AN ABSTRACT OF THE THESIS OF

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Title: High Pressure Induced pH Change and its Effect on the Inactivation of *Lactobacillus plantarum* and *Escherichia coli*

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Microbial lethality is strongly pH dependent in processing regimes including high hydrostatic pressure processing (HPP). HPP induces the dissociation of weak acids that potentially causes a shift in pH in some systems thereby increasing acidity while pressure insensitive buffers do not undergo dissociation. The degree of high pressure induced pH shift depends on the nature of the acid. Furthermore, in the presence of weak organic acids with antimicrobial properties a pH reduction will increase the concentration of the more active protonated form of these acids. The first objective of this study was to determine the differences in lethality between pressure sensitive and pressure insensitive buffers. The second objective was to determine the synergistic effects of antimicrobial organic acids and pressure sensitive buffers in the reduction of microbial population by HPP. *Escherichia coli* and *Lactobacillus plantarum* were utilized to study differences between buffers of low susceptibility (HEPES, ACES, MES) and high susceptibility

(citrate, phosphate) to pH shifts under pressure. In general dissimilar levels of inactivation were observed between pressure sensitive and insensitive buffers. At low pH conditions pressure sensitive buffers achieved higher inactivation levels as was hypothesized. At neutral pH the differences were smaller for *L. plantarum.* However certain combinations (pH 5 and 6) appear to contradict the general trend observed. In the course of this study it was found that *L. plantarum* was less resistant than *E. coli* to HPP processing under most conditions. The effect of antimicrobials (acetic, propionic, sorbic, and benzoic acids) in combination with HPP on a strain of *Lactobacillus plantarum* was studied. The cultures were resuspended in citrate buffers at pH 3 and pH = pK_a of the acids, and HPP treated for one minute over a range of pressures. Survivor curves were fitted using a Weibull equation. Despite conditions in which the organic acid was fully protonated, no microbial effect was observed at pH 3, possibly because of the superior lethal effect of high acidity over the presence of antimicrobials. At pH = pK_a, three of eight conditions showed statistically significant differences from control even though all conditions showed an improvement in lethality. Concentration of antimicrobial had a minimal impact. In all cases, the incorporation of antimicrobial organic acids even at levels below published MIC resulted in a consistent trend of increased lethality under pressure.

High Pressure Induced pH Change and its Effect on the Inactivation of *Lactobacillus plantarum* and *Escherichia coli*

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDIX TABLES

Table | Page

High Pressure Induced pH Change and its Effect on the Inactivation of *Lactobacillus plantarum* and *Escherichia coli*

1. INTRODUCTION

1.1. High Pressure Processing

High pressure processing (HPP) has emerged in the past fifteen years as the leading non-thermal technology for the treatment of foods. Consumer trends indicate an increase in demand for more natural, healthier food products. Contrary to traditional thermal processing, HPP technology has the advantage of low impact on organoleptic properties and nutritional content of the food treated. HPP makes possible the development of products that are less heavily processed, healthier and with a reduced preservative content than previously available. HPP uses pressures ranging from 300 to 700 MPa and low to moderate temperatures to reduce the microbial load of the food and to inactivate enzymes. Additionally HPP can be utilized for rapid freezing and thawing of foods and to achieve texture changes. Fruit juices, oysters, guacamole, and sliced cooked ham are a few examples of HPP treated products currently available to consumers [9, 13, 26, 32, 56, 57].

During processing pressure is transmitted uniformally without occurrences of dead-spots; this characteristic greatly reduces processing time when compared to thermal processing. The energy consumption required for the high pressure

treatment of food is much smaller than that required by traditional thermal processing. Furthermore, the water consumption is much lower than in thermal processing. All of these factors make HPP more environmentally friendly [9, 13, 26, 57].

Although HPP has limited effectivity against bacterial spores, it is efficacious in the inactivation of numerous vegetative pathogenic and spoilage bacteria. When combined with high temperature the sterilization of the food is possible [41]. HPP can be combined with other preservation methods as a hurdle to improve overall efficacy [57].

1.2. pH under High Pressure

Under HPP treatment conditions chemical equilibria are altered. Water is compressed up to 15% at 600 MPa, and the acidity of the system may change. Yet, when pressure is relieved the system returns to the original equilibrium conditions. Thus it is possible to create new and more effective conditions under pressure that would disappear after processing [4, 9, 20, 56].

Le Chatelier's principle states that equilibria subjected to an increase in pressure will shift towards a reduction in volume. A substance will undergo dissociation under pressure if it results in an overall reduction in volume. Acids with negative reaction volumes ($\Delta V°$) are more likely to undergo dissociation under pressure (Table 1.1). A consequence of acid dissociation is a higher concentration of hydrogen (hydronium) ions in solution, thus decreasing pH. The lower pH at pressure can be utilized to further improve the efficacy of HPP as microorganisms tend to be more pressure sensitive in more acidic environments [6, 13, 30, 55].

TABLE 1.1. Reaction Volume of Acids

Acid	pK_a	ΔV° (ml/mol)
phosphate (1^{st})	2.14	-15.5
HEPES	3.00	9.4
citrate (1^{st})	3.03	-10.7
citrate (2^{nd})	4.55	-12.3
MES	6.10	3.9
citrate (3^{rd})	6.10	-22.3
phosphate (2^{nd})	7.20	-28.1
ACES	6.75	4.0

The nature of HPP creates technological difficulties for the measurement of pH. Conventional electrodes are not able to withstand the pressures used in HPP. Furthermore it is technologically difficult to install any type of cable to deliver a signal from inside of the vessel to a recording device on the outside. Pressures of several hundred MPa require extremely strong vessels and sealing mechanisms. A wire pass-through would be a weak point in the structure of the vessel. There have been several attempts at measuring pH indirectly by use of indicator substances that change color or fluorescence with changes in pH. They require a cell with transparent walls (sapphire) that limit the size of the chamber to several milliliters. The accuracy of these methods is questionable and they are inherently limited to the measurement of transparent systems [24, 55].

1.3. Antimicrobial Acids & Pressure

The undissociated form of weak organic acids is widely considered to have antimicrobial properties while the dissociated form possesses minimal, if any, an-

timicrobial activity. It is currently believed that the less polar undissociated form can penetrate the cell membrane more readily. Once inside the cell the acids dissociate, thus reducing internal pH and interrupting cell functions. When the pH is equal to the pK_a 50% of the acid will be protonated. The protonated, or undissociated, form is prevalent in solution when the pH is lower than the pK_a of the acid as can be seen from the Henderson-Hasselbalch equation (Equation 1.1) [6, 14, 17, 42]:

$$pH = pK_a + log\frac{[A^-]}{[HA]} \tag{1.1}$$

If at pressure the pH of the buffer system is lowered then the fraction of protonated acid may increase. The change in equilibrium creates more lethal conditions under high pressure. Once the pressure is lowered to atmospheric conditions the system would return to the original pH.

1.4. Objectives

The goal of this project was to study the interactions between pH and high pressure that improve the efficacy of HPP. The results of this project may allow food processors to utilize the interactions between high pressure and pH for low acid foods. A greater level of microbial inactivation could be attained by the formulation of low acid products for HPP. There are opportunities to develop new products that would take advantage of this synergy.

The first section of the project studied the effect of pressure sensitive and pressure insensitive buffers over a range of pH conditions, from acidic to neutral. The buffers were weak acids that have been reported as pH pressure sensitive and insensitive by Kitamura *et al.* [30]. The goal was to infer the presence, or absence,

of a pressure related pH shift over a range of pressures and acidities. This effect was measured in microbial inactivation of *Lactobacillus plantarum* ATCC 8014, and *Escherichia coli* K12.

The second section of this study aimed to establish the existence of synergy between high pressure and antimicrobial acids, where the presence of antimicrobial acid would increase the degree of microbial inactivation by HPP. *Lactobacillus plantarum* ATCC 8014 was high pressure treated in a pressure sensitive buffer with added antimicrobials. The hypothesis for this section was that a pressure shift can affect the equilibrium of the weak organic acid, which in turn increases the fraction of protonated acid and thus improves the effectiveness of the antimicrobial acid. The resulting conditions are disadvantageous for bacteria and a greater level of inactivation is achieved.

2. LITERATURE REVIEW

2.1. Food Preservation

The preservation of foods has played a central role in human existence as harvested food had to be conserved over long periods of time for later consumption. Heat, cold, drying, and fermenting were the traditional methods of food preservation. Additional common methods were salting, smoking, and pickling of foods [7, 14, 38]. Consumers have the expectation of having foods available year-round, to be safe, and to have a relatively long shelf life. In order to meet consumer demands, a variety of processes are available to expand the shelf life of foods. The spoilage of foods by microorganisms and the prevention of pathogen proliferation in foods are prevented by either chemical or physical methods. Microorganisms only grow under favorable conditions of temperature, water activity, oxygen presence, redox potential and acidity. Preservation of foods can be accomplished by eliminating favorable growth conditions in addition to inactivating microorganisms. Methods of food preservation against microbial growth address one or both aspects [7, 38].

2.1.1. Chemical Additives

There are a wide variety of chemical agents that can be added to food to prevent, or to limit the growth of spoilage and pathogenic microorganisms. Organic acids, sulfites, nitrites affect the exchange of metabolites through the cell membrane, and salt and sugar reduce water activity. Certain flavorings and

spices possess significant antimicrobial activity. Bacteriocins such as nisin are also utilized in foods such as cheese [7, 28, 38].

2.1.2. Physical Methods

Food preservation can be attained through several physical treatments. Drying prevents the growth of microorganisms and the activity of enzymes. A common method of expanding the shelf life of foods is decreasing the level of oxygen. Low pressure storage, vacuum packaging and modified atmosphere packaging are examples of different approaches used to lower oxygen levels. Several radiation techniques are employed to lower the microbial load of foods. These methods include UV light, beta rays, gamma rays, x-rays, and microwaves. Refrigeration and freezing of foods are also common methods of preservation. Low temperature storage slows down the activity of microorganisms thus increasing the shelf life of the product. Emerging nonthermal techniques exist for food treatment such as pulsed electric fields, oscillating magnetic fields, pulsed light and HPP [28, 29].

High temperature is used in the pasteurization and sterilization of foods. The efficacy of thermal treatment is influenced by several factors. Low water activity conditions tend to improve the thermal resistance of microbial cells. Fat, carbohydrate content, and salt concentration determine thermal resistance in addition to delimiting the water activity of the system. High protein content protects microbes from heat inactivation. The growth phase of vegetative microbes determines their thermal tolerance, although there is not a general rule. The resistance of some organisms decreases during exponential growth while others are reported to be more resistance during this growth phase. The acidity of the medium plays

a major role in the thermal resistance of microbes. Foods are legally divided into two groups based on their pH for thermal treatment purposes. High acid foods are those with pH values below 4.6 which prevent germination of *Clostridium botulinum* spores, and low-acid and it is formed by those foods with pH above 4.5. Low acid foods provide a more favorable environment for microbial growth [?]. The resistance of microbes to thermal processing is lowered as the pH decreases, or increases, away from the optimal growth pH. Thus, high acid foods require less heat input to achieve sterilization when compared to neutral pH foods. The presence of antimicrobial compounds increases the effectivity of heat processing. These same factors affect microorganism's resistance to HPP [20, 28, 29].

