Kaprelyants and
Kell, 1992). The uptake of rhodamine 123 is useful not only because it does not require
the use of fixatives to permeabilize the cell, but also because the concentrative uptake is
dependent on an intact and energized cytoplasmic membrane (Back and Kroll, 1991).
This has the great advantage that living cells can be stained and that further physiological
studies may be conducted following staining, if required (Davey et al., 1993).

There are, however, experimental problems with the use of lipophilic cations for
microbial viability determinations. For instance, microbial efflux pumps, causing both
viable and nonviable cells to appear to be nonfluorescent, may pump them out of viable
cells. In addition, although gram-positive bacteria readily concentrate the stain, the
permeability of the stain in gram-negative bacteria is low unless the cells are pretreated
with EDTA (Kaprelyants and Kell, 1992). However, such pretreatment is practically
impossible to standardize, and thus the extent of lipophilic cation accumulation may vary
from experiment to experiment. In addition, in a protocol for viability determination, it is
generally desirable that the number of preprocessing steps be kept minimum in order to
avoid the possibility of affecting the viability of sample.

An alternative approach is the use of lipophilic anions, which, in contrast to
cations, bind to nonviable cells. The lipophilic anion bis-(1,3-dibutylbarbituric acid)
trimethine oxonol, or DiBAC4(3), has been shown to enter eukaryotic membranes only if
the membranes are deenergized (Wilson and Chused, 1985). This stain has been used for
the rapid assessment of microbial responses to antibiotics (Jepras et al., 1997; Mason et
al., 1994, 1995b; Suller et al., 1997), allowing the analysis of heterogeneity within a
microbial population in terms of susceptibility to an antibiotic.
Metabolic activity

In certain circumstances the activity of the cells may be of more interest than membrane integrity. For this purpose, viability stains are used in mammalian cell biology, often as a positive marker in a dual-staining protocol with ethidium bromide or propidium iodide (Aeschbacher et al., 1986). These stains are themselves nonfluorescent and membrane permeant, but are metabolically altered inside the cell to become fluorescent and, under ideal conditions, impermeant. One example is fluorescein diacetate (FDA), which is cleaved by intracellular esterases to produce fluorescein. Dead cells do not stain because they lack enzyme activity and/or the fluorescein diffuses freely through their damaged membranes. Flow cytometric analyses of mammalian cells with this class of dyes are well established (e.g., Aeschbacher et al., 1986; Frey, 1997; Diaper and Edwards (1994a,b) used flow cytometry to detect a variety of viable bacteria after staining with FDA and its derivatives, or with ChemChrome B (Chemunex). Importantly, none of the dyes tested was found to be universal for the detection of viable bacteria. However, ChemChrome B was found to stain the widest number of gram-positive and gram-negative species, whereas the FDA derivatives preferentially stained gram-positive bacteria. Breeuwer et al. (1995) showed that FDA and carboxyfluorescein diacetate (CFDA) penetrated yeast rapidly and that esterase activity was probably most limiting; an energy-dependent efflux of carboxyfluorescein from viable cells was also observed (Breeuwer et al., 1994; Ueckert et al., 1995). It is probable that fluorescein can be pumped out of or leaks rapidly from viable microorganisms, thus giving the appearance of a lack of metabolic activity in cells that are nonetheless viable.
Commercial kits

A variety of kits have been produced specifically for the measurement of viability of specific types of microorganisms. For example, the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes) gives a two-color viability assessment of both gram-positive and gram-negative bacteria, where live cells are labeled green with SYTO 9 and dead cells are labeled red with PI. However, as freely admitted by the company, the kit equates the presence of intact plasma membranes with viability. Thus, “bacteria rendered nonviable by exposure to agents that do not necessarily compromise the integrity of the plasma membrane, usually appear viable by this criterion” (Haugland, 1996). Despite this limitation the kit is becoming widely used in microbiology (Braux et al., 1997; Buchmeier and Libby, 1997; Decamp et al., 1997; Duffy and Sheridan, 1998; Jacobsen et al., 1997; Joux et al., 1997; Korber et al., 1997; Langsrud and Sundheim, 1996; Rigsbee et al., 1997; Swarts et al., 1998; Taghi-Kilani et al., 1996; Terzieva et al., 1996; Virta et al., 1998; Weir et al., 1996). The growing use of such kits reflects, at least in part, their ease of use. In the case of the BacLight kit, the reagents are simultaneously added to the bacterial suspension, which is then analyzed without washing, so “live” and “dead” bacteria can be distinguished and quantitatively detected, rapidly.

Designing a dye-based protocol

When selecting a stain for a particular application there are several factors that need consideration. Some of them are the extinction coefficient, quantum yield, and photostability. These are of general applicability to flow cytometry and fluorescent spectrofluorometry and are discussed in detail elsewhere (Davey and Kell, 1996; Shapiro, 1995).
One factor in the measurement of viability is the toxicity of the stain. Protocols used to access viability should not affect viability. This becomes essential when one wishes to perform further physiological studies on the cells. In this case the toxicity of the stain must be assessed at the concentrations used in the protocol to ensure that they do not have any unwanted effects.

Even where further physiological study is not required, it is generally desirable to use cells that have not been fixed to avoid any possible effect of what one is trying to measure (Fabian et al., 1992; Patonay and Antoine, 1991; Shealy et al., 1995a,b).

When the appropriate stain and excitation source have been selected, it is important to perform a series of experiments to determine the optimum concentration of the stain and the optimum length of time between addition of stain and subsequent analysis. The optimum concentration will inevitably be a compromise between a high one (for maximum signal) and a low one (for specificity). It may be necessary to measure and adjust the cell concentration to ensure that stain uptake is not limiting (Hattori, 1988).

**Estimation of injured cells**

The presence of injured bacteria in populations subjected to stress (e.g., freezing or drying) is very likely. Commonly, such cells have an impaired membrane permeability barrier, which can be tested by membrane-impermeant probes, and NADH-induced respiration procedures using the flow cytometric approaches (MacDonell and Hood, 1982; Mukamolova et al., 1998).

In the case of bacteria, a damaged or leaky membrane may not be a sufficient criterion or defining a cell as nonviable, but it can nevertheless be used as an indication
of stress-induced injury. The permeability barrier of cells, starved for 5 months, was monitored by staining with propidium iodide (MacDonell and Hood, 1982; Mukamolova et al., 1998). It was shown that propidium iodide does not penetrate the cytoplasmic membrane of intact *Micrococcus luteus*, while the administration of 0.5% (v/v) octanol to the cell suspension resulted in 100% of the cells being stained with propidium iodide. Observation of different starved cultures of *M. luteus* showed that resuscitation was not successful in some cultures where the percentage of propidium iodide-positive cells was close to 100%. This indicated a correlation between the state of the permeability barrier and the ability of starved cells to recover, and thus may allow the use of PI staining for discrimination between dormant and dead cells in some populations.
<table>
<thead>
<tr>
<th>Chemical Component</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-positive cell walls</strong></td>
<td></td>
</tr>
<tr>
<td>Peptidoglycan</td>
<td>All species</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td><em>Streptococcus</em> group A, B, C substances</td>
</tr>
<tr>
<td>Teichoic acid</td>
<td></td>
</tr>
<tr>
<td>Ribitol</td>
<td><em>S. aureus, B. subtilis, Lactobacillus</em></td>
</tr>
<tr>
<td>Glycerol</td>
<td><em>S. epidermidis, Lactobacillus</em></td>
</tr>
<tr>
<td>Teichuronic acids (aminogalacturonic or aminomannuronic acid polymers)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>B. licheniformis, M. lysodeikticus</em></td>
</tr>
<tr>
<td>Peptidoglycolipids (muramylpeptide-polysaccharide-mycolates)</td>
<td><em>Corynebacterium</em> spp.,</td>
</tr>
<tr>
<td><strong>Gram-negative cell walls</strong></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>All species</td>
</tr>
<tr>
<td>Lipoprotein</td>
<td><em>E. coli</em> and many enteric bacteria, <em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>Porins (major outer membrane proteins)</td>
<td><em>E. coli, Salmonella typhimurium</em></td>
</tr>
<tr>
<td>Phospholipids and proteins</td>
<td>All species</td>
</tr>
<tr>
<td>Peptidoglycan</td>
<td>Almost all species</td>
</tr>
</tbody>
</table>

Table 1.1: Major Classes of Chemical Components in Bacterial Walls and Envelopes (Beveridge and Davies, 1983).
<table>
<thead>
<tr>
<th>Lipid A</th>
<th>Core</th>
<th>O Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosamine</td>
<td>Ketodeoxyoctonate</td>
<td>Polysaccharide chains;</td>
</tr>
<tr>
<td>β-hydroxymyristate</td>
<td>Phosphoethanolamine</td>
<td>Repeating units of species</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>Heptose,</td>
<td>-specific monosaccharides,</td>
</tr>
<tr>
<td></td>
<td>Glucose, Galactose</td>
<td>e.g. galactose, rhamnose,</td>
</tr>
<tr>
<td></td>
<td>N-acetylglucosamine</td>
<td>mannose and aequose in</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>S. typhimurium</em> LPS.</td>
</tr>
</tbody>
</table>

Table 1.2: The three major, covalently linked regions that form the typical LPS (Wright and Tipper, 1979).
Figure 1.1: Schematic diagram of reversible and irreversible electrical breakdown (Zimmermann, 1986).

(a) Cell membrane with a potential $V_m$
(b) Membrane compression by external electrical field.
(c) Pore formation with reversible breakdown.
(d) Large pores are formed with irreversible breakdown.
Figure 1.2: Electroporation of the cell membrane (Tsong, 1989, 1991).
Figure 1.3: Configuration of a converged electric field-type electrode (Matsumoto et al., 1991).
Figure 1.4. Co-field flow pulsed electric field treatment chamber (Yin et al., 1997).
Figure 1.5: Principle of ohmic heating.
Figure 1.6: Gram-positive cell envelope.
Figure 1.7: Gram-negative cell envelope.
Figure 1.8: Diagrammatic representation of peptidoglycan structures with adjacent glycan strands cross-linked directly from the carboxyterminal D-alanine to the e-amino group of an adjacent tetrapeptide or through a peptide cross bridge, N-acetylmuramic acid; N-acetylglicosamine (Modified from Ghuysen and Hakenbeck, 1994).
Figure 1.9: Structures of cell wall teichoic acids. (A) Ribitol teichoic acid with repeating units of 1,5-phosphodiester linkages of D-ribitol and D-alanyl ester. The glycosyl groups may be an N-acetylglucosaminyl (a or b) as in *S. aureus* or a-glucosyl as in *B. subtilis*. (B) Glycerol teichoic acid with 1,3-phosphodiester linkages of glycerol repeating units. (Modified from Ghuysen and Hakenbeck, 1994).
Molecular formula: C$_{22}$H$_{34}$I$_2$N$_4$
Molecular weight: 668.4

Figure 1.10: Molecular structure, formula, absorption and fluorescence emission spectra of propidium iodide bound to DNA. (http://www.probes.com).
CHAPTER 2

A SPECTROFLUOROMETRIC ASSESSMENT OF BACTERIAL CELL MEMBRANE DAMAGE BY PULSED ELECTRIC FIELD

Abstract

This study was carried out to verify the association between cell injury and death by pulsed electric field (PEF) and the increase in membrane porosity. A fluorescent nucleotide-binding probe, Propidium Iodide (PI), was used to quantify the membrane damage by PEF. Cell suspensions of Lactobacillus leichmannii ATCC 4797, Listeria monocytogenes Scott A and Escherichia coli O157:H7 were subjected to PEF at these conditions: frequency, 1000 Hz; pulse duration time, 3 μs; total treatment time 145.6 μs; and field strengths, 0, 5, 10, 15 and 20 kV/cm. Cells treated or untreated with PEF were stained with PI, and changes in fluorescence intensities were measured by a spectrofluorometer. Increase in field strength (kV/cm) decreased the count of survivors and proportionally increased the fluorescence intensity; this observation may indicate that cell inactivation by PEF is caused by membrane damage. Cells of E. coli O157:H7 were treated with EDTA to remove the outer membrane and sensitize cells to PEF processing. Exposure to PEF caused a similar decrease in counts regardless of the EDTA pre-treatment. Increase in the fluorescence intensity, however, was appreciable in EDTA-PEF
treated, compared with that in cells treated with PEF only. The fluorescence staining technique, therefore, revealed membrane-related injury when EDTA pre-treated cells were PEF-treated. In conclusion, the fluorescence straining technique can be used to access membrane damage associated with PEF treatments and it is potentially useful in determining relative sensitivities of microorganisms to PEF or monitoring the efficacies of such treatments.

