# APPLICATION OF HIGH ELECTRIC FIELD PULSE TECHNIQUE FOR MICROBIAL INACTIVATION IN MILK

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## Abstract

**Milk contains a high level of nutrients such as protein, calcium, vitamin A and vitamin B12 which are beneficial for human consumption. However, the conventional thermal process for reduction of microbial contamination in milk results in destruction of some important nutritional values of the milk. The aim of this research was to study alternative nonthermal processes for reduction of contaminating microbes using high electrical field pulse (HEFP) treatment of ultra filteredmilk. A preliminary investigation of biological and physiochemical characteristics in raw milk was carried out. The milk was first subjected to ultrafiltration treatment and then to HPEF treatment for one minute in a TEFLON treatment chamber at 25°C. The ultrafiltration treatment succeeded in reducing the amount of protein to appropriate amount for HPEF applicationand lipid in the milk. The results from ultra membrane filtration of the raw milk showed a reduction of total solids by approximately 13.05% when the milk was passed through 0.7 and 0.4 µm membranes. The effect of HPEF on bacterial reduction in the ultrafilration treated milk was then studied over the following ranges of applied pulse intensities and number of pulses: applied intensity: 0, 40, 60, 80, and 100 kV/cm, number of pulses: 1, 30, 50, and 150 in the one minute treatment period. The results showed that application of 100 kV/cm and 30 pulses resulted in 9.93% death of *E.coli* but only 7.94% death of *S. Typhimurium.* A statistical analysis showed that increasing the electric Field intensity significantly increased the death percentages of both bacteria with p = 0.039 and p = 0.043, respectively.**

**Keywords: Ultrafiltration, high pulse electric field, Salmonella Typhimurium, *Escherichia coli*, milk**

## Introduction

The use of high pulse electrical fields (HPEF) for microbial inactivation in liquid food is attracting the attention of both academic researchers and food investors. This is because HPEF is a non-thermal pasteurization processing method that can achieve microbial and enzyme

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inactivation while potentially sustaining nutritional values and sensory acceptance. The mechanism for microbial destruction by high electrical fields pulse is cell membrane destruction caused by the formation of a trans-membrane potential which leads to an increase in membrane permeability (Tsong, 1990).

It is known that cow milk contains many nutritional benefits for human consumption, because it contains all essential nutrients, e.g., protein, carbohydrate in the form of lactose, fat, vitamins and minerals (Komorowski and Early, 1992). Ceballos *et al*., (2009) reported that cow milk contains 11.36% total solids, 2.82% proteins, 3.42% fats, 4.47% lactose and 0.65% ash (Ca, P, Mg, Fe, Cu, and Zn).

The inactivation of microbial cells by HPEF depends on many processing parameters, for example, electrical field strength, pulse frequency, treatment time, flow rate and the number of pulses per chamber. The strength of the electrical field that passes through the food is directly proportional to the voltage supplied across the electrodes, and inversely proportional to the distance between the electrodes. Currently, the two mechanisms of necrosis and apoptosis have been suggested for cell destruction (Beebe *et al*., 2013).

Foodborne infections in cow milk have been repeatedly reported for *Salmonella* spp. and human pathogenic verocytotoxin-producing *Escherichia coli* (Mallet *et al*., 2012). This is because of contamination occurring from many sources, for example, from direct contact with the milk, infection of a cow's udder (mastitis), cow diseases (e.g., bovine tuberculosis), bacteria that live on the skin of cows, environment (e.g., feces, dirt, processing equipment), insects, rodents, and other animal vectors. The aim of our study was to reduce the microbial infection in cow milk by non-thermal processes. In the work presented here, the raw milk was first passed through a microfiltration pretreatment in order to reduce some lipid and protein components that would hinder the electrical field treatment. This pretreated milk was then exposed to HPEF with intensities of 0, 40, 60, 80, and 100 kV/cm and number of pulses in 1-minute treatment time of 1, 30, 50, and 150. A physicochemical analysis was then carried out and the reduction of microbial contamination of the treated milk was determined.