2.2. Organic Acid Antimicrobials

Organic acids are generally recognized as safe (GRAS) ingredients. They are commonly added to foods as antimicrobials and acidulants. Saturated fatty acids composed of less than 6 carbon possess antimicrobial activity against gram negative bacteria. Organic acids inhibit bacterial growth by attacking the cell membrane, cell wall, metabolic enzymes, protein synthesis and/or genetic material [4, 44]. The exact mechanism of microbial inhibition is unclear. It has been postulated that antimicrobials function by entering the cell in the undissociated, or protonated, form. The lower polarity of the undissociated form facilitates transport across the membrane. Once inside the cell the cytoplasmic pH (near neutrality) causes the acids to dissociate. The result is an increase in the concentration of hydrogen ions inside the cytoplasm. The cell is forced to expend a large

amount of energy to pump protons across the membrane in order to maintain its internal pH neutral [4, 14, 17, 28, 39, 42, 44].

Cells maintain their internal pH constant by passive and active homeostasis. The cell membrane prevents hydrogen ions from entering the cytoplasm. Additionally a decrease in internal pH will trigger the synthesis of buffering components such as glutamate and citrate. Active homeostasis implies the use of transport systems such as the energy dependent excretion of hydrogen ions. Growth stops when the energy demand created by active homeostasis surpasses the energy provided by catabolism [39].

Alternatively the antimicrobials may accumulate in the cytoplasmic membrane. Under this situation substrate transport and oxidative phosphorylation are uncoupled from the electron transport system [54]. Factors such as pK_a of the acid, the pH of the food and the solubility of the compound affect organic acid efficacy against microbes [14, 28].

2.2.1. Acetic Acid

Acetic acid and its salts are widely used as acidulants and antimicrobials. Aside from its ability to lower pH, acetic acid acts as an antimicrobial . The antimicrobial activity of acetic acid is low compared to other preservatives. It is often utilized in combination with other preservation methods like pasteurization and high salt content [17, 38]. Acetic acid is used in products containing fat, meat, fish, vegetables and fruits. Additionally it is widely used in the manufacture of bakery goods [14, 38]. It is effective against the growth of yeast and bacteria

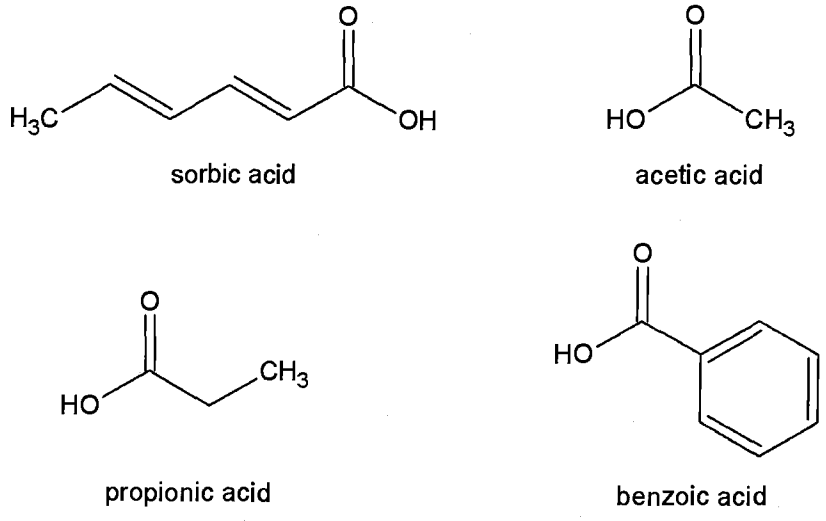

FIGURE 2.1. Chemical Structures of Organic Acids

and against molds to a lesser degree. It has a pK_a value of 4.8 and its minimum inhibitory concentration ranges from 200 to 400 mg/l for bacteria [14, 38].

2.2.2. Sorbic Acid

The antimicrobial activity of sorbates is pH dependent, increasing with acidity. Its inhibitory properties are static and have been linked to the undissociated form of the acid. Sorbates have a higher antimicrobial activity than other organic acids at pH values as high as 5.5. They act synergistically with refrigeration, heat treatment and acids [14, 54]. Sorbic acid enters the cell membrane and inhibits several enzymes necessary for the metabolism of microbes. It is primarily employed against the growth of yeasts and molds. The pK_a of sorbic acid is 4.8

and the minimum inhibitory concentration (MIC) against *Lactobacilli* is 200-700 mg/l [14, 28, 38, 54]. Sorbic acid is widely used in products containing fats, dairy produce, meat, fish, vegetable, fruits and in drinks and bakery goods [38]. It is also used in the pharmaceutical and cosmetic industries [54].

2.2.3. Propionic Acid

Molds, yeast and some bacteria are inhibited by propionic acid and propionates. Like other organic acids, its antimicrobial activity depends on the pH of the system. Propionic acid acts by inhibiting enzymes within the cell. It competes with alanine and other amino acids, preventing microbial growth [14, 38]. Propionates find use mainly in the manufacture of cheese and bakery goods [38]. The MIC range reported for *Lactobacillus plantarum* is 1,000-50,000 mg/l and its pK_a is 4.9 [17, 38].

2.2.4. Benzoic Acid

Benzoic acid and its salts are used mainly as an antimycotic. Like the other antimicrobial organic acids listed here, benzoic acid's inhibitory properties are only present in the undissociated form of the acid [12, 14]. It has been reported to inhibit amino acid uptake in molds and bacteria. It has also been hypothesized that it uncouples substrate transport and oxidative phosphorylation from the electron transport chain. Additionally benzoates can prevent microbial growth through the inhibition of certain enzymes [12, 14, 38]. Sodium benzoate is used in beverages, syrups, margarine, bakery products, preserves,etc. It is also used in

the pharmaceutical and cosmetic industries [12, 14, 38]. Benzoic acid has a pK_a of 4.2 and a reported MIC of 300-1,800 mg/l [38].

2.3. High Pressure Processing

New technologies for processing foods have appeared in recent times in an attempt to fulfill the shortcomings of traditional thermal treatment. Irradiation, pulsed light, pulsed electric fields and HPP are examples of nonthermal processing. The advantages of HPP over other forms of processing are the preservation of organoleptic properties and nutritional content. The result is a 'fresh-like' product that uses a reduced quantity or no chemical preservatives while retaining vitamin content, pigments and flavor compounds. HPP treatment is instantaneous, making scale up simple and straight forward. Although isostatic pressure is homogeneous throughout the vessel, adiabatic heating and cooling creates some temperature gradients that must be taken into consideration when optimizing a high pressure process. The first research on high pressure processing on foods was conducted by Hite in 1899. It was the first demonstration of the capabilities of HPP to inactivate microorganisms in milk. Technological issues limited research and commercial development until the 1980's [4, 9, 26, 28, 56, 57].

High pressure processing is conducted by means of a vessel, a pump and a pressure transferring fluid. The fluid is pumped by an intensifier into the vessel to raise the pressure. For HPP of foods, the fluid is water based and food grade [4, 26, 28, 57]. The most common method of HPP is the batch system where food is placed in a flexible package and then is moved to the high pressure vessel. There exist semicontinuous processes for liquid foods where the food is treated in bulk

and is kept separated from the pressure transmitting fluid in the pressure vessel by means of an isolator. The treated product is sent to an aseptic packaging line after treatment [20, 26].

2.3.1. Applications of HPP

The first commercial applications of high pressure are found in the material science field. Hot isostatic pressing is used in the production of ceramics, steels, superalloys, etc. In this case the pressure transmitting medium is argon gas and the temperatures are much higher (>300°C). High pressure is employed to increase the yield of chemical reactions such as the production of low-density polyethylene (200 MPa, 350°C). High pressure vessels are used to test equipment to be used in high pressure applications such as deep sea research [4]. Apart from applications in the inactivation of microorganisms and denaturation of enzymes, HPP has other interesting capabilities in the freezing and thawing of foods. The phase transition for freezing or thawing can be nearly instantaneous by employing the adiabatic heating and cooling occurring from compression and decompression respectively, combined with freezing point depression under pressure. Uniform ice crystals are the result of homogenous pressure changes and, as a result, phase changes [26, 57]. Adiabatic heating results in a temperature increase of 3°C per 100 MPa increase in pressure for aqueous systems and as high as 9°C per 100 MPa for lipid systems [20].

2.3.2. HPP of Foods

HPP technology consists of subjecting foods to pressures ranging from 200 to 800 MPa. The pressure is transmitted from a pump to a vessel via a water based fluid. The food is packaged in flexible containers before processing. Commercially available products include fruit juices,cured and cooked meats, tomato salsa, oysters, and guacamole. Currently available products treated with HPP also rely on refrigeration for shelf life extension [28, 53, 56].

Certain texture properties of foods can be modified by HPP. Fresh meat can be tenderized in 10 minutes as oppose to the normal two weeks under refrigerated conditions. High pressure is able to modify the structure of starch and protein; the gelation of proteins is possible [4, 26, 57].

2.3.3. Effects of HPP on Microorganisms

It has been reported that yeast and molds are more sensitive to pressure than bacteria. Pressures as low as 200 to 300 MPa are sufficient to inactivate yeast and molds. Gram-positive bacteria are more pressure resistant than gram-negatives. The majority of bacteria can grow under pressures up to 20-30 MPa [4, 28].

Cells lose their mobility under prolonged exposure to high pressure. Species including *E. coli*, *Vibrio*, *Pseudomonas* lose their flagella when subjected to 40 MPa. This loss is reversible in some cases [4].

High pressure can induce the germination of spores. The combination of HPP and high temperature (above 90°C) results in spore inactivation. It is

possible to achieve spore inactivation in a two step process: mild HPP (250 MPa) followed by inactivation of the pressure germinated spores [4, 26].

The degree of microbial inactivation achieved by HPP is dependent on factors including temperature, pH, composition, water activity and osmotic pressure. The more acidic or alkaline the system is, the more effective HPP. Mineral salt solutions and nutrient media appear to protect cells from inactivation by HPP. There is a synergistic effect of pressure and temperature. Increased efficiency of HPP is observed at higher temperatures. Low water activity increases bacterial resistance to HPP [4, 56].

The mechanism of microbial death is not well defined, however, it is believed to be due to enzyme denaturation and cell membrane disruption. HPP denatures cell proteins and enzymes that affect the uptake of nutrients by causing conformational changes in the quaternary and tertiary structures of proteins. The cell membrane is affected by the denaturation of proteins and enzymes. HPP reduces the size of phospholipids in the membrane. The permeability increases causing the leakage of contents. The original permeability of the membrane can be restored if the pressurization conditions are mild enough [4].