**Introduction**

Pulsed Electric Field (PEF) is an emerging food preservation method that is believed to inactivate microorganisms by causing dielectric breakdown of the cell membrane without significant loss of food flavor, color, taste or nutrients (Dunn and Pearlman, 1987; Jia et al., 1999; Knorr et al., 1994; Mertens and Knorr, 1992; Qiu et al., 1998). Processing with PEF involves application of high-voltage electric pulses to food placed between two electrodes. Most liquid foods conduct electricity due to the presence of electrical charge carriers such as ions (Zhang et al., 1995). Because of the short period of discharge (e.g., microseconds or nanoseconds) during the PEF treatment, heating of foods is minimized.

Sale and Hamilton (Sale and Hamilton, 1967, 1968) and Hamilton and Sale (1967) conducted systematic studies on the effect of high electric field on the inactivation of microorganisms. They showed that the electric field strength and total treatment time (which is the product of the number of pulses the product receives and pulse width) are the two most important factors in microbial inactivation. In their experiments, the temperature increase was less than 10°C, and therefore they concluded that microbial
inactivation was not caused by heat generation. Sale and Hamilton (1967) proposed that the electric field caused the loss of membrane function as a semipermeable barrier between the bacterial cell and its environment, which caused cell death. Subsequently, more detailed studies addressed the mechanism of cell inactivation by PEF. Zimmermann (1939) proposed that PEF causes irreversible membrane damage that results in cell death. Benz and Zimmermann (1981) reported that the lipid bilayer might seal after reversible breakdown of cells membrane, i.e., when the pulsed electric field is not strong enough to cause irreversible breakdown. Tsong (1989, 1991) suggested electroporation of cell membrane as a mechanism of cell death by PEF treatment. According to this author, electroporation in a cell membrane can occur both in protein channels and in lipid domains, leading to an osmotic imbalance and subsequently cell death.

Fluorescent stains that bind to intracellular components are useful in determining the viability or the physiological status of microorganisms. Propidium iodide (PI), for example, is a nucleotide-binding probe, which enters only cells with damaged membranes. Thus, PI is often used to label dead cells (Aeschbacher et al., 1986; Arndt-Jovin and Jovin, 1989; Haughland, 1996; Kaneshiro et al., 1993). SYTO 9, another nucleotide-binding probe, diffuses through both intact and damaged membranes and therefore, is used in conjunction with PI as a viability probe for a wide range of microorganisms (Haughland, 1996).

Current interest in alternative food processing technologies (e.g., PEF and high hydrostatic pressure) necessitates in-depth studies on the kinetics and mechanisms of microbial inactivation by these methods. This study was initiated to verify, using fluorescence staining techniques that cell death by PEF results from membrane damage.
Additionally, measurement of membrane damage is proposed as an index of the relative effectiveness of PEF or sensitivity of cell populations. Attempts were made to sensitize a gram-negative bacterium to PEF by destabilizing the outer cell membrane using EDTA treatment. EDTA binds divalent cations (e.g., Mg$^{2+}$), which are known to stabilize the lipopolysaccharide (LPS) layer of gram-negative cell envelope (Neidhardt et al. 1990). Removal of outer membranes by EDTA may enhance the uptake of the fluorescent stain and thus help elucidate the mechanism of action of PEF against bacteria.

Materials and Methods

Microorganisms

*Lactobacillus leichmannii* ATCC 4797, *Escherichia coli* O157:H7, and *Listeria monocytogenes* Scott A were obtained from the culture collection at the Department of Food Science and Technology, Ohio State University (Columbus, OH). Frozen stock cultures were transferred into Trypticase Soy Broth supplemented with 0.6% yeast extract (TSBYE) (Difco, Detroit, MI) for *E. coli* O157:H7, and *L. monocytogenes* Scott A, and Lactobacilli MRS broth (Difco) for *Lb. leichmannii* ATCC 4797. All cultures were incubated at 37°C for 12 hours, otherwise indicated.

Bacterial cell suspension for pulsed electric field treatments

Overnight cultures of *Lb. leichmannii* ATCC 4797 were inoculated into 100 ml Lactobacilli MRS broth at 0.1% level and grown at 37°C to the late exponential phase. *L. monocytogenes* Scott A and *E. coli* O157:H7 were grown in Trypticase Soy broth (TSB; Difco) under conditions similar to those just reported. Cultures were centrifuged for 15
min at 10,000 × g and 20°C and cells were resuspended in 0.85% sterile NaCl solution. Harvested cells were centrifuged again and the pellet was resuspended in 0.1% NaCl (electrical conductivity; 0.2 S/m) to obtain a suspension containing ~10^7 CFU/ml (~0.1 OD_{600} for *Lb. leichmannii* ATCC 4797, 0.05 OD_{600} for *E. coli* O157: H7, and 0.01 for *L. monocytogenes* Scott A).

Cell suspensions were used immediately in PEF treatments and in preparation of untreated (live) or isopropanol-treated (dead) controls. To prepare isopropanol-treated control, 1 ml of the cell suspension was added to 40-ml centrifuge tube containing 20 ml isopropanol (70%). The mixture was incubated at 25°C for 1 hour with mixing every 15 minutes. Treated cells were washed twice by centrifugation for 15 min at 10,000 × g and 20°C and resuspension in filter-sterilized 0.1% NaCl. Isopropanol-treated cells were adjusted to OD_{600} values similar to those of live suspensions.

**Fluorescence labeling with propidium iodide**

To analyze if PEF treatments caused membrane damage, cells treated with PEF and isopropanol, and the untreated control were incubated with propidium iodide (Molecular Probes, Eugene, OR), an impermeant nucleotide-binding probe. Five μl of 20-mM propidium iodide solution in anhydrous dimethyl sulfoxide (DMSO) were mixed with a 3-ml sample of untreated, isopropanol-treated, or PEF-treated cells. Samples were mixed thoroughly by pipetting up and down several times, and incubated in the dark at 25°C for 15 minutes.
Fluorescence spectroscopy

A spectrophotometer (Turner®™ Quantech Filter Fluorometer, Barnstead/Thermolyne, Dubuque, IA), equipped with a 490-nm excitation, and 520-nm and 605-nm emission filters was used in this study. The degree of propidium iodide binding to cells' DNA was measured in fluorescence intensity units. Isopropanol-treated cells gave the highest fluorescence intensity reading; thus fluorescence of all treatments was reported as a percentage of the fluorescence intensity units of this control.

Fluorescence microscopy

Propidium iodide and SYTO 9 (Molecular Probes), a red- and a green-fluorescent nucleic acid stain, was used in this study. Cells treated with isopropanol, PEF, EDTA, and EDTA+PEF and untreated cells were stained with SYTO 9 and propidium iodide and inspected under a fluorescence Microscope as follows. A portion (3 µl) of a solution containing equal volumes of propidium iodide (20 mM in DMSO) and SYTO 9 (3.34 mM in DMSO) was mixed with 1 ml bacterial cell suspension. The mixture was incubated at 22-25°C in the dark for 15 min. Samples of the stained cells (5 µl each) were spotted on a microscope slide and covered with an 18-mm square glass slide cover. Digital images were acquired using a fluorescence microscope (IX-70, Olympus, Millville, NY) equipped with a double bandpass fluorescence optical filter set which is appropriate for simultaneous imaging of fluorescein (excitation 485 nm ± 15, emission 505 nm ± 15) and Texas Red dyes (excitation 585 nm ± 15, emission 625 nm ± 15). This double bandpass optical filter set was suitable for the excitation and emission spectra of SYTO 9 and propidium iodide, respectively. Digital images of the double-strained cells were obtained.
Enumeration of viable cells

Treated and control samples were serially diluted with 0.85% NaCl and dilutions were surface-plated on non-selective agar media plates. L. monocytogenes ScottA and E. coli O157:H7 were plated on Trypticase soy agar supplemented with 0.6% yeast extract (TSAYE), and Lactobacilli MRS agar was used to enumerate Lb. leichmannii ATCC 4797. Cultures were incubated at 37°C and counted after incubation for 24 hours.

Pre-treatment with EDTA

E. coli O157:H7 were treated with EDTA to enhance fluorescent dye uptake and to help reveal the action of PEF on cell membranes. A culture of E. coli O157:H7 (~ 10^9 cfu/ml) was centrifuged and resuspended using 20 mM disodium EDTA (Fisher Chemical, Fair Lawn, NJ) in 0.86% NaCl. The EDTA-cell mixtures were incubated at 37°C for 30 min. Cells were centrifuged at 10,000 x g for 15 min and washed with 0.85% NaCl. Cells were pelleted by centrifugation and suspended in 0.1% NaCl to achieve a final cell population of ~ 10^8 cfu/ml. Untreated, EDTA-treated, PEF-treated, and EDTA plus PEF-treated samples were counted by standard plate counting method on TSAYE. In order to determine the bacterial cell injury, the same samples were also plated on Violet Red Bile Agar (Difco) and TSA + 2% NaCl and incubated at 37°C for 48 hours. Cells treated with EDTA, PEF, and EDTA+PEF and untreated cells were stained with propidium iodide, and fluorescence intensity was analyzed by spectrofluorometer, as indicated earlier.
Pulsed electric field treatment

Cells suspended in 0.1% NaCl (electrical conductivity, 0.2 S/m) were treated with PEF. A pulsed electric field treatment unit with four co-field flow electrodes in series (OSU-PEF Team, The Ohio State University, Columbus, OH) capable of handling 60-ml sample volume was used throughout the study (Fig. 2.1). Equipment and processing parameters were as follows: wave form is bipolar; pulse frequency, 1000 Hz; pulse duration time, 3 μs; number of chambers, 4; field strengths, 0, 5, 10, 15 and 20 kV/cm; electrode diameter, 0.23 cm; electrodes gap, 0.292 cm and product flow rate, 1 ml/s. The sample received ~12 pulses per chamber and total treatment time (145.6 μs) was calculated as follows.

Total treatment time (μs) = Pulse duration (μs) × pulse frequency (pulse/s) × number of chambers × (π /4) × electrode diameter² (cm²) × gap distance (cm) / product flow rate (cm³/s).

Input voltages and the pulse waveform were monitored with a two-channel digital oscilloscope (Tektronix TDS340A, Beaverton, OR). The sample flow rate was controlled by a programmable logic controller (Fig. 2.1). Temperature of the sample before the PEF treatment was 22°C and it did not exceed 35°C after the treatment. A water-bath, adjusted at 22°C, was used to cool the PEF treated samples initially and after the second and fourth treatment chamber. The temperature at the entrance of the first and exit of the fourth treatment chamber was recorded with a dual channel digital thermometer (Tektronix DTM920, Beaverton, OR). The cleaning and disinfecting of the system was
done with a 500 ppm chlorine solution and rinsed with sterile 0.1% NaCl. Immediately after the PEF treatment, bacterial cells were kept in a water-ice bath until further processing.

Data analysis

Counts of survivors (log\textsubscript{10} cfu/ml) were analyzed using the MINITAB statistical program (Minitab Inc., State College, PA). Two-way analysis of variance was performed to compare the effect of the treatments and electric field intensity. Tukey’s student test was used for multiple comparisons of means on significant treatment factors.

Results

Comparison of sensitivities of bacteria to PEF

\textit{Lactobacillus leichmannii} ATCC 4797, a gram-positive spoilage lactic acid bacterium, \textit{Listeria monocytogenes} Scott A, a gram-positive pathogen, and \textit{Escherichia coli} O157:H7, a gram-negative enterohemorrhagic-causing bacterium, were compared for sensitivity to pulsed electric field (PEF). Treatment with PEF inactivated 0.42, 1.2 and 1.5 log \textit{Lb. leichmannii} / ml at 10, 15 and 20 kV/cm, respectively (Fig. 2.2a). Electric field intensities of 5, 10 and 20 kV/cm inactivated 0.11, 0.53, and 2.9 log \textit{E. coli} and 0.12, 1.7, and 2.1 log \textit{L. monocytogenes}, respectively (Fig. 2.2a). Counts (log\textsubscript{10} CFU/ml) of survivors of the three bacteria after PEF treatments were compared statistically at 20 kV/cm. Results show that \textit{Lb. leichmannii} ATCC 4797 was the most resistant, and \textit{E. coli} O157:H7 was the most sensitive to PEF, whereas \textit{L. monocytogenes} Scott A had an intermediate sensitivity. Differences in counts of survivors among the three bacteria were significant (p < 0.05) at this field strength.
When PEF-treated cells were incubated with propidium iodide (PI), the stain reacted with DNA of membrane-damaged cells and produced a fluorescence that varied with the intensity of the PEF treatment (Fig. 2.2b). Relative fluorescence intensity (%) of PEF-treated and PI-stained cells was significantly greater ($p < 0.05$) in \textit{L. monocytogenes} and \textit{E. coli} than in \textit{Lb. leichmannii}. Viability measurements, by plating counting, and fluorescence staining techniques were compared (Fig. 2.2a and 2.2b). It is clear that measurements of cell viability by plate counting and PI-staining followed a similar pattern. Therefore, cell counts (log$_{10}$ CFU/ml) and relative fluorescence intensities of one gram-negative (Fig. 2.3a) and two gram-positive (Fig. 2.3b) PEF-treated bacteria were plotted. PEF-treatments greater than a threshold value (5 kV/cm in case of \textit{L. monocytogenes} and \textit{E. coli} and 10 kV/cm in case of \textit{Lb. leichmannii}) caused a decrease in cell counts that is proportional to the increase in fluorescence intensity; correlation coefficient, $R^2$, was 0.96 for \textit{E. coli} O157:H7, 0.90 for \textit{L. monocytogenes} ScottA and 0.99 for \textit{Lb. leichmannii} ATCC 4797. Therefore, the PI fluorescence staining technique may be used as a rapid method to assess cell viability during PEF treatments.