## Materials and Methods

### Raw Materials

Milk samples obtained from a commercial dairy (Ratchaburi Farm, Thailand) were directly poured into a sterile glass container and kept at a temperature of 4°C. Microbiological media was purchased from Himedia, India. *Escherichia coli* TISTR 780 and *Salmonella Typimurium* ATCC 13311 were purchased from the *Thailand Institute of Scientific and Technological Research.*

### Physical and Chemical analysis of Raw Milk

Triplicate samples of raw milk were analyzed for physical and chemical properties according to the standard methods of the International Standard Organization (ISO) and the standard methods for the examination of dairy products of the American Public Health Association. In detail, the specific gravity of the raw milk at 21°C was measured by Lactodensimeter, the milk turbidity was recorded by spectrophotometer at absorbance of 660 nm, the pH was measured by pH meter, and the conductivity was recorded by a conductivity probe. Total soluble solid was detected by hand refractometer and expressed as °Brix. A colorimetric method measurement of total sugar analysis as hexose and pentose sugar was carried out by the Anthone method. Total protein was determined using the Bradford method with bovine serum albumin as a standard and a carbohydrate analysis was carried out by a phenol-sulfuric assay.

### Microbiological Analysis of Both Treated and Untreated Milk

Treated milk both ultrafiltration with HPEF and a control of untreated raw milk were subjected to microbial analysis. The

number of microbial contaminants in the milk was recorded by standard plate count. *E. coli* and *S. Typimurium* were identified by the two selective media, Eosin Methylene Blue Agar and Brilliant Green Agar Base, respectively.

### Temperature Effect on Rate of Growth

Two sets of six bottles of raw milk were stored at 4°C and 30°C for an incubation time of 6 days. After the incubation, samples were taken at intervals each day and subjected to analysis for microbial growth by the standard plate count agar method with incubation at 32 ± 1°C for 48 ± 3 h. The visible growth was recorded in CFU/ml.

### Pretreatment with Microfiltration

The ultrafiltration pretreatment for reductions of fat and lipids in milk was carried out by passing the milk first through 0.7 µm and then through 0.4 µm micromembrane filter units. Both untreated and treated milk were then checked for lipid content by the Rose-Gottlieb method and protein content by a Bradford assay.

### High Electrical Field Pulse Treatment

Samples of 40 ml ultrafiltrated milk were placed in a TEFLON treatment chamber at 25°C. A high voltage electric field was then applied for one minute with the following ranges of intensities and number of pulses: 0, 40, 60, 80 and 100 kV/cm and 1, 30, 50, and 150 pulses in the one minute treatment period. Treated samples were then kept at 4°C before analysis

### Statistical Analysis

One way ANOVA was used as a statistical test of significance for differences between the microbial reductions in the treated and untreated milk samples. Difference of the means was considered to be significant when $p < 0.05$.

## Results and Discussion

### Rate of Microbial Growth

The microbial growth rates were studied at two temperatures, 30°C and 4°C. As shown in Figure 1, an approximate exponential growth rate was found in raw milk at 30°C with the rate of 0.841 $day^{-1}$ at an initial microbial concentration of $2.65\times10^5$ CFU/ml. In contrast, slow growth was found in the raw milk at 4°C with the rate of growth equal to 5.85×104 CFU/ml/day.