2.3.4. Effects of HPP on Enzymes

The inactivation of enzymes by HPP is highly dependent on the composition of the food. pH, temperature, and the type of enzyme influence the efficacy of enzyme inactivation . HPP has been found to inactivate myrosinase, lipoxygenase, pectinases, alkaline phosphatase, polyphenol oxidase, peroxidase, and lactoperox-

idase [26]. However HPP can increase the activity of polyphenol oxidase five times in pears but not so in apples, bananas or sweet potatoes [4].

Bacterial enzymes can be activated or inactivated by HPP. *E. coli* dehydrogenases activity is lowered after exposure to high pressure. On the other hand, aspartase and cellulase activity is enhanced. High pressure increases the activity of proteolytic enzymes in meat. The inactivation of certain enzymes is reversible, depending on the extent of distortion of the molecule [4].

2.3.5. Mathematical Modeling of Microbial Inactivation by HPP

New technologies for inactivation of microorganisms are compared to thermal processing as a way of assessing their efficacy. Originally, the thermal processing parameters (D, z, F values) were applied to HPP survivor curves in an attempt to standardize the measurement of effectiveness and microbial resistance to pressure [20, 21].

Traditionally the thermal inactivation of microbes has been approximated as a first order kinetics phenomena with respect to time. The plot of the logarithm of survivors against time often follows a linear relationship:

$$\frac{dS(t)}{dt} = -k \cdot S(t) \tag{2.1}$$

where S is the survivor ratio, N/N_0, and k is a rate constant. Equation 2.1 simplifies to:

$$logS(t) = -k \cdot t \tag{2.2}$$

The concept of decimal reduction time, D value, evolved from this linear approximation of survivor plots. It is defined as the time necessary to obtain 1

log reduction in a bacteria culture at a specified temperature. Similarly the z and F values are based on the log-linear approximation as well. These parameters are widely used in the food industry to measure microbial resistance to heat, to assess overall inactivation, and to compare thermal processing regimes. The first-order kinetics approximation has proven itself to be useful in establishing a standard measurement of thermal resistance of microbes. D and Z values allow for comparison between organisms or different food matrices readily permitting adjustment of processing parameters to achieve a target decimal reduction in microbial load [10, 28, 29, 31, 34, 47].

In recent years there has been some challenges to the traditional first-order kinetics approach to thermal inactivation of microbes. The main criticisms can be summarized as first order approximations being overly simplistic. Its parameters are commonly extrapolated several orders of magnitude. The end result is the over processing of foods and hence, a reduction in quality. The first-order approximation performs poorly at describing the numerous examples of deviation from linearity in the literature, not only present in thermal processing but also in nonthermal technologies. For these reasons, the first-order model does not serve well for comparing thermal processing to novel techniques such as HPP [10, 34, 47].

Non first-order inactivation patterns have been explained as the result of a mixture of microbial subpopulations, each following varying first-order mortality kinetics. Peleg *et al.* [45–48] have proposed a non-kinetic mathematical model to describe microbial inactivation. They explain microbial death as a result of a temporal distribution of lethal events. The distributions are not limited and

can be narrow, symmetrical, asymmetrical,skewed, bimodal, etc. They concluded that thermal inactivation curves could be described by a Weibull distribution:

$$logS = -(t/b)^n \tag{2.3}$$

where t is time and n and b are temperature dependent parameters which represent the shape and scale factor of the distribution respectively. The mean ($\bar{t}_c$) and the variance (σ^2_{tc}) are defined as follows [46]:

$$\bar{t}_c = -b \cdot \Gamma[1 + 1/a] \tag{2.4}$$

$$\sigma^2_{tc} = b^2\{\Gamma[1 + 2/a] - (\Gamma[1 + 1/a])^2\} \tag{2.5}$$

$$logS = -b(T) \cdot t^{n(T)} \tag{2.6}$$

Other approaches have been taken to explain non first-order kinetics. A number of more complex mathematical models have been successfully employed to describe asymmetrical sigmoidal microbial growth curves [2, 3, 8, 37]. The following models are frequently cited in literature to describe microbial death behavior as well: (1) the log-logistic model, a four parameter equation, assumes that microorganisms within a population exhibit a range of resistances to a lethal agent [11, 36]; (2) the three parameter modified Gompertz equation is able to describe survivor curves with lag phases, tailing, and linear behaviors [11, 34, 35]; (3) the complex three parameter Baranyi model is capable of appropriately fit non-linear microbial death responses at suboptimal conditions [52].

2.4. pH and Buffers

Acidity plays an important role in the growth and survival of microbes. Microorganisms have an optimum pH for growth but can survive in a organism-

specific range of acidities [17, 39]. Acidity affects the energy metabolism of microbes by increasing the concentration of hydrogen ions and creating gradients across membranes. Low pH also inhibits enzyme activity and the stability of macromolecules [39].

The efficiency of various food preservation techniques is directly affected by pH [4]. pH is commonly kept between 3 and 5 in food processing and it is used as a 'hurdle' with other preservation techniques such as water activity, oxygen availability, preservatives and pasteurization [39].

2.4.1. pH Basics

The Lowry-Brönsted definition of an acid is a substance that tends to donate hydrogen ions whilst a base is a substance that accepts hydrogen ions. Lewis further expanded the definition by identifying acids as those substances that can accept electrons and bases as those capable of donating electrons [6, 49]. pH is determined by the activity of hydrogen ions. In dilute solutions the activity approximates the concentration of hydrogen ions [6, 39, 49]:

$$pH = -log_{10}[H^+] \tag{2.7}$$

Measurement of acidity, or alkalinity, is performed using the pH scale devised by Sörensen where neutrality is defined as pH 7, acids are pH values lower than 7 and alkalis are higher than 7 [6]. The origin of the scale is based on the slight dissociation of pure water:

$$H_2O \rightleftharpoons H^+ + OH^- \tag{2.8}$$

The equilibrium constant, K, has a value of $1.8\text{x}10^{-16}$ M at 25°C.

$$K = \frac{[H^+] \cdot [OH^-]}{[H_2O]} \tag{2.9}$$

If we assume an excess of water in solution, its concentration [H_2O] can be approximated to 55.56 M, the value for pure water. The expression can be simplified to:

$$K_w = K \cdot [H_2O] = [H^+] \cdot [OH^-] = 1 \times 10^{-14} M^2 \tag{2.10}$$

Therefore a neutral system containing the same concentration of H^+ and OH^- would have a pH value of 7 (-$\log_{10}[10^7]$); higher concentrations of H^+ result in pH values below 7 and lower concentrations correspond to pH values above 7. In reality the hydrogen ion does not exist in aqueous solutions. Hydrogen ions react readily with water molecules to form hydronium ions: H_3O^+. This deviation does not affect the calculations presented above [6, 49].

2.4.2. Buffers

Buffers are defined a substances that resist pH change when acid or alkali is added to the solution [49]. The buffering activity is due to the presence of a weak acid and its conjugated base (Equation 2.11, or the presence of a weak base and its conjugated acid (Equation 2.12):

$$HA + H_2O \rightleftharpoons H_3O^+ + A^- \tag{2.11}$$

$$BH^+ + H_2O \rightleftharpoons H_3O^+ + B \tag{2.12}$$

The dissociation constant of an acid is a measure of its strength; a strong acid will have a high K_a value, meaning that there will be more dissociation into A^- and H_3O^+:

$$K_a = \frac{[H^+] \cdot [A^-]}{[HA]} \tag{2.13}$$

Taking the decimal logarithm of both sides of Equation 2.13 we obtain the following expression:

$$-log_{10}[H^+] = -log_{10}K_a + log_{10}\frac{[A^-]}{[HA]} \tag{2.14}$$

Which simplifies to the Henderson-Hasselbalch equation [6, 49]:

$$pH = pK_a + log_{10}\frac{[A^-]}{[HA]} \tag{2.15}$$

When the pH is equal to the pK_a, the concentration of acid is equal to the concentration of the conjugated base. Addition of acid or base will shift that equilibrium. Buffers have the capacity to prevent abrupt changes in pH by acting as a sink or as a source of H^+ . Weak acids and bases act as buffers when the pH is at or near their pK_a. The further away the pH is from the pK_a, the lower the buffering effect of the weak acid or base. Buffers have varying degrees of effectivity against changes in pH. The buffering capacity (β) measures the ability to resist changes in pH [6]:

$$\beta = 2.303 \cdot \frac{K_a \cdot C_t \cdot [H^+]}{(K_a + [H^+])^2} \tag{2.16}$$

where C_t is the total concentration of acid and base. From Equation 2.16 it can be concluded that the buffering capacity depends on the K_a, the pH and the concentration of the buffer [6].

The ionic strength of the solution, I, also affects the buffering action of weak acids. It describes the overall ionic properties of a solution and it is defined by Equation 2.17 [6, 49].

$$I = \frac{1}{2} \cdot \sum(c_i \cdot z^2) \tag{2.17}$$

where c_i is the concentration of a particular ion and z is its charge. Ionic strength affects the pK_a of the buffer. Addition of a salt to a solution will alter the pK_a in the following manner:

$$pK'_a = pK_a + (2 \cdot z_a - 1) \cdot [\frac{A \cdot \sqrt{I}}{1 + \sqrt{I}} - 0.1 \cdot I] \tag{2.18}$$

where pK_a' is the modified pK_a value, z_a is the charge on the conjugated acid species and A is a temperature dependent constant approximately equal to 0.5 [6].

2.4.3. Good's Buffers

In 1967, Good *et al.* [22] published a description of a series of buffering substances for use in biological experiments. The buffers were aminoethane and aminopropane sulfonic acids such as ACES (N-(2-acetamido)-2-aminoethanesulfonic acid), HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane-sulfonic acid), and MES (2-morpholinoethanesulfonic acid). These substances have anionic and cationic sites (zwitterionic) that makes them very water soluble. Good's buffers are especially indicated for biological environments due to their pK_a lying within the physiological pH range (pH 6-10). They are also highly soluble in water, their cell membrane permeability is low, they have low metal chelating capability and high chemical stability, they are non-toxic, and they do not interfere with biological processes [16, 22, 23, 51]. They are also insensitive to the pressure driven dissociation due to their positive values of $\Delta V°$ (Table 1.1).

2.5. pH and High Pressure Processing

Pressures of several thousand bar reduce the volume of water. For example water can be compressed 15% at 6000 bar (600 MPa). It follows that those reactions that involve a increase or a decrease in volume are affected by high pressure [4, 20].

High pressure alters the pH of the medium as seen in deep sea research where the pH of sea water is 8.10 at 1 atm but drops to 7.87 at a pressure of 1,100 atm [4].