**Detection of cell injury by fluorescence staining**

When bacterial cells were treated with PEF at 5 kV/cm, a negligible decrease in cell count was observed. At this low field intensity, however, fluorescence was noticeably greater in case of PEF treated than in un-treated cells (Fig. 2.2 and 2.3). This increase in fluorescence of the population that remained largely viable may indicate cell injury that is caused by repairable membrane damage of the type found during the electroporation process for introduction of foreign DNA (Tsong, 1989, 1991). To test this
hypothesis, *E. coli* O157:H7 was treated with EDTA to destabilize the outer membrane (Neidhardt et al. 1990) and to render cells prone to injury by PEF treatment. Untreated and EDTA-treated cells were PEF-processed and fluorescence-stained using PI. Processing with PEF inactivated EDTA-treated and untreated cells equally (*p* > 0.05) (Fig. 2.4). However, fluorescence, following PEF processing and PI staining, was appreciably greater (*p* < 0.05) in EDTA-treated than in untreated cells (Fig. 2.4). These results show that PEF caused cell injury, and fluorescence staining with propidium iodide is a suitable technique for detecting sublethal and lethal membrane damage by PEF. Cell injury, however, was not detectable by plating on selective and non-selective agar media (Fig. 2.5).

**Fluorescent microscopy**

A mixture of SYTO 9 and propidium iodide, a green- and a red-fluorescent nucleic acid stain, respectively, is used commercially for bacterial viability testing (Molecular Probes). According to the manufacturer, SYTO 9 labels all bacteria with intact and damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9-stain fluorescence when both dyes are mixed. The excitation/emission spectra for these dyes are about 480 nm / 500 nm for SYTO 9 stain and 490 nm/635 nm for propidium iodide. Digital images of PI- and SYTO 9-labeled, PEF-treated bacterial cells (Fig. 2.6a) are compared to those of a similarly stained mixture of dead (isopropyl-treated) and live cells (Fig. 2.6b). The background in both treatments remained virtually dark, with no evidence of cell-free
fluorescent substances. Such images may provide a visual means of measuring the efficacy of PEF treatment or comparing the PEF sensitivities of different bacterial strains.

Discussion

Fluorescence staining techniques provide a powerful tool to investigate cells in the live, dead, and intermediate states. These techniques are particularly valuable in investigating the viability and injury of foodborne pathogens during treatments with novel processing technologies. The nucleotide binding probes, propidium iodide and SYTO 9, were used in this study to verify the association between cell death by PEF and membrane damage. Samples of gram-positive and gram-negative bacteria were treated with PEF, strained with PI, and viabilities using plate-counting and staining techniques were compared. Strong correlations (R^2 = 0.90 to 0.99) were observed between log_{10} survivors and relative fluorescence intensity (%) of bacteria treated with PEF at a greater than a threshold field intensity (5-10 kV/cm). Since fluorescence is a measure of PI binding to cell DNA, and PI binds only to damaged and dead cells (Haughland, 1996), it is concluded that death of bacterial cells by PEF treatment is most likely caused by membrane damage. Our study provides an additional support to the earlier theories about the mechanism of microbial inactivation by PEF treatment. According to Hamilton and Sale (1967), evidence of membrane damage during PEF treatments includes cell lysis, leakage of intracellular contents, loss of the ability of Escherichia coli to plasmolyze in a hypertonic medium, and release of β-galactosidase. The authors reported that PEF treatments caused the loss of cell motility and synthesis of β-galactosidase in bacteria. These investigators proposed that PEF caused the death of bacteria when they irreversibly lose the function of cell membrane as a semipermeable barrier.
Tsong (1989, 1991) reported that PEF causes the pore formation in protein channels and the lipid domain of a cell membrane. The opening and closing of protein channels are reported to be depending on the transmembrane electric potential (Tsien and Tsong, 1981). When PEF is applied, voltage-sensitive protein channels open before the transmembrane potential reaches the breakdown potential of the lipid bilayer, i.e., 150-500 mV. The opening of these voltage-sensitive protein channels may not be enough to prevent a continuous increase of transmembrane potential to reach the breakdown potential of the lipid bilayer. Once protein channels open, irreversible denaturation may occur by electrical heating (Joule heating) or electrical modifications in the functional groups of protein channels.

Counting survivors of a processing treatment on non-selective and selective agar media provides an estimate of the degree of cell injury (Kalchayanand et al., 1998; Kim, 1998). However, there were no differences in counts of *E. coli* O157:H7 on non-selective (TSAYE) and two selective plating media (TSA + 2% NaCl, and VRBA) for cells treated at 15 kV/cm (*p > 0.05*) (Fig. 2.5). When a similar pair of media was used in a previous study to enumerate high-pressure treated *L. monocytogenes*, the percentage of survivors sustaining sublethal injury increased with increasing severity of the treatment (Simpson and Gilmour, 1997). Russell et al. (2000) and Simpson et al. (1999), however, reported that inactivation by pulsed electric field is an “all or nothing” event in which little or no sublethal injury occurred before bacterial cells become completely inactivated. Our data show that plating on selective/non-selective media pairs may not be suitable for detection of sublethal injury by PEF (Fig. 2.4 and 2.5). Alternatively, fluorescence staining technique with propidium iodide or similar stains may be used to detect sublethally injured bacteria.
It is generally accepted that pulsed electric field shows its lethal effects by causing pore formation in the cytoplasmic cell membranes (Castro et al., 1993; Tsong, 1989, 1991). This is supported by the present study in which increasing the pulsed electric field strength led to greater relative fluorescence intensities (Fig. 2.2 and 2.4). Nucleic acid stains such as propidium iodide (PI) and ethidium bromide (EB) are generally excluded by intact plasma membranes and their uptake is often used to indicate cell death (Aeschchbacher et al., 1986; Bohmer, 1985; Green et al., 1994; Grogan and Collins, 1990; Jones, 1987; Kaneshiro et al., 1993; Lapinsky et al., 1991; Lopez-Amaros et al., 1995; Schmid et al., 1992). The applicability of excluded dyes for viability determinations needs to be carefully considered for each type of microorganism (Jernaes and Steen, 1994; Lewis, 1994). Propidium iodide, ethidium bromide, acridine orange, proflavin, and quinacrine are some of the most commonly used noncovalent nucleic acid dyes, which bind to RNA as well as DNA (Arndt-Jovin and Jovin, 1989). The positively charged forms of these dyes may also bind electrostatically to other macromolecules such as proteins, polysaccharides, or glycosaminoglycans, and to membranes (Arndt-Jovin and Jovin, 1989). Propidium iodide and ethidium bromide bind to DNA of any base composition and may insert from either the major or the minor groove with little or no sequence preference (Neidle and Berman, 1983). These dyes are commonly used as a chromosome counter-stain and as a stain for damaged and dead cells (Arndt-Jovin and Jovin, 1989). Fluorescent stain kits are available commercially for measuring viability of bacterial cells (e.g., LIVE/DEAD BacLight Bacterial Viability Kit of Molecular Probes). These kits, however, equate the presence of intact plasma membranes with viability. According to Haugland (1996), "bacteria rendered nonviable by exposure to agents that do not necessarily compromise the integrity of the plasma membrane, usually appear viable by this criterion.”
The electric field strengths used in this study were generally lower than those applied in studies of PEF pasteurization of liquid foods; electric field strengths of 20-45 kV/cm have been used (Vega-Mercado et al., 1997). Additionally, a threshold value of ~15 kV/cm for irreversible membrane breakdown has been reported (Hamilton and Sale, 1967; Sale and Hamilton, 1967, 1968). In this study, the inactivation threshold for L. monocytogenes and E. coli O157:H7 was >5 kV/cm and for Lb. leichmannii was >10 kV/cm. Cell injury, however, was detectable at all electric field intensity values.

In conclusion, cells stained with PI after PEF treatment produce fluorescence that is proportional to the intensity of applied electric field and degree of cell inactivation. Therefore, fluorescence staining is suitable for rapidly monitoring of PEF treatments. PEF treatments at 5-10 kV/cm may not affect cell viability but cause appreciable cell injury that is detectable by the PI staining technique. Combining EDTA with PEF substantially increases membrane damage, as measured by PI uptake, without increasing appreciably cell inactivation as measured by plate counting technique. Staining with PI offers a rapid method of assessing relative sensitivity of organisms to PEF, which may substitute the time-consuming plate counting. Results of these rapid tests can also be conformed by fluorescence microscopy.
References


Figure 2.1: Pulsed electric field bench-scale processing unit and fluid/sample handling system, adapted from the "User's Manual of OSU-4C Pulsed Electric Field System and Fluid/Sample Handling Operation" (OSU-PEF Team, Food Science and Technology, The Ohio State University, Columbus, OH). Figure is not drawn to scale.
Figure 2.2: Inactivation of *Lactobacillus leichmannii*, *Escherichia coli* and *Listeria monocytogenes* at different intensities of pulsed electric field treatments, and associated increase in fluorescence of treated cells when stained with propidium iodide. Results are averages of two independent trials. (◆): *Lb. leichmannii*, (■): *E. coli*, (▲): *L. monocytogenes*. 
Figure 2.3: Relationship between cell inactivation and associated increase in fluorescence when cells of *Escherichia coli* O157: H7, *Lactobacillus leichmannii* ATCC 4797, and *Listeria monocytogenes* Scott A were treated with different intensities of pulsed electric field. Results are averages of two independent trials. (a) *E. coli* O157: H7; (b) *Lb. leichmannii* ATCC 4797 (—♦—), and *L. monocytogenes* Scott A (—▲—).
Figure 2.4: Inactivation of *Escherichia coli* O157: H7, with or without EDTA at different intensities of pulsed electric field, and the associated increase in relative fluorescence intensities (RFI) of treated cells when stained with propidium iodide. (Δ): Log_{10} CFU/ml when EDTA absent; (○): Log_{10} CFU/ml when EDTA present; (▲): RFI when EDTA absent, (●): RFI when EDTA present.
Figure 2.5: Counts of *Escherichia coli* O157:H7, which was pre-treated with EDTA (20 mM), processed with pulsed electric field (15 kV/cm), and plated on selective (VRBA and TSA+2% NaCl) and non-selective (TSAYE) agar media. Results are averages of two independent trials. Empty bar: TSAYE; dotted bar: VRBA; hatched bar: TSA + 2% NaCl.
CHAPTER 3

SENSITIZATION OF ESCHERICHIA COLI O157:H7 AND
LISTERIA MONOCYTOGENES SCOTT A TO PULSED ELECTRIC FIELD

Abstract

*Escherichia coli* O157:H7 (~ 10^8 CFU/ml) was pre-treated with disodium ethylene diamine tetraacetic acid (EDTA), lysozyme, and their combination before processing with pulsed electric field at 15 kV/cm. The PEF treatment alone reduced the population of *E. coli* O157:H7 by 1.8 log_{10} CFU/ml. Pretreatment with lysozyme did not enhance the efficacy of PEF against *E. coli* O157:H7. However, when PEF was applied to cells pre-treated with EDTA, a small but significant increase in inactivation (0.3 log_{10} cfu/ml, p < 0.05) was observed, compared with PEF treatment alone. Treated cells were stained with propidium iodide (PI), and changes in fluorescence intensities were measured by a spectrofluorometer. Treatment with lysozyme, EDTA or their combination did not increase the relative fluorescence intensities when compared to untreated cells. However, application of PEF after the pre-treatments, compared to the PEF treatment alone, increased appreciably the relative fluorescence intensity of cells. Therefore, lysozyme and EDTA pre-treatments increased cells’ susceptibility to injury by PEF.