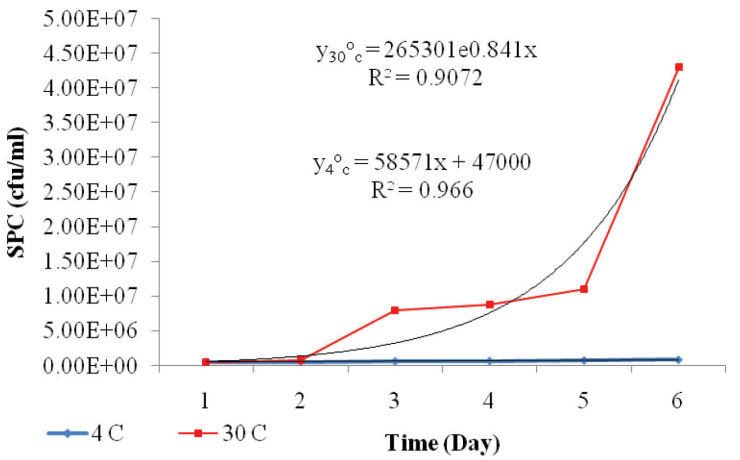


**Figure 1. Rates of microbial growth under different incubation temperatures**

**Physicochemical Changes in Raw Milk**

The results of a physicochemical analysis of raw milk that passed through the ultrafiltration process are shown in Table 1. The results show that there was only a small change in specific gravity, turbidity, pH and electro-conductivity, but a larger change in nutritional contents. The reduction in nutritional contents of the pretreated milk which passed through 0.7 μm and 0.4 μm membranes were as follows: hexose 7.08%, pentose 6.61%, total protein 63.90% and carbohydrate 15.29%. Asshown in Table 1, a statistically significant reduction of 11.54% occurred in total solid content.

**HEFP Effect to Microbial Reduction in Raw Milk**

To investigate the effect of HPEF on microbial reduction, the raw milk samples exposed to different intensities and different numbers of pulses were identified for total microbial content by using nutrient agar (Figure 2(a)) and for *E. coli* and *S. Typimurium* content by using EMB and BGA media (Figure 2(b, c)) Further identifications were also done by gram strains as shown in Figure 2(d, e). It was found that the morphological *E. coli* cell in raw milk was gram negative, and typically rod-shaped with length of 2.0 μm length and diameter of 0.5 μm. The *S. Typimurium* was also gram negative, and typically rod-shaped with length around 2 to 5 μm and diameter around 0.7 to 1.5 μm. The measured initial concentrations of *E. coliand S. Typhimuriumwere* $1.51\times10^5$ and $1.68\times10^6$ CFU/ml.

The effect of intensity (applied intensities: 0, 40, 60, 80, and 100 kV/cm) was then studied on the ultrafilration treated milk and the raw milk for varying numbers of pulses: 1, 30, 50, and 150 pulses. For the HPEF treatment of the ultrafiltered milk it was found that at 40 kV/cm the death rate of *S. Typimurium* increased by 1.5 times when the number of pulses was increased by 3 times (Figure 3). For raw milk it was found that for a fixed number of 30 pulses the death rate of *S. Typimurium* increased as the electrical intensity was increased. For example, the death rate at 100 kV/cm was 24.14% higher than at 60 kV/cm. For the HPEF treatment of ultrafiltered milk it was found that at 40 kV/cm the death rate of *E. coli* increased by 2 times when the number of pulses was

**Table 1. Physicochemical parameters of raw milk passed through ultrafiltration**

| Parameter | Before | After |
|---|---|---|
| Specific gravity | 1.028 | 1.021 |
| Turbidity | 3.291 | 3.186 |
| pH | 6.7 | 6.63 |
| Electro conductivity (μs) | 5.35 | 5.06 |
| Total Soluble Solid (°Brix) | 9 | 9 |
| Alcohol | - | - |
| Total Solid % | 16.420 | 14.524 |
| Sugar (g/ml) | | 8.497 |
| - Hexose (g/ml) | 9.145 | 8.539 |
| - Pentose (g/ml) | 9.144 | 1.155 |
| Total Protein | 3.200 | 0.8946 |
| Total Lipid (g) | 0.9263 | |
| Carbohydrate (g/ml) | 27.73 | 23.490 |

increased by 3 times (Figure 4). For raw milk it was found that for a fixed number of 30 pulses the death rate of *E. coli* increased as the intensity was increased. For example, the death rate at 100 kV/cm was 20% higher than that at 60 kV/cm.

Although, as stated above, there were appreciable increases in the death rates for both *S. Typimurium* (1.5 times) and *E. coli* (2 times) in the ultrafiltered milk when the number of pulses was increased by 3 times at HPEF intensities of 40 kV/cm, it was found that at 100 kV/cm there were only quite small increases of 1.15 times in the death rates even when the number of pulses was increased 5 times.