Kitamura *et al.* [30] describe a calculation for the pressure dependency of pH from a known reaction volume value. pK_a is pressure dependent as can be seen in the following equation:

$$\frac{dpK_a}{dP} = -\frac{\Delta V^o(P)}{R \cdot T} \tag{2.19}$$

where $\Delta V^o(P)$ is the pressure dependent reaction volume at infinite dilution of the dissociation reaction described in Equation 2.11, R is the ideal gas constant, and T is temperature. For pressures below 8,000 bar, Equation 2.19 can be integrated as follows:

$$(pK_a)_P - (pK_a)_L = -\frac{P \cdot \Delta V^o(P)}{R \cdot T \cdot (1 + b \cdot P)} \tag{2.20}$$

where ΔV^o is the reaction volume at atmospheric pressure and b is equal to $9.2X10^{-5}$ bar^{-1}. Contributions from activity coefficients are negligible. The subscript P indicates conditions at pressure and subscript L refers to atmospheric conditions. The change in pH at pressure can be estimated:

$$(pH)_P - (pH)_L = -\frac{1.75X10^{-5}\Delta V^o P}{1 + 9.2X10^{-5}P)} - 354.3(2z-1)\sqrt{I}[(\sqrt{\frac{\rho}{\varepsilon^3}})_P - (\sqrt{\frac{\rho}{\varepsilon^3}})_L] \tag{2.21}$$

where ρ is the density of water, ε is the dielectric constant of water, I is the ionic strength of the solution.

Electrostriction plays an important role in the pressure dependency of pK_a and pH change. The concept of electrostriction refers to the contraction in volume that occurs when an ion of certain charge and radius is immersed from a vacuum into a dielectric [33]. As pressure increases water is more densely packed around ions than around undissociated molecules and there is a decrease in volume. This phenomenom occurs when acids dissociate, which in turn reduces the pH of the solution [15, 24, 25, 30, 55].

In addition to changes in the dissociation constant of acids, the activity coefficient is also affected by high pressures. The two phenomena affect the change in pH under high pressure. For dilute solutions at atmospheric conditions, the activity of hydrogen ions can be approximated to be equal to the concentration of H^+. Ions in solution interact with other ions. The result is that ions behave as if their concentration were lower. The activity of the ion is defined as follows [6, 55] :

$$a_i = \gamma \cdot c_i \tag{2.22}$$

where γ is the activity coefficient and c_i is the concentration [55].

It was found that the protonation of zwitterions was negligible except for HEPPS (N-2-hydroxyethylpiperazinepropanesulfonic acid). Zwitterions (Good's buffers) have a reduced sensitivity to pressure induced pH shifts.

Technological hurdles prevent the direct measurement of pH under pressure. A method of pH measurements for pressures up to 250 MPa was published by Hayert *et al.* [24]. It involved measurement of pH by means of the fluorescent

compound fluorescein through a small volume sapphire windowed pressure cell. This compound exhibit fluorescence at different wavelengths depending on pH. Spectra at wavelengths ranging from 300 to 660 nm was recorded and correlated to pH. The shift of pH under pressure (200 MPa) was demonstrated for a variety of substances including water (ΔpH=-0.31) and orthophosphate (ΔpH=-0.92), acetate (ΔpH=-0.40), and potassic buffers (ΔpH=-0.16).

Stippl *et al.* [55] recently published a procedure for monitoring pH under high pressure. The method is based on a mixture of 16 indicator dyes that change color as a response to pH. They make use of a sapphire window and a spectrometer to measure changes in color. The system is able to measure pH at a maximum pressure of 450 MPa with an accuracy of 0.24 pH units. Interactions between indicator dyes and food ingredients as well as the effect of temperature were not taken into account.

3. MATERIALS AND METHODS

3.1. Bacteria Cultures

Two bacteria were used in this project. The first one, *Escherichia coli* K12, was donated by Dr. Mark Daeschel, Department of Food Science and Technology, Oregon State University, Corvallis, OR. The culture was kept on a trypticase soy agar slant (BBL^{TM} Trypticase^{TM} Soy Broth and Bacto^{TM} Agar, Becton, Dickinson and Company, Sparks, MD). The second culture was *Lactobacillus plantarum* ATCC 8014, obtained from the American Type Culture Collection, Manassas, VA. It was kept on De Man, Rogosa, and Sharpe agar slants (Lactobacilli MRS Broth, EMD Chemicals, Inc., Darmstadt, Germany and Bacto^{TM} Agar).

For each experimental replicate, a single colony of a bacterium was picked from the agar slant and transferred to a test tube containing 20 ml of the appropriate media. The culture was incubated for 24 hours at 35°C. After the incubation period, 0.2 ml of culture was transferred to a second 20 ml tube and incubated for 24 hours under the same conditions. The cultures were then centrifuged at 7,000 RPM for five minutes at 2°C by use of a Sorvall Superspeed RC2-B apparatus (Kendro, Asheville, NC). The supernatant was aseptically removed and the bacteria were resuspended in sterile filtered water (Milli-Q, Millipore, Billerica, MA). After a second centrifugation under the same conditions, they were resuspended in the appropriate buffer solution.

Pressure processing of the cultures were performed by transferring 3 ml of the cultures to polyethylene bags made of 2 millimeter tube stock (J.C. Danczac, Inc., Westfield, MA). The bags were sealed by use of an impulse sealer (TEW

Electric Heating Equipment Co., Ltd., Taipei, Taiwan) and then placed on ice until pressure treatment.

The bacteria population of the cultures and survivors was enumerated by using an Autoplate 4000 (Spiral Biotech, Inc., Norwood, MA). The plates were made with the appropriate media for each bacterium, as indicated above. The plates were incubated at 35°C. *Escherichia coli* K12 was incubated for 24 hours before counting while *Lactobacillus plantarum* ATCC 8014 was incubated for 48 hours due to its slower growth.

3.2. Buffer Preparation

All the buffers utilized during this study were 0.09 M strength. They were made by dissolving the specific amount of each acid in Milli-Q water and then adjusting pH by addition of 5 N sodium hydroxide (VWR International, West Chester, PA).

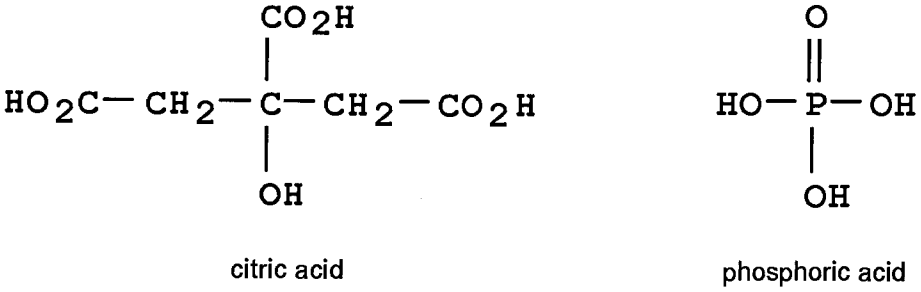

FIGURE 3.1. Chemical Structures of Citric and Phosphoric Acid Buffers

The study of pH shift pressure sensitivity employed a series of buffers: HEPES, sodium salt (sodium N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate;

MES (2-(N-morpholino)ethane-sulfonic acid, monohydrate); ACES (N-(2-acetamido)-2-amino-ethanesulfonic Acid); citric acid, anhydrous (OmniPur, EM Science, Darmstad, Germany); and phosphoric acid (EMD Chemicals Inc., Darmstadt, Germany) (Figure 3.2 and 3.1). In the case of HEPES, sodium salt, the pH was adjusted by titrating with sodium hydroxide or 1 M hydrochloric acid (J.T. Baker Mallinckrodt Baker, Inc., Phillipsburg, NJ) as described by Kitamura *et al.* [30].

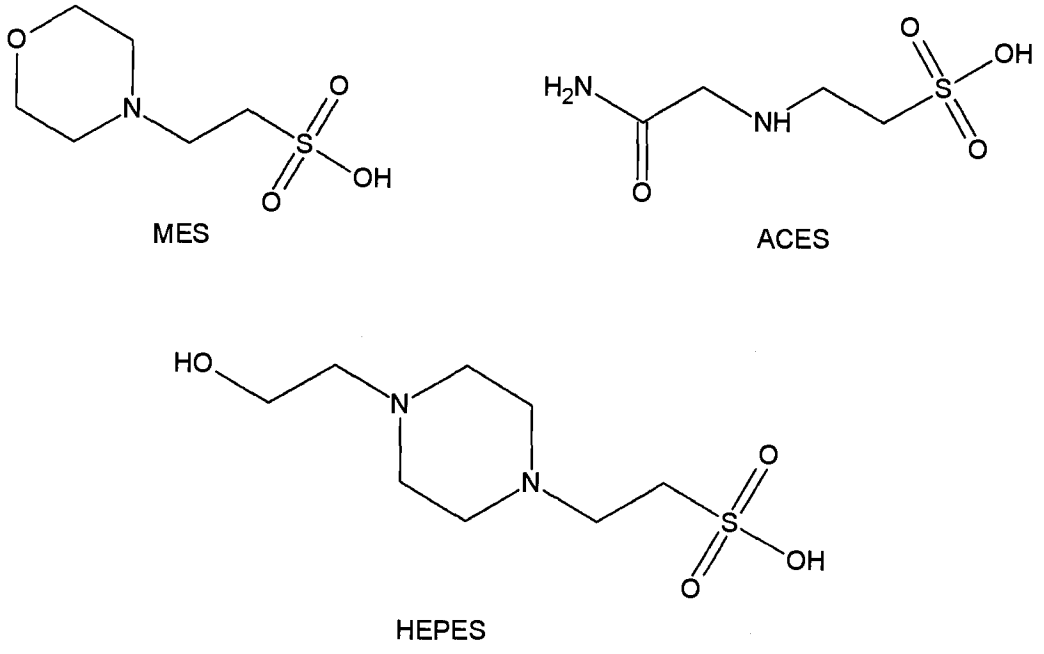

FIGURE 3.2. Chemical Structures of Good's Hydrogen Ion Buffers

The study of the interaction of antimicrobial acids and pH shift made use of citrate buffer (0.09 M) with organic acids added at two levels, high and low (within and below minimum inhibitory concentrations, Table 3.2). The antimicrobial organic acids were propionic acid (Mallinckrodt AR, Mallinckrodt Baker,

Inc., Paris, KY); acetic acid (EM Science, Darmstadt, Germany); sorbic acid (J.T. Baker Mallinckrodt Baker, Inc., Phillipsburg, NJ); and benzoic acid (EMD Chemicals Inc., Darmstadt, Germany). The antimicrobial organic acids were added at the specified concentrations before adjusting the pH by addition of 5 N sodium hydroxide.

3.3. High Pressure Equipment

The experiments were conducted using a 20 l high pressure vessel (National Forge Company, Irvine, PA) in conjunction with a 40 HP Flow International 7X-6000 intensifier (Flow International Corp., South Kent, WA).