The effect of different growth temperatures on the susceptibility of *Listeria monocytogenes* Scott A to PEF was investigated. When *L. monocytogenes* Scott A was
grown at 7°C, PEF treatment at 20 and 25 kV/cm inactivated 1.4 and 5.4 log_{10} CFU/ml, respectively. However, cells grown at 22°C were inactivated 1.2 and 2.0 log_{10} CFU/ml only. The greatest inactivation (3.3 and 6.1 log_{10} CFU/ml, respectively) was observed when *L. monocytogenes* Scott A was grown at 37°C before the PEF treatment. In conclusion, cells of *E. coli* O157:H7 suffered sublethal membrane damage by PEF when they were pre-treated with EDTA and lysozyme. Cultures of *L. monocytogenes* Scott A incubated at the pathogen's optimum growth temperature (37°C) produced cells with most susceptibility to PEF treatment.

**Introduction**

Pulsed electric field (PEF) is an emerging nonthermal processing technology with potential applications in preserving pumpable foods such as milk, liquid whole egg, fruit juices, and pea soup (Reina et al., 1997; Calderon-Miranda et al., 1999a; Calderon-Miranda et al., 1999b; Simpson et al., 1995; Sitzmann, 1995; Vega-Mercado et al., 1996). PEF inactivates vegetative microbial cells by causing damage to their cytoplasmic membranes. However, PEF is ineffective against bacterial endospores as well as mold ascospores (Knorr et al., 1994).

Efficacy of PEF varies with the microorganism and its physiological status at the time of treatment. Critical electric field intensity (E_{c}), i.e., the smallest electric field intensity causing detectable inactivation, indicates the relative resistance of a microorganism to the PEF. According to Hulsheger (1983), E_{c} for gram-negative bacteria was lower than that for gram-positive, in accordance with the smaller PEF resistance of the former. Recently, Jeyamkondan et al. (1999) noted that low transmembrane potential
decreases the $E_c$. The fluidity of cell membrane and transmembrane potential may change at different growth temperatures. Currently, no literature is available on the effect of different growth temperatures on the susceptibility of microorganisms to PEF treatments.

A combination of factors or hurdles can be applied to accomplish food preservation. Each hurdle contributes to the stability and safety of the food product. The use of several hurdles in combination may act additively or synergistically (Leistner, 1992, Wagner and Moberg, 1989). PEF, for example, can be combined with other preservation factors to increase the microbial inactivation (Hulsheger et al., 1981; Jayaram et al., 1992); Kalchayanand et al., 1994; Liu et al., 1997). PEF treatments and moderately lethal temperatures (~ 50 to 60°C) exhibit synergistic lethality against microorganisms (Jayaram et al., 1992; Dunn and Pearlman, 1987). Hulsheger et al. (1981) suggested that high treatment temperatures change the cell membrane fluidity and permeability, which increase the susceptibility of microbial cells to the mechanical disruption by PEF.

The action of PEF on the cytoplasmic membrane may be enhanced when bacterial cell envelope is weakened. Lysozyme and disodium ethylene diamine tetraacetic acid (EDTA) are some of the reagents that alter cell envelope. Lysozyme enhances lysis of bacteria by enzymatic hydrolysis of peptidoglycans found in the cell wall of gram-positive and some gram-negative bacteria (Facon and Skura, 1996). In gram-negative bacteria, however, the peptidoglycans are protected by a layer of lipopolysaccharide (LPS), and therefore are not accessssible to lysozyme under normal conditions. The LPS layer protects the cell against the enzymatic activity of lysozyme and prevents penetration of hydrophobic antibiotics (Ohno and Morrison, 1989). Pre-treatment with EDTA
enhanced the activity of lysozyme against gram-negative bacteria in tris buffer at pH 8.0 (Wooley and Blue, 1975). Lysozyme is used as a food preservative in some countries (Proctor and Cunningham, 1988). Synergy among apolactoferrin, lysozyme and EDTA against *Salmonella enteritidis* has been demonstrated in 1% peptone medium (Facon and Skura, 1996). The LPS is strongly anionic layer that is stabilized by Ca$^{2+}$ and Mg$^{2+}$. Disruption of the outer membrane by polycations or chelating agents such as EDTA can be demonstrated by the increased sensitivity of bacteria to antibiotics, lysozyme or bile salts (Nikaido and Vaara, 1987).

Factors that may sensitize pathogens to PEF treatment are investigated in this study. The research was done specifically to determine the efficacy of PEF on *E. coli* O157:H7 whose outer membrane is altered by various chemical (EDTA, lysozyme) treatments. Additionally, influence of growth temperatures on the sensitization of *Listeria monocytogenes* Scott A to PEF treatments was also investigated.

**Materials and Methods**

**Microorganisms**

*Escherichia coli* O157:H7 and *Listeria monocytogenes* Scott A were obtained from the culture collection at the Department of Food Science and Technology, the Ohio State University (Columbus, OH). Frozen stock cultures were transferred into trypticase soy broth supplemented with 0.6% yeast extract (TSBYE) (Difco, Detroit, MI). After two successive transfers under similar conditions, the bacterial cells were harvested by centrifugation at 5000 × g for 10 min (Sorvall RC-5B Superspeed Centrifuge, DuPont Instruments, Wilmington, DE). The cells were washed twice with 0.85% NaCl and
suspended in 0.1% NaCl solution prior to PEF treatments. The inoculum sizes were estimated by measuring absorbance at 600 nm ($A_{600}$) and calculating approximate counts from the standard curve for absorbance versus bacterial counts.

**Pre-treatment of *Escherichia coli* O157:H7 with EDTA and lysozyme**

*E. coli* O157:H7 were pre-treated with EDTA, lysozyme and EDTA + lysozyme prior to application of PEF (Fig. 3.1). A 12-h culture of *E. coli* O157:H7 (~$10^9$ CFU/ml) was centrifuged and resuspended using 20 mM disodium EDTA (Fisher Chemical, Fair Lawn, NJ) in 0.85% NaCl. The EDTA-cell mixtures were incubated at 37°C for 30 min. Cells were centrifuged at 5000 × g for 10 min (DuPont Instruments, Wilmington, DE) and washed twice with 0.85% NaCl. Cells were pelleted by centrifugation and resuspended in 0.1% NaCl to achieve a final cell population of ~$10^8$ CFU/ml. The former procedure was repeated for treatment of cells with lysozyme (20 μg/ml of culture) (Boehringer Mannheim, GmbH Germany) and the combined treatment with EDTA and lysozyme.

**Growth conditions of *L. monocytogenes* Scott A**

Effect of different growth temperatures on the inactivation of *L. monocytogenes* Scott A by PEF was investigated. The bacterium was inoculated, at the rate of $10^3$ CFU/mL, into 500-mL TSBYE and the mixture was incubated at 7, 22 or 37°C. Cultures at mid-log phase ($A_{600} = 0.6$) were centrifuged for 10 min at 5000 × g and the same temperatures at which the original culture was incubated. The resulting pellets were resuspended in 0.1% NaCl solutions at 22°C. The cell density was adjusted to ~$10^8$ ($A_{600} = 0.05$) using 0.1% NaCl at 22°C and the suspensions were treated with PEF immediately.

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Pulsed electric field treatment

Cells suspended in 0.1% NaCl (electrical conductivity, 0.2 S/m) at 22°C were treated with PEF. A pulsed electric field treatment unit with four co-field flow electrodes in series (OSU-Nonthermal Food Processing Laboratory, The Ohio State University, Columbus, OH) capable of handling 60-ml sample volume was used throughout the study. Processing and equipment parameters were as follows: waveform is bipolar; pulse frequency, 1000 Hz; pulse duration time (pulse width), 3 μs; number of chambers, 4; electric field strengths, 15, 20 and 25 kV/cm; electrode diameter, 0.23 cm; electrodes gap, 0.292 cm and product flow rate, 1 ml/s. The sample received ~12 pulses per chamber and total treatment time (145.6 μs) was calculated as follows.

Total treatment time (μs) = Pulse duration (μs) × pulse frequency (pulse/s) × number of chambers × (π / 4) × electrode diameter² (cm²) × gap distance (cm) / product flow rate (cm³/s).

Input voltages and the pulse waveform were monitored with a two-channel digital oscilloscope (Tektronix TDS340A, Beaverton, OR). A programmable logic controller controlled the sample flow rate. Temperature of the sample before the PEF treatment was 22°C. A water-bath, adjusted at 22°C, was used to maintain the temperature of samples initially and after the second and fourth treatment chamber. The temperature at the entrance of the first and exit of the fourth treatment chamber was recorded with a dual channel digital thermometer (Tektronix DTM920, Beaverton, OR). The cleaning and disinfecting of the system was done with a 500 ppm chlorine solution and rinsed with sterile 0.1% NaCl. Immediately after the PEF treatment, bacterial cells were kept in a water-ice bath until further processing.
Fluorescence labeling with propidium iodide

To analyze if PEF, with or without the pre-treatments, damaged the membranes of the pathogenic bacteria, cells were stained with propidium iodide (Molecular Probes, Eugene, OR), an impermeant nucleotide-binding probe. Five μl of 20-mM propidium iodide solution in anhydrous dimethyl sulfoxide (DMSO) were mixed with a 3-ml sample of untreated (negative control), isopropanol-treated (positive control), or PEF-treated cells. Samples were mixed thoroughly by pipetting up and down several times, and incubated in the dark at 25°C for 15 minutes. Fluorescence intensity of stained cells was determined using a spectrofluorometer (Turner® Quantech Filter Fluorometer, Barnstead/Thermolyne, Dubuque, IA), equipped with a 490-nm excitation, and 520-nm and 605-nm emission filters. Isopropanol-treated cells gave the highest fluorescence intensity reading; thus fluorescence of all treatments was reported as a percentage of the fluorescence intensity units of this control.

Enumeration of viable cells

Treated and control samples were serially diluted in 0.85% NaCl and dilutions were surface-plated on non-selective and selective agar media plates. For non-selective plating, E. coli O157:H7 and L. monocytogenes Scott A were plated on trypticase soy agar supplemented with 0.6% yeast extract (TSAYE) (Difco), and plates were incubated at 37°C and counted after incubation for 24 hours. For E. coli O157:H7, the same samples were also plated on violet red bile agar (Difco) and incubated at 37°C for 48 hours in order to determine the bacterial cell injury.
Transmission electron microscopy

*Escherichia coli* O157:H7 (~ $10^8$ CFU/ml) was untreated, pre-treated with EDTA, lysozyme, and their combination. Cells were processed with pulsed electric field at 15 kV/cm as described earlier and fixed in 2% paraformaldehyde and 2% glutaraldehyde solution in 0.1 M phosphate buffer at pH 7.1. Cells in the suspension were stored overnight at 4°C, rinsed three times in phosphate buffer and resuspended in 2% agarose. The gel was allowed to solidify by placing the mixture in ice water bath. The cellular portions were cut out into one-mm cubes into buffer. Osmium tetra oxide (1%) in buffer was used for post-fixation at 22°C, followed by rinsing three times in distilled water. The bloc was stained in aqueous 1% uranyl acetate for 1 hour and rinsed three times in water. The cells were dehydrated through graded series of ethanol for 10 minutes at each step (70, 80, 95 and 100%). The cells were further exposed to propylene oxide for 10 min and propylene oxide + Spurr resin (1:1 ratio) for 1 hour. The bloc was kept in propylene oxide+Spurr resin (1:2 ratio) overnight. The bloc was rinsed two times in fresh Spurr resin over 6 h and embedded in Spurr to polymerize overnight in a 60°C oven. Sections containing the cells were cut on a Reichert ultra cut E ultramicrotome with a diamond knife at 80 nm. They were stained with uranyl acetate and Reynolds lead citrate to be examined on Philips CM12 transmission electron microscope at 60 kV.

Data analysis

Counts of survivors ($\log_{10}$ CFU/ml) were analyzed using the MINITAB statistical program (Minitab Inc., State College, PA). Two-way analysis of variance was performed to compare the effect of the type of treatment. Tukey's student test was used for multiple comparison of means on significant treatment factors.
Results and Discussion

In this study, we investigated the potential sensitization of *Escherichia coli* O157:H7 to PEF following a pre-treatment with EDTA (20mM), lysozyme (20 µg/ml cell culture), and EDTA plus lysozyme (20 mM and 20 µg/ml, respectively). Additionally, sensitivity of *Listeria monocytogenes*, which has been grown at different temperatures, to PEF, was investigated.