A comparison of the bacterial death rates are shown in Figure 5. At 40 kV/cm and 50 pulses there was no difference in the percentages of death of *E. coli* and S. *Typimurium*. At 40 kV/cm and 150 pulses, the death percentage for *E. coli* was 1.5 times higher than that of *S. Typimurium*. The results in Figure 5 show that *E. coli* death percentage

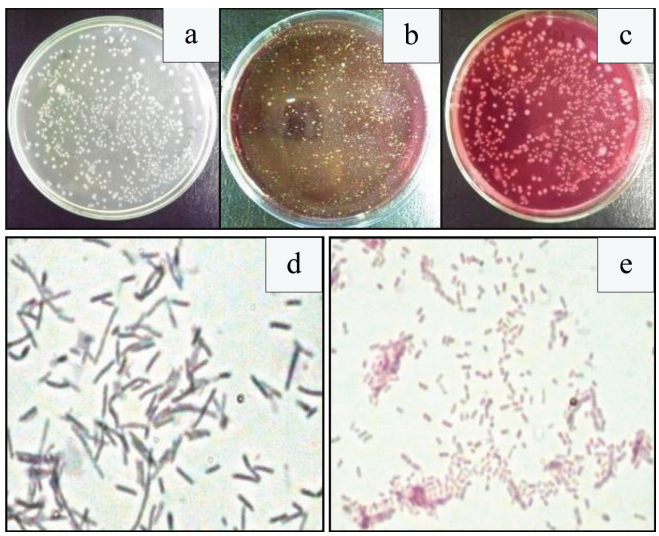


**Figure 2. Characteristics of microbial growth in general and enrichment media. (a) nutrient agar, (b) Eosin Methylene Blue agar, (c) Brilliant Green Agar Base. Gram stains from microscope 1000X as (d) *Escherichia coli* (e) *Salmonella Typhimurium* from screening of raw milk**

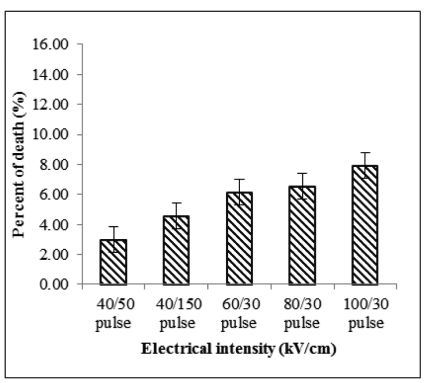


**Figure 3. Effect of HPEF on *Samonella Typhimurium* death**

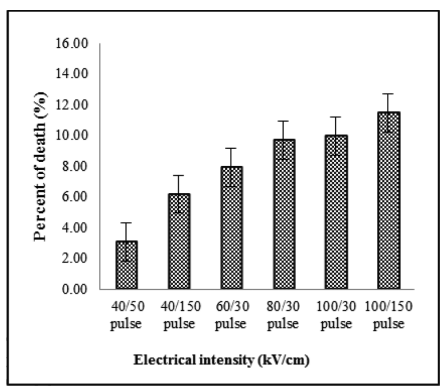


**Figure 4. Effect of HPEF on *Escherichia coli* death**

increases faster than death percentage of *S. Typimurim* as electrical intensity is increased. Statistical analysis of the effects of an increase in electrical intensity on death percentage showed significant effects in both bacteria with p = 0.039 and p = 0.043, respectively.

It is suggested that cell death occurs when the pulsed electric fields destroy the cell walls and allow the contents of the cell to be released. Pothakamury *et al*. (1997) have reported experiments which show that cell surfaces become increasingly rough as the electric field intensity is increased even if the number of pulses is not changed. A possible mechanism for the cell wall destruction is that the pulsed field causes an electric-mechanical compression which produces a separation of charges between the inside and outside of the microbial cells in the milk. This separation could then produce small holes in the membrane and a leakage of cellular contents. Therefore, an increase in field intensity could increase microbial inactivation because the corresponding increase in electrico-mechanical compression results in increased cell wall destruction.