3.4. Experimental Design

The pressure sensitive buffers selected for this study were phosphate and citrate, while insensitive buffers were the following sulfonic acids: MES, ACES and HEPES in sodium salt form, see Table 1.1. All treatments were one minute in length and approximately 25°C at pressure. The conditions of pH values 5, 6, and 7 were treated at the same pressure (580 MPa), the maximum operating pressure of the equipment used. The pressures were chosen to cause a population reduction less than 8 log units, to permit comparison between pressure sensitive and insensitive conditions. The pressure treatments were selected based on the acidity of the system to yield a level of inactivation that would allow differentiation between buffers, e.g. pressure treatment at lower pH was more effective than at neutral pH, thus lower pressures were used for high acidity buffers. Processing conditions are listed in Table 3.1. Three replicates of each combination of

organism, pH and buffer were conducted. The replicates were pressured treated in a randomized fashion. The bags containing the cultures were kept on ice until treatment.

The HPP system utilized in the experiments lacks *in situ* temperature measurement and control capabilities. The temperature of the cultures at pressure was controlled by isolating the bags from the vessel by placing them inside a 1 l plastic screw top bottle filled with water. The temperature of water increases 3°C per 100 MPa increment [20], therefore the water temperature in the bottle was adjusted accordingly to compensate for adiabatic heating. The bottle was then placed inside a nylon bag filled with water that was temperature adjusted as well. The target temperature at all pressures was 25°C. The temperature of the water inside the bottle was measured before and after processing to estimate the temperature at pressure. The bags were placed on ice immediately after processing.

TABLE 3.1. Pressure Sensitivity Study Experimental Conditions

Buffer Type	pH 3 (350 MPa)	pH 4 (450 MPa)	pH 5 (586 MPa)	pH 6 (586 MPa)	pH 7 (586 MPa)
Pressure Insensitive	HEPES	HEPES	MES	MES ACES	ACES
Pressure Sensitive	citrate phosphate	citrate	citrate	citrate phosphate	phosphate

The second section of this project studied the possible interactions between weak organic acids with known antimicrobial properties and the pressure induced pH shift. The lower acidity reached at pressure may induced a higher concentration of protonated organic acid, the form with the antimicrobial activity.

Lactobacillus plantarum ATCC 8014 was grown and resuspended in citrate buffer containing an antimicrobial organic acid. The antimicrobials were used at two different concentrations, one within the published minimum inhibitory concentrations (MIC) and one below it. The antimicrobials were used at two pH levels, a pH equal to the pK_a of the acid and a pH lower than the pK_a. When the pH is the same as the pK_a of the acid, there are equal concentrations of the protonated and the dissociated forms in solution (see Equation 2.15). The further the pH of the system is below the pK_a of the organic acid, the higher the proportion of the undissociated form in solution.

TABLE 3.2. Antimicrobial Experimental Conditions

Antimicrobial	pK_a	MIC (mg/l)	Low (mg/l)	High (mg/l)	pH-1	pH-2
Benzoic	4.2	300-1,800	150	1,500	3	4.2
Acetic	4.8	200-400	100	400	3	4.8
Sorbic	4.8	200-700	100	500	3	4.8
Propionic	4.9	1,000-50,000	150	1,500	3	4.9
Control	-	-	0	0	3	4.2, 4.8, 4.9

3.5. Data Analysis

3.5.1. Buffer Pressure Sensitivity

The level of bacterial inactivation achieved when cultures were treated in pressure sensitive buffer was compared to that of the pressure insensitive buffer for all the treatment conditions. Results were reported as bacterial population reduction in logarithm units. Three replicates of each treatment were conducted. More

replicates were conducted if there were data points suspected to be outliers. A two-sample t-test, assuming equal variance, was calculated using Microsoft Excel to assess differences between pressure sensitive and insensitive buffers. Since the efficacy of HPP increases with decreasing pH, the processing pressures changed for the pH conditions (Table 3.1). Only results within the same processing conditions of pressure and pH could be compared.

3.5.2. Antimicrobials

The degree of bacterial reduction was calculated as the decimal logarithm of survivors: $\log(S) = \log(N/N_0)$, where N_0 was the original bacterial count of the culture (CFU/ml) and N was the bacterial population after treatment. The Weibull equation (Equation 3.1) was fitted to survivor versus pressure data using NLREG [43].

$$log(S) = -\frac{1}{a} \cdot P^b \tag{3.1}$$

The coefficients a and b calculated by NLREG were used as starting values for statistical comparison using the PROC NLINMIXED in SAS [50].

4. RESULTS AND DISCUSSION

4.1. Pressure Induced pH Shift Sensitivity

Weak acids act as buffers when the pH of the system is at or near its pK_a. The dissociation constant of weak acids is pressure dependent and can shift with pressure changes. The dependency of the pK_a on pressure is dictated by its reaction volume ($\Delta V°$) (Equation 2.19), a measurement of the degree of electrostriction or volume change caused by a increase in pressure. It follows that pH drops as a result of an increase in the dissociation of weak acids under pressure [15, 24, 25, 30, 33, 55]. Therefore pressure can temporarily reduce acidity and increase the efficacy of HPP, see Section 2.1.2. This study aims to establish the effect of pressure induced pH shifts on the inactivation of microorganisms by HPP. A reduction of pH would manifest itself as an increase in microbial inactivation.

Buffers were chosen based upon their pK_a and $\Delta V°$ values. Negative$\Delta V°$ indicates a tendency towards dissociation under pressure, releasing free hydrogen ions into the solution and thus increasing acidity. The closer the $\Delta V°$ to 0, the less pressure sensitive the buffer. *Lactobacillus plantarum* ATCC 8014 (gram-positive) and *Escherichia coli* K12 (gram-negative) were pressure treated in sensitive and insensitive buffers.

Since weak acids can only buffer when the pH of the system is near their pK_a value, some constraints existed. Only buffers of similar pK_a could be compared to each other. Furthermore, lowering the pH of the system can significantly reduce the pressure needed to inactivate microorganisms thus pressure treatments near neutrality required higher pressures than high acidity treatments.

Pressure sensitive and pressure insensitive buffers of similar pK_a values were compared against each other (Table 3.2). The cultures were suspended in the selected buffers and treated at pressures that would result in population reductions between one and eight log units. While higher levels of inactivation were possible, they lay outside the detection limit of the enumeration technique used.

Experimental data from the pH shift sensitivity study are presented in Figures 4.1 and 4.2. The inactivation of the bacteria is reported as population reduction in logarithm units. It was observed that lower pH values required a lower pressure to achieve the same degree of inactivation for both bacteria species. This point corroborates the observation that higher acidity increases the efficacy of HPP.

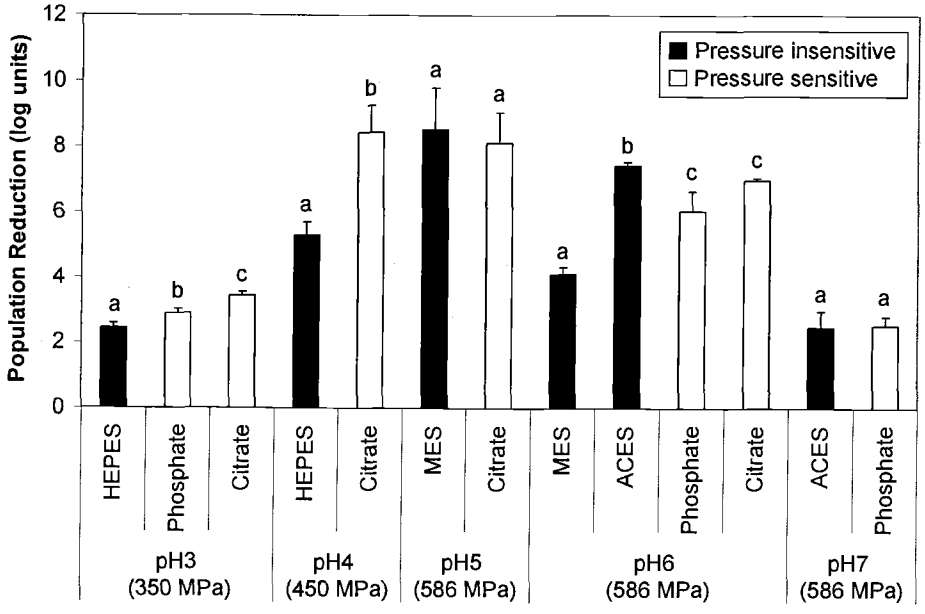

FIGURE 4.1. Reduction of microbial population of *L. plantarum* ATCC 8014. Subscripts with different letters are significantly different ($p<0.05$)within each pH increment; error bars represent +1 standard deviation, n=3-6

At pH 3 and 4 cultures of *L. plantarum* ATCC 8014 and *E. coli* K12 were more susceptible to HPP when treated in pressure sensitive buffer. Cultures of *Lactobacillus plantarum* suspended in citrate buffer were more sensitive to pressure than cultures treated in phosphate buffer. This effect was not observed in the case of *E. coli* and there were no significant differences between the two pressure sensitive buffers at pH 3. The inactivation of *E. coli* in citrate buffer was higher than in HEPES, however there were no differences between phosphate and HEPES. The results demonstrate the impact of the nature of the buffering agent in the inactivation of microorganisms. Even though the buffers were equal in acidity, there were significant differences in the level of inactivation attained that can be attributed to the type of buffer and in turn to a shift in pH.

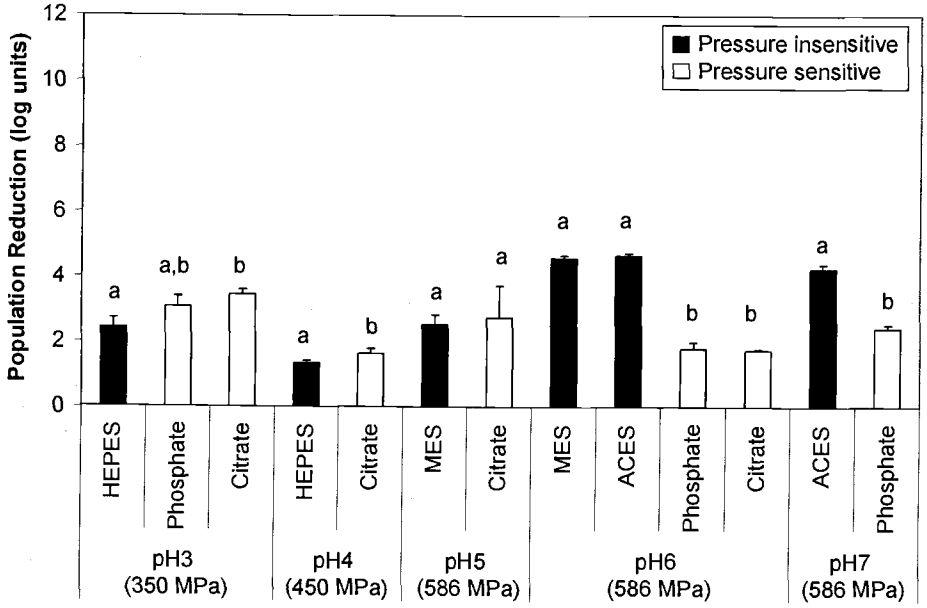

FIGURE 4.2. Reduction of microbial population of *E. coli* K12. Subscripts with different letters are significantly different ($p<0.05$) within each pH increment; error bars represent +1 standard deviation, n=3-6

At pH 5 neither bacteria demonstrated significant differences between pressure sensitive and insensitive buffers. In the case of *Escherichia coli* K12, pressure insensitive ACES (pH 6 and 7) and MES (pH 6) demonstrated a higher degree of inactivation than citrate and phosphate. *Lactobacillus plantarum*on the other hand, was most susceptible to ACES at pH 6, followed by the pressure sensitive buffers. The lowest inactivation level was observed when it was treated in MES. At pH 7 there were no differences between ACES and phosphate. Overall, there were small differences in lethality observed at the higher pH conditions. This can be attributed to the lesser impact of a pH change near neutral pH conditions, i.e. a small shift in pH would not bring the acidity of the system to adverse conditions for the bacteria and thus similar inactivation levels would be expected.