Sensitization of *Escherichia coli* O157:H7 to PEF

PEF (15 kV/cm) decreased the population of *E. coli* O157:H7 that has been pre-treated with EDTA, lysozyme, and EDTA plus lysozyme by 1.8, 2.1, 1.8, and 2.5 logs, respectively (p < 0.05) (Fig. 3.2). Lysozyme, EDTA or their combination did not have any antimicrobial activity against *E. coli* O157:H7 when compared to untreated control (p > 0.05). PEF treatment alone however, reduced the population of *E. coli* O157:H7 significantly by 1.8 log_{10} CFU/ml (p < 0.05). Combination of lysozyme and PEF treatment did not significantly enhance the inactivation of *E. coli* O157:H7 when compared to PEF treated cells (p > 0.05). However, when PEF was applied to EDTA-treated cells, a small but significant (p < 0.05) increase in inactivation was observed, compared to that observed with PEF treated only; PEF only decreased 1.8 log_{10} CFU/ml and PEF following EDTA treatment inactivated 2.1 log_{10} CFU/ml. Greater inactivation (2.5 log_{10} CFU/ml) was obtained when PEF was applied to EDTA plus lysozyme treated *E. coli* O157:H7 cells (p < 0.05).
Treated and untreated *E. coli* O157:H7 were stained with propidium iodide, and changes in fluorescence intensities were measured by a spectrofluorometer. Treatment with lysozyme, EDTA, EDTA plus lysozyme and PEF alone significantly (\( p < 0.05 \)) increased relative fluorescence intensities when compared to untreated cells (Fig. 3.3). When PEF treatment was combined with EDTA, lysozyme, and EDTA + lysozyme, relative fluorescence intensities were substantially greater than that noticed for the single treatments (Fig. 3.3). Kaprelyants and Kell (1992) reported that the permeability of propidium iodide (PI) and other lipophilic cations (e.g. acridine orange and rhodamine 123) into gram-negative bacterial cells is low unless the cells are pretreated with EDTA. Our results indicated that pre-treatment with lysozyme and EDTA not only increased cell injury (sublethal membrane damage) during the PEF treatment; it also helped reveal this type of injury by the fluorescent staining technique.

Treatment of gram-negative bacteria with EDTA detaches magnesium ions from the outer membrane and results in the destabilization of the lipopolysaccharide layer. Increase in outer membrane permeability may sensitize cells and enhance the inactivation by PEF. Ineffectiveness of the lysozyme pre-treatment is likely related to the outer membrane barrier that surrounds and protects the peptidoglycan layer. Lysozyme catalyzes the lysis of bacteria by hydrolyzing the \( \beta \)-linkage between muramic acid and glycosamine of the glycopolysaccharides of cell wall (murein layer). Gram-positive bacteria are lysed in an environment with low osmotic pressure when treated with lysozyme. Gram-negative bacteria such as *S. typhimurium*, *E. coli* O157:H7, and *Campylobacter jejuni* are resistant to the antimicrobial activity of lysozyme due to the presence of the outer membrane (Hughey et al., 1987). The outer membrane can be made
permeable to lysozyme by the use of the bivalent ion chelator EDTA, which loosens the structure of LPS, or by freezing and thawing the cells (Neidhardt et al., 1990). EDTA may destabilize the outer membrane of \textit{E. coli} O157:H7 and this destabilization may allow further damage by both lysozyme and PEF. This may explain the increased efficacy of PEF in causing membrane injury (Fig. 3.3) after the combined treatments with EDTA and lysozyme compared to only EDTA or lysozyme treatments of \textit{E. coli}.

The majority of commercially prepared lysozyme is purified from hen egg white. Lysozyme presently has a small number of applications in the food industry with the major usage involving the prevention of \textit{Clostridium tyrobutyricum} spore outgrowth in hard cheeses (Wasserfall and Tueber, 1979). Lysozyme has been demonstrated to be active throughout a wide pH range of 4 - 10 (Davies et al., 1969).

**Sensitization of \textit{Listeria monocytogenes} to PEF**

PEF at 20 and 25 kV/cm significantly (p < 0.05) decreased the population of \textit{L. monocytogenes} Scott A, grown at 7°C by 1.4 and 5.4 \( \log_{10} \) CFU/ml, respectively (Fig. 3.4). Cells grown at 22°C were inactivated 1.2 and 2.0 \( \log_{10} \) CFU/ml, respectively. The greatest inactivation (3.3 and 6.1 \( \log_{10} \) CFU/ml) was obtained when \textit{L. monocytogenes} was grown at 37°C before treatment with 20 and 25 kV/cm electric field intensities, respectively. There was no significant difference in inactivation between cultures grown at 7 and 22°C before PEF treatment at 20 kV/cm (p > 0.05), however, a significant difference (p < 0.05) between these two incubation temperatures was observed with the 25 kV/cm PEF treatment. During the PEF treatments at 20 and 25 kV/cm, final sample temperatures were 35 and 48°C, respectively.
Cultures of *L. monocytogenes* that were grown at 7 to 37°C and PEF treated at 20 kV/cm were stained with PI and changes in fluorescence intensity were monitored. Relative fluorescence intensities increased significantly (*p* < 0.05) when incubation temperature increased (Fig. 3.5). Incubation at 37°C caused the largest increase in cell inactivation and relative fluorescence intensity among cultures grown at 7-37°C before PEF treatment (Fig. 3.5). Although no significant difference in inactivation between cells grown at 7 and 22°C (*p* > 0.05) was observed, the relative fluorescence intensities of same cultures were statistically different (*p* < 0.05). Therefore, data at 20 kV/cm show that resistance of *L. monocytogenes* to PEF is high when the bacterium grew at low incubation temperatures. Injury *L. monocytogenes* with PEF, as indicated by PI uptake, increased consistently with the increase in the incubation temperatures.

Fatty acid composition of cytoplasmic membrane plays a major role in determining membrane fluidity, especially in bacteria, which typically lack sterols in their membranes (Annous et al., 1997). In order for growth to occur at low temperatures, the cytoplasmic membrane must retain sufficient fluidity to ensure the necessary physical state, required for membrane structure and function (Sinesky, 1974). The fatty acid composition of *L. monocytogenes* is dominated to an unusual extend by branched-chain fatty acids, and at low temperatures anteiso-C_{15:0} becomes the major fatty acid (Edgcomb et al. 2000). Jones et al. (1997) reported that *L. monocytogenes* had a lower proportion of anteiso-C_{17:0} and a higher proportion of anteiso-C_{15:0} and short chain fatty acids when grown in continuous culture at 10°C compared to 30°C. According to Mastronicolis et al. (1998), anteiso-C_{15:0} increased in all lipid classes in *L. monocytogenes* grown at 6°C. Relative rigidity of membranes associated with high incubation temperatures may
account for the observed increase in sensitivity to PEF of cultures grown at 37°C. It is likely that repair of PEF-damaged membranes is more readily accomplished in fluid than in rigid membranes. This hypothesis may justify the greater decrease in viability and increase in PI uptake by cells grown at 37°C than that at 7 or 22°C and then treated with PEF. At high electric field intensity (25 kV/cm), resistance of *L. monocytogenes* to PEF was greatest when the bacterium was grown at 22°C, than at 7 or 37°C. PEF treatment at 25 kV/cm increased sample temperature to an injurious level (48°C). High PEF inactivation in cells grown at 7°C may have resulted from the sensitivity of these cells to the PEF-temperature combination.

According to Hulsheger et al. (1981), the medium temperature and PEF treatment synergistically enhanced the microbial inactivation. Higher treatment temperatures increased the susceptibility of microbial cells to mechanical damage by PEF. Vega-Mercado et al. (1996) studied the effect of temperature on the inactivation of *E. coli* in pea soup by PEF. A change in temperature from 32 to 55°C decreased the population of *E. coli* by 1 to 6.5 log$_{10}$ CFU/ml, respectively. When process temperature was changed from 5 or 10°C to 25°C, a higher lethal effect of PEF treatment was accomplished. This might be due to the increase in the electrical conductivity of solution at higher temperature (Marquez et al., 1997). Hulsheger et al. (1981) reported a synergistic lethal effect of medium temperature with PEF treatment. Contrary to our earlier hypothesis, these authors suggested that high treatment temperatures increased the cell membrane fluidity and permeability, which increased the susceptibility of the cell to mechanical disruption during the PEF treatment. Increasing the medium temperature may also decrease the transmembrane potential of the cell membrane and cause thermal-injury, resulting in a higher inactivation (Pothakumary et al., 1996).
Transmission electron microscopy

Untreated cells of *E. coli* O157:H7 suspended in 0.1% NaCl presented a smooth cell wall (Fig. 3.6). Cells of *E. coli* O157:H7 exposed to lysozyme (20 μg/ml of cell suspension) and EDTA (20 mM) in 0.1% NaCl exhibited limited morphological differences from untreated cells (Fig. 3.7). PEF treatment alone (15 kV/cm) caused the disruption of cell membrane and the damage was mostly noticeable at cell ends (Fig. 3.8). However, when lysozyme and EDTA pre-treated cells were exposed to PEF (15 kV/cm), the cell wall of *E. coli* O157:H7 lost its smoothness and uniformity (Fig. 3.9a and b). Delves-Broughton (1990) and Castro et al. (1993) reported that the lack of cytoplasm could be induced by loss of membrane functions due to PEF or by risin, which is known to induce leakage of cellular contents.

In conclusion, efficacy of PEF did not increase appreciably when EDTA and lysozyme prior to the electrical treatment altered the cell envelope of *E. coli* O157:H7. However, fluorescence staining technique revealed substantial sublethal membrane damage when the cell envelope was altered prior to the PEF treatment. When *E. coli* O157:H7 pre-treated with lysozyme and EDTA was exposed to PEF and examined by transmission electron microscopy, integrity of cell membrane was greatly compromised.

Incubation of *L. monocytogenes* Scott A at optimum growth temperature (37°C) resulted in a culture that is most susceptible to the lethal and injurious effect of PEF. However, growth of these cultures at the most optimum temperature for cell repair (22°C) increased appreciably the resistance of the pathogen to PEF treatment. These results may help in building appropriate hurdle combinations to enhance the efficacy of PEF in food processing.
References


Figure 3.1: Experimental procedure for the pre-treatment of *Escherichia coli* O157:H7 with EDTA (20 mM) and/or lysozyme (20 μg/ml) prior to pulsed electric field treatment (15 kV/cm), and fluorescence staining with propidium iodide (5 μg/ml of cell suspension) after PEF treatment.
Figure 3.2: Inactivation of EDTA-treated (20 mM), lysozyme-treated (20 μg/ml of culture) and EDTA + lysozyme treated *Escherichia coli* O157:H7 (~ 1.5 x 10^8 CFU/ml) in 0.1% NaCl by pulsed electric field (15 kV/cm). Counts obtained by plating on trypticase soy agar supplemented with 0.6% yeast extract (TSAYE) (A and C), and on violet red bile agar (VRBA) (B and D).
Figure 3.3: Relative fluorescence intensities of untreated, lysozyme-treated (20μg/ml of culture), EDTA-treated (20 mM), and lysozyme + EDTA treated *Escherichia coli* O157:H7 (~ 10^8 CFU/ml, initially) after processing by pulsed electric field (15 kV/cm) staining with 20 mM propidium iodide. No PEF applied (empty bar); PEF applied (solid bar).
Figure 3.4: Inactivation of *L. monocytogenes* Scott A by pulsed electric fields treatments (20 and 25 kV/cm) when cultures were grown at 7, 22, and 37°C.
Figure 3.5: Changes in counts and relative fluorescence intensities of propidium iodide-stained *L. monocytogenes* Scott A (~10^8, initially) which was incubated at different growth temperatures and treated with pulsed electric fields (20 kV/cm). Counts, log_{10} CFU/ml (○); relative relative fluorescence intensities (%) (■).
Figure 3.6: Untreated control *E. coli* O157:H7 cell.
Figure 3.7: *E. coli* O157:H7 cells pre-treated with lysozyme (20 µg/ml of cell suspension) and EDTA (20 mM).
Figure 3.8: *E. coli* O157:H7 cell treated with pulsed electric field (15 kV/cm).
Figure 3.9: E. coli O157:H7 cells pre-treated with lysozyme (20 μg/ml of cell suspension) and EDTA (20 mM) and exposed to PEF (15 kV/cm).
CHAPTER 4

INACTIVATION OF ESCHERICHIA COLI O157:H7, LISTERIA MONOCYTOGENES AND LACTOBACILLUS LEICHMANNII BY COMBINATION OF OZONE AND PULSED ELECTRIC FIELD

Abstract

Pulsed electric field (PEF) and ozone technologies are non-thermal processing methods with potential applications in the food industry. This research was performed to explore the potential synergy between ozone and PEF treatments against selected foodborne bacteria. Cells of Lactobacillus leichmannii ATCC 4797, Escherichia coli O157:H7, and Listeria monocytogenes Scott A were suspended in 0.1% NaCl and treated with ozone, PEF and ozone plus PEF. Cells were treated with 0.25-1.00 μg ozone/ml cell suspension, PEF at 10-30 kV/cm, and selected combinations of ozone and PEF. Synergy between ozone and PEF varied with the treatment level and the bacterium treated. Lb. leichmannii, treated with PEF (20 kV/cm) after exposure to 0.75 and 1.00 μg/ml ozone, was inactivated by 7.1, and 7.2 log_{10} CFU/ml, respectively. However, ozone at 0.75 and 1.00 μg/ml and PEF at 20 kV/cm inactivated 2.2, 3.6 and 1.3 log_{10} CFU/ml, respectively. Similarly, ozone at 0.5, and 0.75 μg/ml inactivated 0.5, 1.8 log_{10} E. coli CFU/ml, PEF at 15 kV/cm inactivated 1.8 log_{10} CFU/ml, and ozone at 0.5 and 0.75 μg/ml followed by PEF (15kV/cm) inactivated by 2.9, and 3.6 log_{10} CFU/ml, respectively. Population of L.
monocytogenes decreased 0.1, 0.5, 3.0, 3.9, and 0.8 log_{10} CFU/ml when treated with 0.25, 0.5, and 0.75 μg ozone/ml, and PEF (15 kV/cm), respectively. However, when the bacterium was treated with 15 kV/cm, after exposure to 0.25, 0.5, and 0.75 μg ozone/ml, 1.7, 2.0, and 3.9 log_{10} CFU/ml were killed, respectively. In conclusion, exposure of Lb. leichmannii, E. coli and L. monocytogenes to ozone followed by the PEF treatment showed a synergistic bactericidal effect. This synergy was more apparent at mild than severe doses of ozone, and when the combination treatment was applied to Lb. leichmannii than to E. coli or L. monocytogenes.