Normally, cell size and shape of the microorganisms have been used to explain different degrees of inactivation of different microbes (Qin *et al*., 1998) However, in this case, both *E. coli* and *S. Typhimurium* are gram negative bacteria which are similar in size and shape. The 30% higher cell death in *E. coli* than *S. Typimurium* could be due to differences in orientation of cells with respect to the external electrical field causing different numbers of target cells to be affected.

The number of cell deaths for both types of bacteria in our experiments was lower than expected when compared with many previous reports of effects of the HPEF treatment of simulated milk ultrafiltrate (SMUF). Martin *et al.* 1997 applied HPEF treatment at 35 kV/cm and 64 pulses to milk inoculated with *E. coli* and reported a reduction of approximately 2 log cycles. Several investigators have also reported *E. coli* reductions in SMUF of about 6 and 9 log cycles from HPEF treatments at 60 kV/cm and 50 pulses and at 70 kV/cm and 80 pulses (Zhang *et al*., 1995; Qin *et al*., 1998).

Our results suggest that the milk, especially the milk composition (fat content of approximately 3.38%), could help to protect the microorganisms from the pulsed electric field. This shielding of microorganisms by milk composition has also been reported previously by Deeth and Datta (2011) who studied bacteria destruction in milk and found that the destruction in milk containing 3.5% fat was less than that in milk containing 1.5% fat. Milk fat is excreted in the form of small

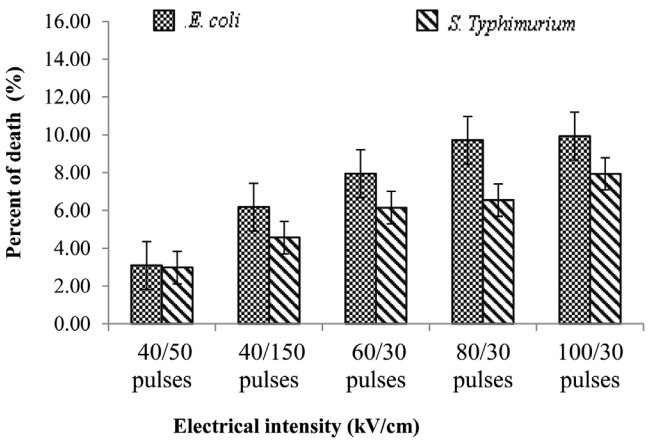


**Figure 5. Death percentage comparison between two microbial strains**

droplets, which in cow milk range from 1 to 12 μm in diameter with a mean of about 3 μm. Triacylglycerols are the predominant lipids in bovine milk, accounting for 97-98% of total lipid. The remaining lipids are diacylglycerols, monoacylglycerols, phospholipids, free fatty acids, and cholesterol and its esters (Muir, 1992).

All of these kinds of fat could have protective effects on free microbial cells and/or on bacterial cells adsorbed on the proteins and fat droplets. Furthermore, milk contains many components which conduct electricity, for example, ions, proteins and minerals. The proteins in milk fall into two distinct types, caseins (82.2%) and whey proteins (17.8%) (Huffman and Harper, 1999). The degree of microbial inactivation achievable by HPEF is therefore expected to decrease with increasing conductivity (or decreasing resistivity). The reported resistivity of milk ranges from 1.3 to 3.1 Ω (Deeth and Datta, 2011). The conductivity of the charged particles could therefore create many problems such as electrical field distortion during the HPEF treatment. However, although our results showed a reduced effectiveness of HPEF for microbial inactivation in milk compared with some previous studies, we still found a positive effect for HPEF.

It is also worth noting that some investigators (Zhang *et al*., 1994; Zhang *et al*., 1995) have reported that a high initial concentration of inoculums negatively affects microbial inactivation as in the case of *E. coli*. However, it has also been reported (Zhang *et al*., 1995) that an initial concentration of $1.15\times10^{3}$-$7.14\times10^{8}$ CFU/ml in SMUF did not affect the inactivation rates obtained. As stated previously, the measured initial concentrations in our experiments were for *E.coliand S. Typhimuriumwere* $1.51\times10^{5}$ and $1.68\times10^{6}$ CFU/ml.