The nature of a buffer plays an important role in the efficacy of HPP and should be taken into consideration when designing HPP experiments. In commercial applications, food developers could make use of the more lethal conditions that exist under pressure as a result of a shift in pH. Shorter treatment times or lower pressures would lead to lower processing conditions as the life of seals and other equipment would be extended. Additionally a food product could be formulated at a higher pH to take advantage of the pH shift, achieving the same degree of microbial inactivation as a more acidic product. The food system would recover the original acidity after processing.

Escherichia coli K12 and *Lactobacillus plantarum* ATCC 8014 showed similar resistance to pressure at pH 7 / 586 MPa and pH 3 / 350 MPa. The other conditions studied demonstrated a higher resistance for *Escherichia coli* K12. *Lactobacillus plantarum* ATCC 8014 was expected to have a higher resistance to HPP

since it is gram-positive, see Section 2.3.3. The discrepancy is possibly due to the particular strain of *Lactobacillus plantarum.*

4.2. pH Shift and Antimicrobials

As was demonstrated earlier, microbial inactivation under pressure is influenced by the type of buffer used and the organism's sensitivity to pressure. pH shifts under high pressures increase the acidity of the system leading to an increase in inactivation. The use of organic acids with known antimicrobial properties could lead to an improvement in the efficacy of HPP. The undissociated form of organic acids has been demonstrated to have antimicrobial activity, while the dissociated form does not. It has been postulated that the lower polarity facilitates transport of the protonated acid across the cell membrane. Once inside, the acid inhibits enzymes and transport across the membrane, see Section 2.2. Antimicrobials are used in combination with other preservation methods as a hurdle to improve overall microbial inactivation. The higher activity of the protonated form indicates the important role of acidity in the efficacy of organic acids as antimicrobials. When the pH of the system is equal to the pK_a of the acid there are equal proportions of undissociated and dissociated acid. As the pH is lowered, there is more acid in the undissociated form and less in the protonated form. Thus pH and pK_a dictate the extent of dissociation of the organic acid and its efficacy as an antimicrobial.

The second section of the project examines the possible interactions of pressure induced pH shift and the antimicrobial activity of weak organic acids. A hypothesis being proposed for this section is that the lower acidity under pressure

may shift the equilibrium of the antimicrobial acid increasing the concentration of the protonated form of the acid, and thus the larger concentration of protonated antimicrobial would result in an increase in efficacy of HPP. Antimicrobials were added to a pressure sensitive buffer at two concentration levels, one below the reported MIC and one within. *Lactobacillus plantarum* ATCC 8014 was used to compare the inactivation levels achieved by the presence of added antimicrobials and a control buffer solution.

To study the effect of pH shift, the pressure treatments were performed at two pH levels. One was treated at pH 3, a condition acidic enough that the majority of the acid would be in the undissociated form. The second condition was at pH equal to the pK_a of the acid, where half of the acid is in the dissociated form at atmospheric conditions.

The data are presented in Figures 4.4 - 4.10. The logarithm of the ratio of survivors (log S) is plotted against pressure. The data were fitted with a Weibull equation (Equation 2.3) using the software package NLREG. The PROC NLMIXED procedure in SAS was used to compare the parameters of the Weibull equation to assess differences from control.

The Weibull equation does not explain tailing nor estimates maximum overall inactivation. More complex non-linear mathematical models are capable of these estimates (refer to Section 2.3.5). These sigmoidal mathematical expressions obtain higher R^2 values due to the combination of low number of data points per set and higher number of parameters in the equation. However, the simpler two-parameter Weibull equation more appropriately described the inactivation data recorded in large part because sigmoidal behavior was not observed in the data.

$$logS = -(1/a) \cdot (P)^b \tag{4.1}$$

The parameters a and b from the Weibull equation can be used to calculate the required pressure for a specified number of population reduction in logarithm units. For example the pressure in MPa needed for 1 log reduction is the b^{th} root of parameter a: $a^{1/b}$. Similarly, the pressure needed for 5 decimal reductions would be $(5 \cdot a)^{1/b}$.

The two levels of each antimicrobial were plotted along with a control where no antimicrobial was added. The acidity of the system clearly had an important effect on the efficacy of HPP, with lower pH values required less pressure to achieve the same degree of microbial inactivation.

The parameter b and the pressure needed for a 1 log reduction for each condition were compared to the control curve as the probability of difference occurring by chance. The results are listed in Tables 4.1 - 4.4. At pH 3, there were no significant differences (NSD) from control for any of the antimicrobials (Table 4.1, Figures 4.4 - 4.3). It appears that the acidity at pH 3 may have a larger impact on microbial inactivation than the presence of an antimicrobial acid, granting all conditions equally effective at this pH level.

At pH 4.2, only the low level (150 ppm) of benzoic demonstrated any difference from control about the $a^{1/b}$ parameter, 15 MPa lower than control ($p=0.0837$), see Table 4.2.

Acetic acid did not have an effect at pH 4.8. Sorbic acid, however, demonstrated a strong difference from control for both parameters at the low concentration ($p<0.05$). At the high level, there were a strong difference from control for b ($p<0.05$) but not so for $a^{1/b}$($p=0.0506$). There were no significant differences at pH 4.9 for propionic acid, see Table 4.4 and Figure 4.10.

For each antimicrobial the results from high concentration were compared to the parameters from the low concentration conditions within the same pH. The results are summarized in Table 4.5. There were no significant differences found between high and low concentrations for any of the antimicrobials except for propionic acid.

Even though the statistical analysis did not show significant differences for most of the conditions (other than sorbic and benzoic acids), it can be seen from the survivor plots that the presence of the antimicrobial had an impact on the shape of the curve. When the pH was equal to the pK_a the processing pressure required to reach a 5 decimal population reduction was reduced in all instances where organic acids were added. This implies a reduction of microbial resistance in the presence of the antimicrobial organic acids. It is possible that no differences were found either due to the low number of observations or to the rapid drop in bacterial inactivation in the 400 to 500 MPa range. The b coefficient is a measure of how much pressure increase is needed for a change in inactivation. It was observed that the addition of antimicrobial when pH was equal to pK_a increased the value of b substantially with the exception of propionic acid at 150 ppm (Table 4.2 - 4.4). Concentration, on the other hand, appeared to have a minimal, if any, impact on inactivation. Both high and low concentrations had similar effects except for the case of propionic acid. The effect of antimicrobial presence may have a upper limit above which there ceases to be an increase in inactivation with higher concentrations of antimicrobials. More intriguing was the fact that levels below the MIC produced significant results.

The lack of statistical difference from control may also arise from the acids themselves undergoing dissociation under pressure due to electrostriction effects

as described in Section 2.5. The increase in pH could have a lesser effect than electrostriction under high pressure. The dissociated organic acids would not have an antimicrobial effect and thus no differences would be observed from control.

TABLE 4.1. Weibull Parameters at pH 3

Antimicrobial	(ppm)	a	b	Difference from control	$a^{(1/b)}$	Difference from control	R_a^2
control	0	1.17E+20	8.304	-	261	-	0.9130
acetic	100	7.01E+23	9.891	NSD	258	NSD	0.8997
acetic	400	1.97E+14	5.950	NSD	253	NSD	0.7805
sorbic	100	1.12E+23	9.573	NSD	256	NSD	0.9948
sorbic	500	5.94E+22	9.450	NSD	257	NSD	0.9897
propionic	150	8.27E+24	10.242	NSD	271	NSD	0.9958
propionic	1500	3.10E+24	10.056	NSD	273	NSD	0.9943
benzoic	150	5.45E+23	9.852	NSD	257	NSD	0.9934
benzoic	1500	4.17E+22	9.424	NSD	251	NSD	0.9988

TABLE 4.2. Weibull Parameters at pH 4.2

Antimicrobial	(ppm)	a	b	Difference from control	$a^{(1/b)}$	Difference from control	R_a^2
none	0	3.38E+22	8.780	-	368	-	0.9964
benzoic	150	5.64E+25	10.107	NSD	353	0.0837	0.9732
benzoic	1500	2.82E+26	10.390	NSD	351	NSD	0.8689

TABLE 4.3. Weibull Parameters at pH 4.8

Antimicrobial	(ppm)	a	b	Difference from control	$a^{(1/b)}$	Difference from control	R_a^2
none	0	8.97E+18	7.332	-	384	-	0.9657
acetic	100	3.47E+28	10.963	NSD	401	NSD	0.9754
acetic	400	2.77E+30	11.685	NSD	403	NSD	0.9423
sorbic	100	8.21E+42	16.359	0.0147	420	0.0220	0.9913
sorbic	500	1.43E+39	14.960	0.0184	414	0.0506	0.9895

TABLE 4.4. Weibull Parameters at pH 4.9

Antimicrobial	(ppm)	a	b	Difference from control	$a^{(1/b)}$	Difference from control	R_a^2
none	0	3.79E+17	6.712	-	416	-	0.9513
propionic	150	2.36E+16	6.365	NSD	374	NSD	0.9436
propionic	1500	2.12E+31	11.936	NSD	421	NSD	0.9895

TABLE 4.5. Comparison of High and Low Antimicrobial Concentration Levels

Antimicrobial	pH	Difference between high and low levels ($a^{(1/b)}$)	Difference between high and low levels (b)
acetic	3.0	NSD	NSD
acetic	4.8	NSD	NSD
sorbic	3.0	NSD	NSD
sorbic	4.8	NSD	NSD
propionic	3.0	NSD	NSD
propionic	4.9	0.0261	NSD
benzoic	3.0	NSD	NSD
benzoic	4.2	NSD	NSD