Introduction

Consumers' demand for minimally processed foods or fresh-like products with reduced use of chemical additives led to substantial developments in nonthermal food preservation technologies (Mertens and Knorr, 1992). Pulsed electric field, high hydrostatic pressure, high voltage arc discharge, oscillating magnetic field pulses, intense pulsed light, ultraviolet light, ultrasound, X-rays, and microwave processing are some of the newly developed technologies. The food industry is also interested in potent antimicrobials (e.g., ozone) as alternatives to conventional sanitizers.

Pulsed electric field (PEF) treatment is one of the emerging food processing technologies. PEF treatment effectively inactivated bacteria and yeast in orange juice, milk, skim milk, yogurt, liquid egg and pea soup (Vega-Mercado et al., 1997). However, PEF is ineffective against bacterial and mold spores. Sale and Hamilton (1967, 1968) and Hamilton and Sale (1967) demonstrated that microbial inactivation was non-thermal and PEF exerted its lethal effect by causing irreversible loss of membrane function.
Ozone is a very potent antimicrobial agent, capable of destroying bacteria, bacterial spores, protozoan cysts and viruses at relatively low concentration and in short exposure time when applied to the pure cell suspensions (Giese and Christensen, 1954; Kim, 1998; Scott and Lesher, 1963). However, presence of organic matter (e.g., food components) increases effective doses of ozone; these high doses of ozone are detrimental to the sensory attributes of foods. Depending on the commodities and ozonation conditions, more than 5 logs of food microflora were eliminated (Kim, 1998; Kim et al., 1999).

In today's food industry, the use of combination of factors is becoming a popular food preservation technique. When liquid whole eggs are thermally pasteurized, addition of antimicrobials enhances the safety of the product (Delves-Broughton et al., 1992; Roberts, 1989). Combining selected hurdles in a food may provide an additive or a synergistic effect against pathogens of concern. Since PEF and ozone both act on cell membranes, synergistic effects might be expected. Therefore, the purpose of this research was to explore potential enhancement of inactivation of selected foodborne bacteria by PEF when they are pretreated with ozone.

**Materials and Methods**

**Microorganisms**

*Lactobacillus leichmannii* ATCC 4797, *Escherichia coli* O157:H7, and *Listeria monocytogenes* Scott A were obtained from the culture collection at the Department of Food Science and Technology, The Ohio State University (Columbus, OH). Frozen stock cultures were transferred into Trypticase Soy Broth supplemented with 0.6% yeast extract (TSBYE) (Difco, Detroit, MI) for *E. coli* O157:H7, and *L. monocytogenes* Scott A, and 107
lactobacilli MRS broth (Difco) for *Lb. leichmannii* ATCC 4797. After two successive transfers under similar conditions, bacterial cells were harvested by centrifugation at 5000 × g for 10 min (Sorvall RC-5B Superspeed Centrifuge, DuPont Instruments, Wilmington, DE). The cells were washed twice with 0.85% NaCl and suspended in 0.1% NaCl solution prior to PEF and ozone treatments. The inoculum sizes were estimated by measuring absorbance at 600 nm (A₆₀₀) and calculating approximate counts from the standard curve for absorbance versus bacterial counts. The initial microbial inocula for use in the experiments were approximately ~10⁸ CFU/ml for *E. coli* O157:H7 and *L. monocytogenes* Scott A, and ~10⁷ for *Lb. leichmannii* ATCC 4797 cultures.

**Preparation of ozone demand-free glassware and water**

All glassware was washed with a mild detergent and thoroughly rinsed with hot tap water and deionized water. They were then autoclaved and dried to remove volatile organic compounds. Ozone demand-free water was prepared by ozonating deionized water. The water was then autoclaved at 121°C for 15 min to remove the residual ozone and stored in sealed ozone demand-free glass containers until needed (Korick et al., 1990).

**Ozone production**

Ozone (1.1 mM; ca. 2.5%, v/v; ca. 3.65%, w/w) was produced from purified, extra dry oxygen by an ozone generator (U.S. Filter/Polymetrics T-816, San Jose, CA). A stainless-steel sparger with 10 μm pore size (Solvent Inlet Filter, Fisher Scientific, Fair Lawn, NJ) was used for bubbling ozone in cell suspension. The amount of ozone
produced from the generator was determined by the method described by Shechter (1973) and the applied ozone dose for the reaction was calculated. All experimental work with ozone gas was done in a chemical hood. Excess ozone was destroyed by a heated catalyst or neutralized by diverting the gas stream into a reservoir containing 2% potassium iodine solution.

**Inactivation by ozone**

A batch-type reaction system (Fig. 4.1) was used for the ozone inactivation studies as follows: cells suspended in 0.1% NaCl solution (200 ml) were treated with gaseous ozone at a predetermined rate (ozone concentration; 52.8 mg/l, flow rate; 11.4 ml/min). Rates of ozonation were adjusted to achieve 0.25, 0.5, 0.75 or 1.00 μg ozone/ml cell suspension. All test solutions were continuously mixed during the treatment at 100 rpm with a stirrer. The temperature during the ozone treatment was 22 °C. Ozone dose applied in the reactor was calculated as follows:

\[
\text{mg ozone gas/ml of sample} = \left\{\frac{\text{ozone concentration} \times \text{flow rate} \times \text{contact time}}{\text{volume of cell suspension} \times 1000}\right\}
\]

**PEF treatment**

Cells suspended in 0.1% NaCl (electrical conductivity, 0.2 S/m) were treated with PEF. A pulsed electric field treatment unit with four co-field flow electrodes in series (OSU-PEF Team, The Ohio State University, Columbus, OH) capable of handling 60-ml sample volume (Fig. 4.2) was used throughout the study. Equipment and processing
parameters were as follows: bipolar wave form; pulse frequency, 1000 Hz; pulse duration time, 3 μs; number of chambers, 4; field strengths, 0 to 30 kV/cm; electrode diameter, 0.23 cm; electrodes gap, 0.292 cm and product flow rate, 1 ml/s. The sample received ~12 pulses per chamber and total treatment time (145.6 μs) was calculated as follows.

Total treatment time (μs) = (Pulse duration (μs) × pulse frequency (pulse/s) × number of chambers × (π / 4) × electrode diameter² (cm²) × gap distance (cm) / product flow rate (cm³/s)).

Input voltages and the pulse waveform were monitored with a two-channel digital oscilloscope (Tektronix TDS340A, Beaverton, OR). A programmable logic controller was used to control the flow rate of treated samples (Fig. 4.2). Temperature of the sample before the PEF treatment was 22°C and it did not exceed 35°C after PEF treatments with 5, 10, 15, and 20 kV/cm. However, the temperature exceeded 35°C when electric field intensities were more than 20 kV/cm. A water-bath, adjusted at 22°C, was used to cool the PEF treated samples initially and after the second and fourth treatment chamber. The temperature at the inlet of the first and outlet of the fourth treatment chamber was recorded with a dual channel digital thermometer (Tektronix DTM920, Beaverton, OR). The cleaning and disinfecting of the system was done with a 500 ppm chlorine solution and rinsed with sterile 0.1% NaCl.
Enumeration of viable and injured cells

The cell suspension from each treatment was serially diluted with 0.85% NaCl. From the selected dilution, 100-μl in duplicate was surface plated simultaneously on non-selective and selective agar plates. The non-selective medium for *L. monocytogenes* Scott A and *E. coli* O157:H7 was trypticase soy agar (Difco) supplemented with 0.6% yeast extract (TSAYE). The selective agar media were violet red bile agar (VRBA, Difco) (Kalchayanand et al., 1998), and trypticase soy agar supplemented with 5% NaCl (including 0.5% NaCl in the original formula of media), respectively. The plates were incubated at 37°C for 48 h and used to determine viable (CFU in TSAYE agar) and non-injured (corresponding CFU in selective agar) cells in the population. The difference between the viable and non-injured cells was used to estimate injured survivors.

Statistical analysis

All experiments were run twice. Population inactivated after the treatments were analyzed using MINITAB statistical program (Minitab Inc., State College, PA). One-way analysis of variance was performed to compare the effect of the treatments. Synergistic effects between the ozone and the PEF treatment for the inactivation of *Lb. leichmannii*, *E. coli* O157:H7 and *L. monocytogenes* ScottA, were evaluated (Fig. 4.3-4.5). The differences in inactivation between ozone-treated and ozone-PEF treated cell suspension (e.g., segments II or III, Fig. 4.3) were compared using Tukey's range test with those between the untreated and PEF-treated samples (segment I). If II or III is significantly greater than I, this shows a significant synergistic effect. If II or III is not significantly different from I, this indicates an additive effect.
Results

According to an earlier study (Kim, 1998), when bacterial cells are treated with different concentrations of ozone, mildly lethal doses cause the greatest degree of cell injury. Therefore, we screened different ozone doses and electric field intensities and chose intermediate ozone dose and electric field intensity for synergistic studies so that the combination would still result in countable cell populations and measurable synergy (Table 4.1). Based on the screening study with ozone and PEF, ozone doses (0.75 and 1.00 μg ozone/ml cell suspension) and 20 kV/cm electric field intensity were chosen for the synergistic effects of ozone and PEF against Lactobacillus leichmannii ATCC 4797. For Escherichia coli O157:H7, 15 kV/cm electric field intensity and 0.5, and 0.75 μg ozone/ml cell suspension were selected. Ozone doses at 0.25, 0.5 and 0.75 μg ozone/ml and 15 kV/cm were selected for the synergistic study to inactivate Listeria monocytogenes Scott A.

Inactivation by ozone and pulsed electric field

Ozone treatment at 0.75 and 1.00 μg/ml significantly (p < 0.05) reduced the population of Lb. leichmannii ATCC 4797 (~3 × 10^7) by 2.2 and 3.6 log, respectively (Table 4.1). Pulsed electric field treatment at 10, 15, 20, 25, and 30 kV/cm inactivated 0.7, 1.0, 1.3, 2.8, and 4.8 log cell population, respectively (Table 4.1). All PEF treatments significantly decreased the cell population (p < 0.05). The temperature monitored during processing of samples by PEF (10, 15, and 20 kV/cm) did not exceed 35°C. However, the
temperature exceeded 35°C when electric field intensities were higher than 20 kV/cm, therefore, these high field intensities were not used in the synergistic study.

When *E. coli* O157:H7 was treated with 0.25, 0.5, 0.75 and 1.00 μg ozone/ml, 0.1, 0.3, 1.8 and 3.7 log_{10} CFU/ml were inactivated, compared to the control treatment, respectively (Table 4.1). Ozone treatment at 0.25 and 0.5 μg/ml did not decrease the population of *E. coli* O157:H7 significantly (p > 0.05). However, significant population decrease was observed when 0.75 and 1.00 μg ozone/ml were applied (p < 0.05). Cell inactivation increased as ozone dose increased. When *E. coli* O157:H7 was subjected to PEF treatments at 10, 15, and 20 kV/cm, counts decreased 0.5, 1.8, and 4.5 log_{10} CFU/ml, respectively (Table 4.1). All the PEF treatments significantly reduced the population of *E. coli* O157:H7 (p < 0.05).