In conclusion, the present study has shown that inactivation of pathogenic bacteria could be achieved with the current laboratory scale HPEF generator and treatment conditions. However, only a small destruction of microbial cells was found due to the complex composition of milk which has high viscosity, low resistivity and contains proteins and fat. This type of medium hinders the inactivation of microbial cells.

For future work, different microorganisms and different milk compositions should be studied in order to determine the optimum conditions for HPEF treatment which will achieve the maximum microbial inactivation and meet the pasteurization standard.

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## References

Deeth, H.C. and Datta, N. (2011). Non-thermal technologies: pulsed electric field technology and ultrasonication, encyclopedia of dairy ciences (econddition), p. 738-743.

Huffman, L.M. and Harper, J.W. (1999). Symposium: marketing dairy value through technology. Maximizing the value of milk through separation technologies. J. Dairy Sci., 82:2238-2244.

Komorowski, E.S. and Early, R. (1992). Liquid milk and cream. In:The Technology of Dairy Products. Early, R. (ed). VCH Publishers, Inc., NY, p. 1.

Mallet, A., Guéguen, M., Kauffmann, F., Chesneau, C., Sesboué. A.,and Desmasures. N. (2012). Quantitative and qualitative microbial analysis of raw milk reveals substantial diversity influenced by herd management practices. Int. Dairy J., 27:13-21.

Martı´n, O., Qin, B.L., Chang, F.J., Barbosa-Ca´novas, G.V.,and Swanson,B.G. (1997). Inactivation of *Escherichia coli* in skim milk by high intensity pulsed electric fields. J. Food Process. Engin., 20:317-336.

Muir, D.D. (1992). Milk chemistry and nutritive value. In: The Technology of Dairy Products. Early, R. (ed).VCH Publishers, Inc., NY, p. 24-33.

Pothakamury, U.R., Barbosa-Ca´novas, G.V., Swanson, B.G., and Spence, K.D. (1997). Ultrastructural changes in *Staphylococcus aureus* treated with

pulsed electric fields. Food Sci. Technol. Int., 3:113-121.

Qin, B.L., Barbosa-Ca´novas, G.V., Swanson, B.G., Pedrow, P.D.,and Olsen, R.G. (1998). Inactivating micro-organisms using a pulsed electric field continuous treatment system. IEEE Trans. Indus. Applicat., 34:43-50.

Qin, B.L., Pothakamury, U.R., Vega, H., Mart´n, O., Barbosa-Ca´novas.G.V., and Swanson, B.G. (1995). Food pasteurization using high-intensity pulsed electric fields. Food Technol., 49:55-60.

Sanz Ceballos, L., Ramos Morales, E., Torre Adarve, G.D.l., Dı´az Castro, J., Pé rez Martı´nez. L.,and Sanz Sampelayo, M.R. (2009). Composition of goat and cow milk produced under similar conditions and analyzed by identical methodology. J. Food Compos. Anal., 22:322-329.

Stephen, J., Beebe Nova, M.,Sain, L.,and Ren, W. (2013). Induction of cell death mechanisms and apoptosis by nanosecond pulsed electric fields (nsPEFs).Cells, 2:136-162.

Tsong, T.Y. (1990). Electrical modulation of membrane proteins: enforced conformational oscillations and biological energy signals. Annu. Rev. Biophys. Chem.,19:83-106.

Zhang, Q., Monsalve-Gonzı´lez, A., Barbosa-Ca´novas, G.V., and Swanson. B.G. (1994). Inactivation of *Escherichia coli* and *Sacharomyces cerevisiae* by pulsed electric fields under controlled temperature conditions. Transactions of the ASAE, 37:581-587.

Zhang, Q., Qin, B.L., Barbosa-Ca´novas, G.V., and Swanson, B.G. (1995). Inactivation of *Escherichia coli* for food pasteurization by high-strength pulsed electric fields. J. Food Process. Preser., 19:103-118.