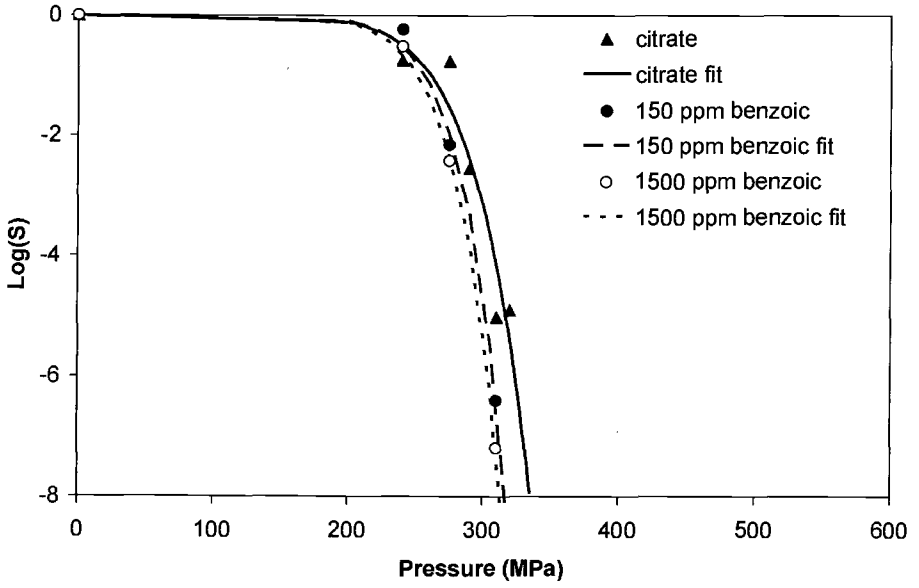

FIGURE 4.3. Survivor Plot of *L. plantarum* ATCC 8014 at pH 3, benzoic acid

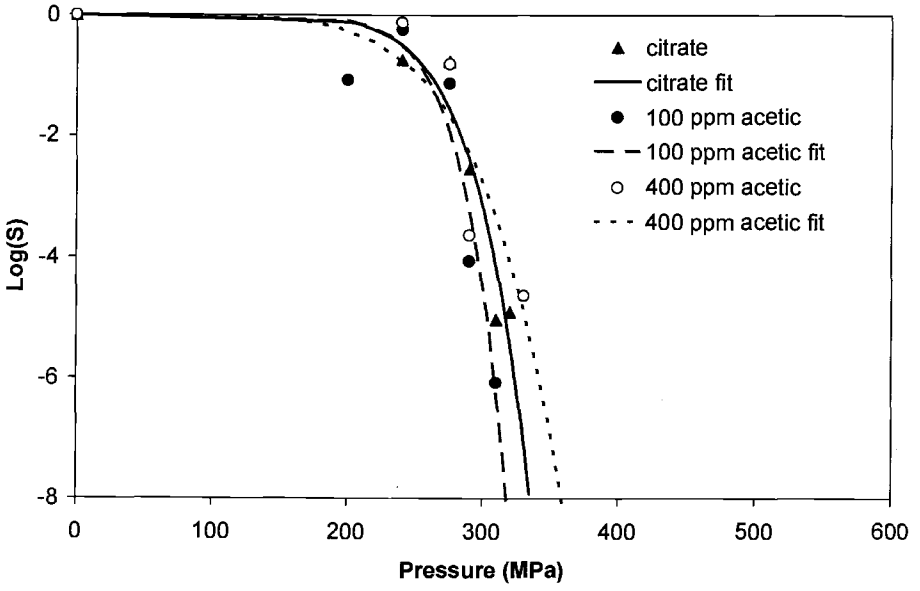

FIGURE 4.4. Survivor Plot of *L. plantarum* ATCC 8014 at pH 3, acetic acid

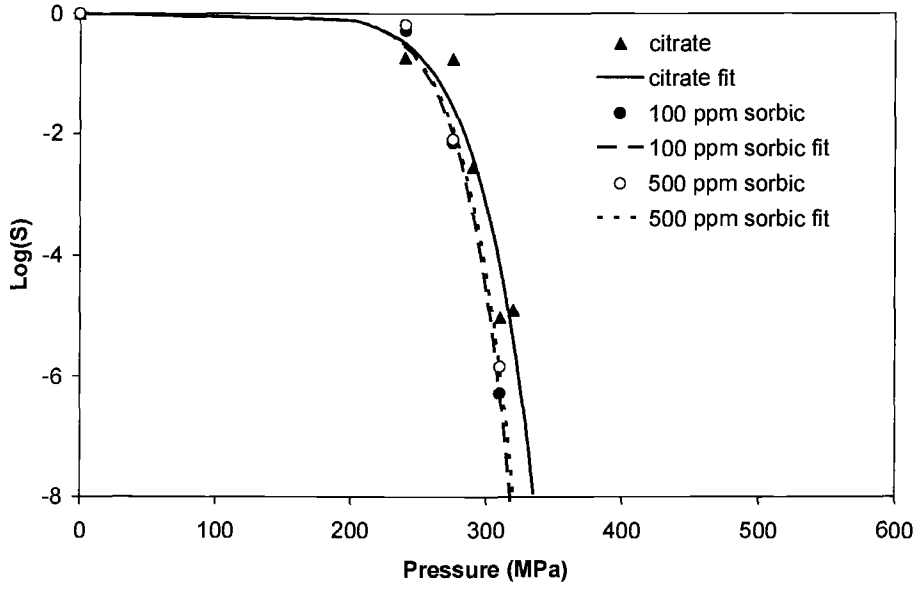

FIGURE 4.5. Survivor Plot of *L. plantarum* ATCC 8014 at pH 3, sorbic acid

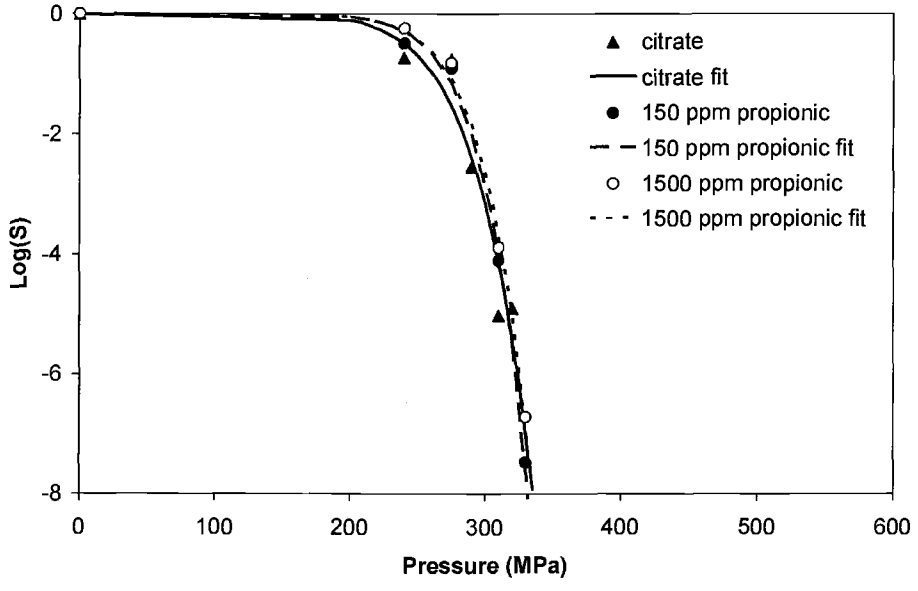

FIGURE 4.6. Survivor Plot of *L. plantarum* ATCC 8014 at pH 3, propionic acid

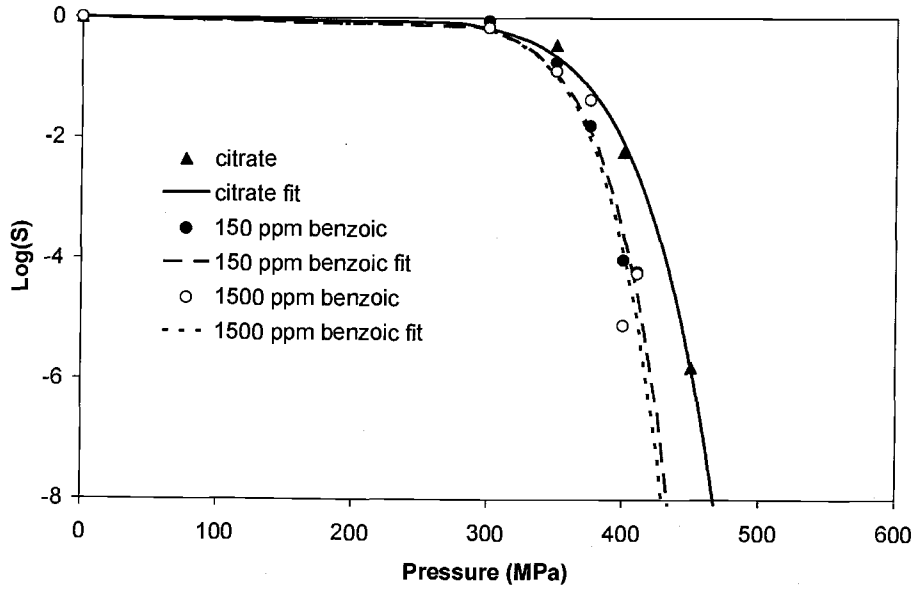

FIGURE 4.7. Survivor Plot of *L. plantarum* ATCC 8014 at pH 4.2, benzoic acid

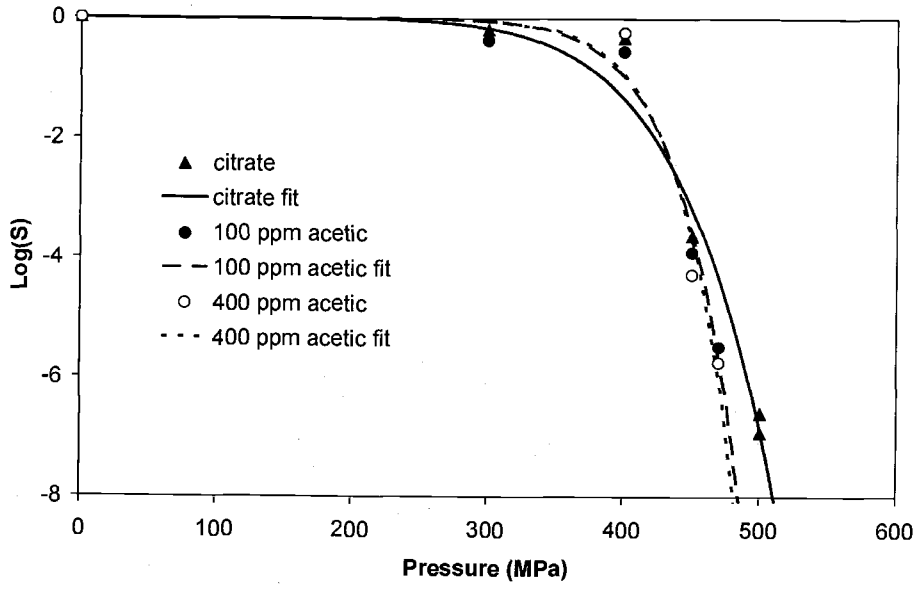

FIGURE 4.8. Survivor Plot of *L. plantarum* ATCC 8014 at pH 4.8, acetic acid

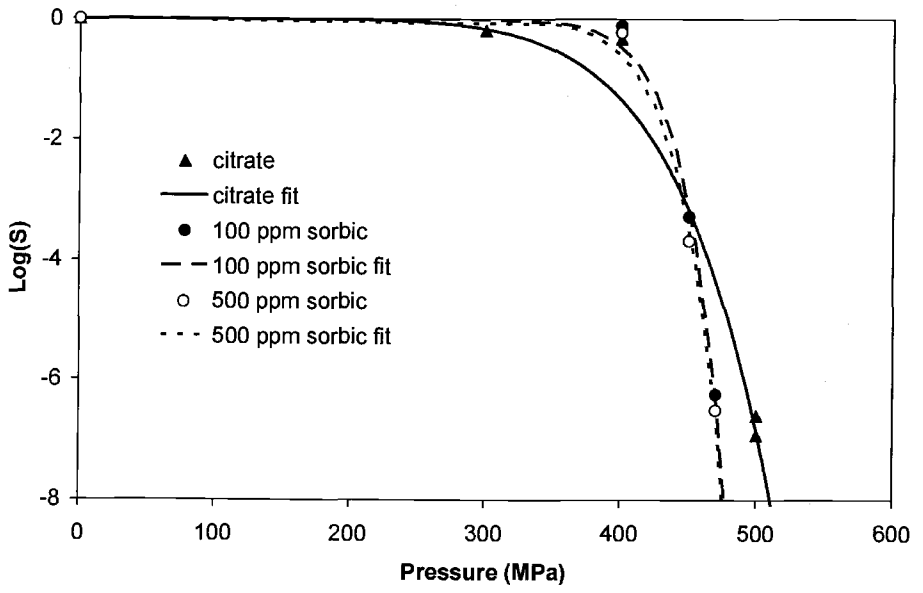

FIGURE 4.9. Survivor Plot of *L. plantarum* ATCC 8014 at pH 4.8, sorbic acid

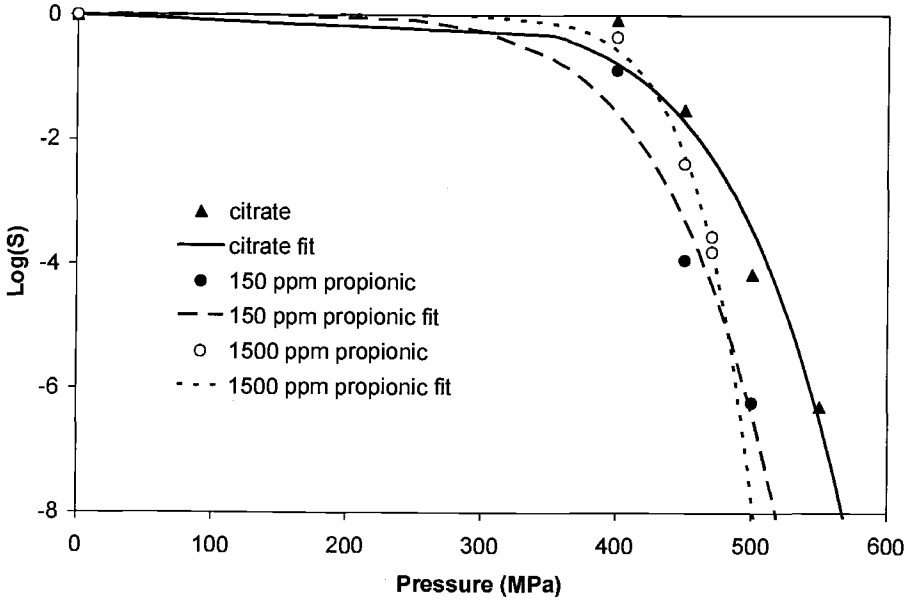

FIGURE 4.10. Survivor Plot of *L. plantarum* ATCC 8014 at pH 4.9, propionic acid

5. CONCLUSIONS

Weak acids of negative reaction volume ($\Delta V°$) may undergo a shift in pH under high pressure conditions, leading to an increase in acidity. The microbial inactivation of HPP is directly affected by pH, increasing with higher levels of acidity. Therefore the use of pressure sensitive buffers in HPP may lead to an increase in bacterial inactivation compared to treatment in pressure insensitive buffers. The results show a greater level of inactivation when pressure sensitive buffers were used at lower pH conditions, thus proving the impact of buffer pressure sensitivity on HPP. At pH 6 and 7 a greater degree of inactivation of *E. coli* was achieved with the pressure insensitive buffer. At pH 6, only one of the pressure insensitive buffers (ACES) results in greater inactivation than the pressure sensitive buffers for *L. plantarum*. At pH 7, there is no difference between the two types of buffer. There was no difference in inactivation between buffers at pH 5 for both bacteria. *Escherichia coli* demonstrated a greater resistant to pressure treatment than *L. plantarum* under the majority of the processing conditions investigated. At the highest and lowest acidity conditions the degree of inactivation was similar for both bacteria. Buffer characteristics can affect the results of high hydrostatic pressure microbial inactivation studies; therefore the effect of buffer in addition to pH needs to be taken into consideration when performing or interpreting bacterial pressure inactivation studies. In commercial applications, the shift of pH may be utilized by food developers to increase microbial lethality of an HPP process. The food system would recover the original acidity after processing.

Addition of antimicrobial organic acids may be utilized to further improve the effect of pH shift under pressure. The antimicrobial properties of organic acids