When cells of *L. monocytogenes* Scott A (~ 10^8 CFU/ml) were treated with four different ozone doses (0.25, 0.5, 0.75, and 1.00 μg ozone/ml), the population decreased 0.1, 0.5, 3.0, and 3.9 log_{10} CFU/ml, respectively (Table 4.1). The ozone dose at 0.25 μg/ml did not significantly decrease the population of *L. monocytogenes* Scott A (p > 0.05) but significantly greater inactivation was observed at ≥ 0.5 μg ozone/ml cell suspension (p < 0.05). As the case of *Lb. leichmannii* ATCC 4797 and *E. coli* O157:H7, inactivation of *L. monocytogenes* Scott A also was enhanced as the applied ozone dose increased. Electric field intensities of 10, 15, and 20 kV/cm significantly (p < 0.05) decreased the population of *L. monocytogenes* Scott, by 0.70, 0.8, and 1.1 log_{10} CFU/ml, respectively (Table 4.1).
Synergistic effects of ozone and pulsed electric field

Treatment of *Lb. leichmannii* by PEF (20 kV/cm) after exposure to 0.75 and 1.0 μg ozone/ml significantly (*p < 0.05*) decreased the counts by 7.1, and 7.2 log₁₀ CFU/ml, respectively. In Fig. 4.3, the difference of inactivation resulting from ozone only and ozone combined with PEF is indicated by I, II, or III. Segments II and III are significantly greater (*p < 0.05*) than I, therefore, the lethality of PEF treatment was synergistically enhanced by ozone treatment. However, synergy of PEF with ozone was greater at lower (II) than higher (III) ozone dose.

Combining PEF treatment (15 kV/cm) with 0.5 μg ozone/ml cell suspension caused an additive, but not synergistic effects against *E. coli* O157:H7 since segment II is not significantly greater than segment I (*p > 0.05*) (Fig. 4.4a). However, segment III was significantly (*p < 0.05*) greater than segment I, indicating that ozone at 0.75 μg/ml and PEF at 15 kV/cm synergistically decreased *E. coli* O157:H7 count by 3.6 log₁₀ CFU/ml.

The PEF treatment (15 kV/cm) combined with 0.25 and 0.5 μg ozone/ml cell suspension synergistically inactivated the population of *L. monocytogenes* Scott A by 1.7 and 2 log₁₀ CFU/ml since segment II and III were significantly greater than the segment I (*p < 0.05*) (Fig. 4.5a). The combination of 0.75 μg ozone/ml with 15 kV/cm of electric field strength inactivated 3.9 log₁₀ CFU/ml, however, there is no evidence of additive or synergistic effect at this combination since segment IV was significantly smaller than segment I (*p > 0.05*).
Cell injury by ozone, PEF and their combinations

Cell suspensions of *E. coli* O157:H7 were first subjected to one of two ozone concentrations (0.5 or 0.75 μg/ml of cell suspension), then treated with PEF (15 kV/cm) and enumerated for total survivors on a non-selective medium, TSAYE, and for non-injured survivors on a selective medium, TSA + 5% NaCl (Fig. 4.4b). In general, cell death increased with an increase in ozone dose (Fig. 4.4, solid lines) and an appreciable proportion of cell population was injured at 0.5 and 0.75 μg ozone/ml of cell suspension (Fig. 4.4b, shaded area). PEF treatment alone did not injure *E. coli* O157:H7 significantly since segment IV was not significantly greater than segment I (p > 0.05) (Fig. 4.4b). The cell injury increased from 0.2 to 0.6 log₁₀ CFU/ml as the applied ozone dose increased from 0 to 0.75 μg ozone/ml as indicated by shaded area in Fig. 4.4b. Combination of ozone at 0.5 and 0.75 μg ozone/ml with PEF (15 kV/cm), compared to ozone only, did not significantly injure *E. coli* O157:H7, since segments V and VI were not significantly greater than segments II and III, respectively (p > 0.05) (Fig. 4.4b).

The pattern of cell injury of *L. monocytogenes* Scott A was somewhat different from that for *E. coli* O157:H7. Very small portions of the cell population were injured by low ozone dose (0.25 μg/ml cell suspension). PEF treatment, however, injured 90% of cell population (Fig. 4.5b). As the cell inactivation increased with increase of ozone dose, the degree of injury also increased. The maximum cell injury was obtained at 0.5 μg ozone/ml cell suspension (Fig. 4.5b). Treatment with 0.5 μg ozone/ml followed by PEF injured about 99% of the population (~2 log₁₀ CFU/ml). However, combination of high dose of ozone (0.75 μg/ml) with PEF did not result in measurable cell injury.
Discussion

We demonstrated that certain concentrations of ozone and intensities of PEF are effective treatments against *Lactobacillus leichmannii* ATCC 4791, *Escherichia coli* O157:H7 and *Listeria monocytogenes* Scott A. Combinations of ozone and PEF treatments caused a lethality that was greater than the sum of lethalities of ozone and PEF when applied individually. These synergistic effects between ozone and PEF were substantial under most of the tested experimental conditions. Synergy between ozone and PEF may have resulted from cell injury during the ozone treatment and rapid inactivation of injured cells when they were subsequently treated with PEF. Therefore, ozone, PEF, and ozone-PEF treated cells were plated onto selective and non-selective agar media and results are shown (Fig. 4.4 and 4.5). Cells of *E. coli* O157:H7 were injured minimally at 0.5 µg ozone/ml, but appreciable injury was observed at 0.75 µg ozone/ml (Fig. 4.4b). This may explain the synergistic effect of 0.75 µg ozone/ml in combination with PEF and lack of synergy when the combination included a treatment with 0.5 µg ozone/ml. PEF alone did not cause appreciable cell injury.

Injury of *L. monocytogenes* was most prominent at 0.5 µg ozone/ml (Fig. 4.5b, segment III). Interestingly, synergy between ozone and PEF in inactivating this pathogen also is most apparent at the intermediate concentrations of ozone (0.25 and 0.5 µg ozone/ml) (Fig. 4.5a). Therefore, injury by ozone may have sensitized *L. monocytogenes* to PEF, resulting in a synergistic effect during the combined treatment. *L. monocytogenes*, which was treated with PEF, only exhibited cell injury (segment V, compared to I; Fig. 4.5b). Additionally, the combination of 0.5 µg ozone/ml and PEF
synergistically enhanced cell injury. This may indicate that pre-treatment of *L. monocytogenes* with ozone enhanced the ability of PEF to cause cell injury. These data may also indicate that ozone and PEF injured *Listeria* cells at different sites. Kim (1998) tested the injury of *Pseudomonas fluorescens*, *E. coli* O157:H7, *Leuconostoc mesenteroides*, and *L. monocytogenes* Scott A by ozone. He found that the degree of injury varied depending on the microorganism, ozone concentration and exposure time. He concluded that the maximum injury occurred at ozone concentrations that caused mild lethality.

Ohshima et al. (1997) treated *E. coli* with combinations of PEF and ozone. They reported that simultaneous application of PEF and ozone synergistically inactivated *E. coli*. Their data, however, show that ozone and PEF combinations had an additive rather than a synergistic effect.

PEF technology can be used as a terminal treatment or in combination with other hurdles such as nisin (Calderon-Miranda et al., 1999; Pol et al., 2000), benzoic and sorbic acids (Liu et al. 1997), low pH (Vega-Mercado et al., 1996) and ozone (Ohshima et al., 1997). For optimized food preservation systems by hurdle technologies, the mode of action of each hurdle should be understood. According to Zimmermann (1986), an external electric field induces an additional transmembrane potential larger than the natural membrane potential. When the overall membrane potential reaches to a critical value of ~1 V, dielectric rupture takes place. This rupture causes disorientation in the membrane structure, resulting in formation of pores and increasing the permeability of cell membrane. Depending on the electrical field strength, pulse duration and pulse number, permeabilization can be either reversible or irreversible (Benz and Zimmermann, 1981).
In general, ozone molecules rapidly react with bacteria leading to death of cells. Ozone is believed to cause the oxidation of lipids on the cell envelope of bacteria (Murray et al., 1965; Scott and Lesher, 1963). Further oxidation leads to leakage of intracellular cell contents and damage of genetic material and to death of microorganisms (Prat and Cier, 1968; Scott, 1975).

Although ozone is highly effective against microorganisms in pure cell suspensions, it is unlikely to be used directly in foods. Organic constituents of food compete with microorganisms for ozone, and thus high doses of this agent may be needed for effective elimination of microorganisms. These levels of ozone may alter sensory attributes, and adversely affect the acceptability of foods. Ozone, however, may be conveniently used to disinfect the surfaces of fruits such as apples and oranges. Application of ozone on the surface of fruits can significantly reduce the microbial flora (Achen, 2000). When juice extracted from ozone-washed fruits is treated with PEF, a synergistic effect may be observed.

Pulsed electric field technology alone may not sterilize liquid foods. When foods are pre-treated with ozone before applying PEF, greater degree of kill and safety can be assured. Application of low dosage ozone followed by low-pulsed electric field treatment can be an alternative food preservation system.

In summary, combinations of ozone and PEF synergistically enhanced the inactivation of a spoilage microorganism, Lb. leichmannii ATCC 4797, and two emerging foodborne pathogens, E. coli O157:H7 and L. monocytogenes Scott A. Our results may assist the recent efforts in developing PEF and other non-thermal pasteurization methods for liquid foods. Future studies are required for combined
preservation systems to elucidate their modes of action against targeted microorganisms
This may allow optimized preservation systems and provide evidence to regulatory
agencies to enable authorization of these novel combined technologies for use in the food
industry.

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### Counts of cells (Reduction in LOG CFU/ml)\(^a\)

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\(^a\) Data points are averages of two trials.
\(^b\) Standard deviation.
\(^c\) Not determined

Table 4.1: Inactivation of *Lb. leichmannii* ATCC 4797, \(\sim 3 \times 10^7\) CFU/ml, *E. coli* O157:H7, \(\sim 2 \times 10^8\) CFU/ml, and *L. monocytogenes* Scott A, \(\sim 2 \times 10^8\) CFU/ml, suspended in 0.1% NaCl after treatment with ozone and pulsed electric field.
Figure 4.1: Inactivation of microorganisms by ozone, experimental setup (Modified from Kim, 1998).
A. Blank (0.1% NaCl)
B. Inoculated sample
C. Treated sample
D. Waste

Figure 4.2: Pulsed electric field bench-scale processing unit and fluid/sample handling system, adapted from the "User's Manual of OSU-4C Pulsed Electric Field System and Fluid/Sample Handling Operation" (OSU-PEF Team, Food Science and Technology, The Ohio State University, Columbus, OH). Figure is not drawn to scale.
Figure 4.3: Inactivation of *Lactobacillus leichmannii* ATCC 4797 by combination of ozone (0.75 and 1.00 μg/ml cell suspension) and pulsed electric field (20 kV/cm). Results are averages of two independent trials. Differences between ozone and the combination treatment (segments I-III) with different superscripts are significantly different (p < 0.05). Ozone only (-○-), and ozone + PEF-treated (-□-).
Figure 4.4: Inactivation (a) and injury [shaded area in (b)] of *Escherichia coli* O157:H7 by combinations of ozone (0.5, and 0.75 μg/ml of cell suspension) and pulsed electric field (15 kV/cm). Results are averages of two independent trials. Differences between ozone and the combination treatment (segments I-III, Fig. 4a) with different superscripts are significantly different (p < 0.05). Ozone treated cells plated on TSAYE (●—●); ozone/PEF-treated cells plated on TSAYE (■—■); ozone treated cells plated on VRBA (●—●); ozone/PEF-treated cells plated on VRBA (■—■).
Figure 4.5: Inactivation (a) and injury [shaded area in (b)] of *Listeria monocytogenes* Scott A by combinations of ozone (0.25, 0.50, and 0.75 μg/ml of cell suspension) and pulsed electric field (15 kV/cm). Results are averages of two independent trials. Differences between ozone and the combination treatment (segments I-IV, Fig. 5a) with different superscripts are significantly different (p < 0.05). Ozone treated cells plated on TSAEY (●——●); ozone/PEF-treated cells plated on TSAEY (■——■); ozone treated cells plated on TSA + 5% NaCl (●——●); ozone/PEF-treated cells plated on TSA + 5% NaCl (■——■).
CHAPTER 5

GROWTH KINETICS OF \textit{Lactococcus lactis} subsp. \textit{lactis} ATCC 11454 BY SUBLETHAL OHMIC HEATING

Abstract

Use of ohmic heating to enhance bacterial growth is a novel approach with potential applications in food fermentations. The goal of this investigation was to study the effects of sublethal ohmic heating on the growth of \textit{L. lactis} subsp. \textit{lactis} ATCC 11454 and nisin production by this bacterium. \textit{L. lactis} subsp. \textit{lactis} was inoculated into M17 broth supplemented with 0.5\% glucose in a fermenter vessel and incubated at 25, 30, and 37\(^\circ\)C with agitation. Incubation temperature was supplied by ohmic or conventional heating. An electrical current at 15 V or 30V was applied to heat the broth by sublethal ohmic heating. Lag period, minimum generation time, maximum growth, and pH changes were determined during fermentation. Nisin activity, glucose consumption, and lactic acid production were also determined. Lag period of \textit{L. lactis} decreased 2.5, 1.8, and 1.56 hours by sublethal-ohmic heating when compared with conventional heating at 25, 30, and 37\(^\circ\)C, respectively. Sublethal ohmic, compared with conventional, heating resulted in lower nisin activity in the fermented medium. Electrical current increased during the fermentation probably due to the production of lactic acid.
and other polar components. The measurement of electrical current when ohmic heating is applied at a constant voltage is potentially useful in monitoring the growth of the culture. It might be desirable in some food fermentations to apply electric current to decrease the lag period of cultures at the initial stage of growth, and then apply conventional heating at the later stages of growth. This phenomenon may considerably shorten the processing time in cheese and yogurt making, and also other type of industrial fermentations.

Introduction

Recent interest of industry has resulted in renewed attention towards technologies utilizing electricity in foods. Most of the research using electrical energy hold promise of microbial inactivation and provide high quality, and shelf-stable foods. Use of electricity for microbial inactivation in foods is not a new approach. However, there is not sufficient data on how the electricity affects microorganisms. Most recent studies (Castro et al., 1993; Ho et al., 1995; Liu et al., 1997; Martin-Belloso et al., 1997; Vega-Mercado et al., 1997) dealt with microbial inactivation by using high electric pulses. Palaniappan et al. (1992) reported that there was no significant lethality by electricity at electric field strength commonly associated with electrical (ohmic) heating.

Ohmic heating involves passing an electrical current at an appropriate voltage throughout the liquid or liquid-particulate food. Ohmic heating is considered as a novel food processing technique (Fryer et al., 1993) used for liquid food pasteurization. The growth kinetics of Lactobacillus acidophilus OSU133, which produces a bacteriocin
known as Lactacin A, was studied by Cho et al. (1996) with application of both conventional and ohmic heating. The effect of non-lethal electricity was investigated on the growth parameters and changes in pH during the fermentation. The lag period for *L. acidophilus* was affected by the method of heating. There was a 94% decrease in lag period under low-voltage ohmic heating (30°C, 15V) when compared with conventional heating method. The electrical current enhanced the early stages of growth, but inhibited the late stages of growth. Ohmic heating resulted in higher pH and lower bacteriocin activity in the fermented medium. These authors suggested that the lag period decreased by ohmic heating at 30°C could be beneficially used in the food industry to shorten the time for processing cheese and yogurt. However, electrical current decreased and showed some fluctuations during the fermentation due to the noise from the electric source, the high ionic strength of medium, and lack of sufficiently accurate and sensitive measuring devices at the level of current in their study.

The effects and interactions of sublethal ohmic heating on the growth and metabolic activity of microorganisms have not been studied in details. Firstenberg et al. (1984) used impedance measurement techniques to monitor the microbial growth and metabolic activities. Parrot (1992) and Wolcott et al. (1969) reported that the changes in the composition of the medium following growth of microorganisms are associated with the changes of impedance. Rowley (1972) reported that electrical current generated by electrochemical reactions enhanced the growth of bacteria. Results showed that bacterial counts were 100 fold higher than controls. Rowley (1972) concluded that electrical current can be used to enhance or inhibit the growth of microorganisms depending on the experimental conditions.
The objective of this study is to investigate the effect of sublethal ohmic electrical treatment on the growth kinetics of *L. lactis* subsp. *lactis* ATCC 11454, and possible changes in metabolic activity of the culture.

**Materials and Methods**

**Microorganisms**

_*Lactococcus lactis* subsp. *lactis* ATCC 11454 was obtained from the culture collection of the food microbiology laboratory of the Department of Food Science and Technology, the Ohio State University. The culture was maintained as frozen stock at -80°C in M17 broth (Difco, Detroit, MI) containing 10% glycerol (Sigma Chemical Co., St. Louis, MO). Before every experimental run, the culture was inoculated into M17 broth containing 0.5% glucose (GM17) at 1% level and incubated at 30°C for 12 h, followed by a second transfer under the same conditions.

_*Lactobacillus delbrueckii* subsp. *leichmanii* ATCC 4797 was used as an indicator microorganism to measure the activity of nisin produced by *L. lactis* subsp. *lactis* ATCC 11454. The culture was maintained as frozen stock at -80°C in MRS broth (Difco, Detroit, MI) with 10% glycerol (Sigma Chemical Co., St. Louis, MO). The culture was inoculated into MRS broth and incubated at 37°C for 12 h before experiments.

**Ohmically heated fermenter and experimental setup**

A 5-L fermenter vessel (2-L working volume) was used throughout the experiments. The vessel lid was equipped with ports for pH probe, thermocouple, inoculation, water circulation coil, medium circulation, and two stainless steel plate electrodes. The electrode gap was 14 cm and the cross-sectional area was 73.5 cm². Metal surfaces were coated with epoxylite for electrical insulation and inertness.
A schematic diagram of the fermenter equipped with ohmic heating is shown in Fig. 5.1. A teflon coated thermocouple was used to measure the temperature in the fermenter vessel and was connected to the controller (MIC 200 series, The Partlow Co., New Hartford, NY). A steam sterilizable pH probe (Ingold Electrodes, Inc., Wilmington, MA) was used to measure the pH of the medium during the fermentation. A water bath (Haake, Germany) was used to maintain constant temperature during conventional and sublethal ohmic heating. A double-beam spectrophotometer (model 100-60, Hitachi, Japan) was used to measure the absorbance of medium at 600 nm. The spectrophotometer was equipped with 2 cuvettes (cells), one for measuring the absorbance of the fermented medium and the other containing autoclaved M17 broth as a reference sample. Voltage and current transducers were used to measure voltage and current. Isolation transformer, regulation transformer and variable transformers were used to supply constant voltage during ohmic heating experiments. The time, temperature, pH, OD<sub>600</sub>, voltage and current were collected by a data logger that was connected to an IBM-type personal computer. For conventional heating studies, a similar experimental design was used, however, the device to supply electricity was excluded from the setup.

**Conventional and ohmic fermentation**

Fermentations were performed in a fermenter equipped with conventional or ohmic heating instruments. Ohmic heating was obtained by passing alternating current of 60 Hz such that a constant voltage (15 or 30 V) was supplied. A constant temperature was maintained during fermentation under ohmic heating by intermittently circulating cooling water through the water circulation coil. The temperatures of the cooling water
were 10°C and 5°C at the low voltage (15 V), and high voltage (30 V) ohmic heating, respectively. For conventional heating, the fermenter was maintained at a constant temperature by continuously circulating controlled-temperature water through the water circulation coil. Three temperatures (25, 30, and 37°C) were tested in these experiments. The fluctuations around the set temperatures were ± 0.2 for conventional and ohmic heating. Average room temperature during these fermentations was 25.4°C.

The fermentation vessel was filled with 2.0 L of M17 broth (Difco, Detroit, MI) containing 0.5% glucose (GM17), and sterilized at 121°C for 15 min. The medium in the fermenter was agitated for 15 min prior to inoculation using magnetic stirrer. The culture of *L. lactis* subsp. *lactis* ATCC 11454, which was prepared as shown earlier, was inoculated at 0.001% (v/v) of the medium volume. Data (time, temperature, pH, OD<sub>600</sub>, voltage, and current) were collected during fermentation at 15 min intervals by a microcomputer. Changes in metabolic activity were determined in samples from the fermentations at 30°C; these samples were also collected at 15 min intervals.

**Assay methods**

Samples of fermented medium were taken by an extract collector device linked to fermenter vessel and kept at 4°C refrigerator. Samples were taken continuously by the extract collector in every one hour and collected in test tubes. Samples were centrifuged at 12,000 × g for 20 min. Supernatants were filtered through a 0.4 μm-pore-size filter. Total decreases in the concentrations of glucose and increases in lactic acid were measured by the HPLC system which consisted of a pump, an autosampler, an ion exclusion column (HPX-87H, Bio-Rad, Richmond, CA), a programmable
multiwavelength detector (Waters, model 490), a differential refractometer (Waters, model 410), and a 386-class microcomputer loaded with a data acquisition software (Maxima 820 workstation, Waters). Nisin activity in culture filtrate was analyzed by spot on lawn assay (Fig. 5.2). Detailed procedures about nisin and HPLS assays can be found elsewhere (Lewis et al., 1992; Liao et al., 1994; and Yang et al., 1991).

**Results and Discussion**

**Characteristics of growth**

Differences in the growth characteristics of *Lactococcus lactis* subsp. *lactis* ATCC 11454 were observed when M17 broth was fermented under conventional and sublethal ohmic heating (Fig. 5.3, 5.4, and Table 5.1). The lag period was greater under conventional than sublethal ohmic heating. It appears that electric current enhanced the early stage of growth of *L. lactis*. Alternating electrical voltages at 15 and 30 V were applied to heat the broth directly during fermentation. Decreasing lag phase of *L. lactis* with 15 and 30 V may be a useful application in the early stages of fermentations in food, and another industrial type of fermentations.

Electrical current increased during the fermentation probably due to the production of lactic acid and polar components produced which may enhance the conductivity of M17 broth medium. We believe that the alternating current affected the polar nutrients in the medium and helped the intake of these nutrients into the cells. Another possibility may be the induction of bacterial enzymes by sublethal electricity.
Lag period, minimum generation time and maximum growth were estimated by applying Gompertz mathematical model (Zwietering et al., 1990; Breidt et al., 1994). Lag period of *L. lactis* decreased 2.5, 1.8, and 1.56 h by low-voltage ohmic heating when compared with conventional heating at 25, 30, and 37°C, respectively (Table 5.1).

**Metabolic activity**

Little differences in pH changes were observed between sublethal ohmic and conventional heating methods at 30°C with 15 and 30 V.

**Changes in electrical current**

The electrical current passing in the fermented medium, under a constant voltage (15 and 30V), increased with the progress of growth and metabolic activity. Most of the changes in the current occurred after lactic acid was produced in detectable quantities. Cho et al. (1995) reported that most of the current changes occurred at the early exponential growth phase of *Lactobacillus acidophilus*. In this study, electrical current decreased while the fermentation was proceeding. Cho et al. (1996) reported that there were some variability in the measurement of current caused by the noise from the electric source, the high ionic strength of medium, and lack of sufficiently accurate and sensitive measuring devices at the level of current in their study. This may be due to the experimental setup that they used to monitor the parameters. Our results showed that electrical current increased possibly due to the production of lactic acid and the associated increase in electrical conductivity. Electrical current followed a closed relationship with the production of lactic acid and metabolic activity of the culture. This
increase in electrical current provided evidence that heating broth medium by sublethal ohmic heating may be useful in the food industry. It may be desirable in some food fermentations to monitor the growth of culture rapidly during the fermentation. Monitoring growth by the changes in electric current will be useful in fermentations where the optical density is impossible to measure (e.g., milk fermentation).

**Bacteriocin (nisin) Activity**

*Lactococcus lactis* subsp. *lactis* ATCC 11454 produces an antimicrobial bacteriocin (polypeptide) known as nisin. Nisin production was delayed, and lesser activity was determined in fermented medium by sublethal ohmic, compared with the conventional heating method at 30°C (Fig. 54).

**Conclusion**

Lag period of *L. lactis* decreased 2.5, 1.8, and 1.56 hours by low-voltage ohmic heating when compared with conventional heating at 25, 30, and 37°C, respectively. Sublethal ohmic, compared with conventional, heating resulted in lower nisin activity in the fermented medium. Electrical current (I) increased during the fermentation probably due to the production of lactic acid (increase in [H+]i) and other polar components.

It might be desirable in some food fermentations to apply electric current to decrease the lag period of cultures at the initial stage of growth, and then apply conventional heating at the later stages of growth. The measurement of electrical current when ohmic heating is applied at a constant voltage may be used to monitor the growth of culture instead of plate counting or measuring absorbance.
References


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<th>Minimum generation Time (h)</th>
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Table 5.1: Growth parameters for *Lactococcus lactis* subsp. *lactis* under conventional and ohmic heating.
Figure 5.1: Experimental setup for ohmic heating.
Figure 5.2: Spot on lawn bacteriocin assay.
Figure 5.3: Changes in growth, measured as OD600, and current during fermentation of M17 by Lactococcus lactis subsp. lactis ATCC 11454 at 30°C under ohmic and conventional heating.
Figure 5.4: Changes in nisin activity from *Lactococcus lactis* subsp. *lactis* ATCC 11454 at 30 °C under ohmic and conventional heating methods. (■) Ohmic heating (15 V), (▲) ohmic heating (30 V), and (◆) conventional heating.
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