lay on the protonated form of the acid, which is promoted by lower pH levels. A shift towards higher acidity under pressure could lead to a higher proportion of undissociated acid, thus increasing the efficacy of HPP. There were no antimicrobial effects in bacterial inactivation at pH 3 for any of the organic acids, possibly due to the large impact of acidity on microbial inactivation. Even though the presence of antimicrobial acids improved lethality over the control, there were no statistically significant differences found for most of the organic acids. Sorbic acid was found to impart the largest impact on the lethality of HPP when compared to a control ($p<0.05$). There were some differences found, as well, for benzoic at 150 ppm ($p=0.08$). Concentration of antimicrobial did not have a strong impact. Only propionic acid demonstrated a difference in microbial inactivation between the high and the low concentrations ($p<0.05$) for pH = pK_a. Pressure treatments with antimicrobials added at a level below the published MIC had similar effect in pressure inactivation of bacteria as pressure treatment with antimicrobials added at concentrations within the MIC. The lack of statistical difference could possible be due to the low number of observations per survivor curve. Alternatively, pressure may induce the dissociation of organic acids due to electrostriction, limiting the antimicrobial activity of the acids.

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APPENDICES

APPENDIX A. Temperature Data

TABLE A-1. Temperature Data and Estimated Temperature at Pressure for pH Shift Experiments

		P (MPa)	T_o (°C)	T_f (°C)	Avg, T (°C)	ΔT (°C)			T at press. (°C)		
pH 3	#1	350	14.9	16.6	15.8	1.7	Avg.	2.3	25.6	Avg.	25.4
	#2	350	14.2	16.9	15.6	2.7	SD	0.55	25.4	SD	0.18
	#3	350	14.1	16.7	15.4	2.6	CV (%)	23.6	25.2	CV (%)	0.69
pH 4	#1	450	11.3	15.1	13.2	3.8	Avg.	4.1	25.8	Avg.	25.9
	#2	450	11.1	15.3	13.2	4.2	SD	0.23	25.8	SD	0.17
	#3	450	11.4	15.6	13.5	4.2	CV (%)	5.68	26.1	CV (%)	0.67
pH 5	#1	586	6.8	10.7	8.8	3.9	Avg.	3.1	25.2	Avg.	25.0
	#2	586	7.5	9.9	8.7	2.4	SD	0.75	25.1	SD	0.16
	#3	586	6.9	10	8.5	3.1	CV (%)	24.0	24.9	CV (%)	0.64
	#1	586	6.9	11	9.0	4.1	Avg.	3.8	25.4	Avg.	25.1
	#2	586	6.7	10.7	8.7	4.0	SD	0.38	25.1	SD	0.23
	#3	586	6.8	10.2	8.5	3.4	CV (%)	9.9	24.9	CV (%)	0.90
	#1	586	6.6	10.2	8.4	3.6	Avg.	4.4	24.8	Avg.	25.2
	#2	586	6.5	11.6	9.1	5.1	SD	0.76	25.5	SD	0.33
	#3	586	6.5	11.1	8.8	4.6	CV (%)	17.2	25.2	CV (%)	1.30
pH 6	#1	586	7.0	11.1	9.1	4.1	Avg.	3.2	25.5	Avg.	24.9
	#2	586	6.7	9.1	7.9	2.4	SD	0.85	24.3	SD	0.58
	#3	586	7.1	10.2	8.7	3.1	CV (%)	26.7	25.1	CV (%)	2.34
	#1	586	6.6	11.4	9.0	4.8	Avg.	5.6	25.4	Avg.	26.0
	#2	586	7.0	12.6	9.8	5.6	SD	0.85	26.2	SD	0.48
	#3	586	6.6	13.1	9.9	6.5	CV (%)	15.1	26.3	CV (%)	1.84
pH 7	#1	586	6.9	12.1	9.5	5.2	Avg.	4.6	25.9	Avg.	25.5
	#2	586	6.8	11.6	9.2	4.8	SD	0.72	25.6	SD	0.46
	#3	586	6.7	10.5	8.6	3.8	CV (%)	15.7	25.0	CV (%)	1.80
					Avg.	3.90		Avg.	25.4		
					SD	1.13		SD	0.47		
					CV (%)	28.9		CV (%)	1.87		

TABLE A-2. Temperature Data and Estimated Temperature at Pressure for Antimicrobial Study

	P (Mpa)	T_o (°C)	T_f (°C)	Avg T (°C)	ΔT (°C)	T at P (°C)
pH 3 citrate	200	17.0	15.5	16.3	-1.5	21.9
	240	18.3	17.8	18.1	-0.5	24.8
	275	17.3	18.6	18.0	1.3	25.7
	290	17.0	15.5	16.3	-1.5	24.4
	310	16.2	17.9	17.1	1.7	25.7
	320	16.0	16.3	16.2	0.3	25.1
pH 3 100 ppm acetic acid	200	19.5	14.7	17.1	-4.8	22.7
	240	18.3	17.8	18.1	-0.5	24.8
	275	17.3	18.6	18.0	1.3	25.7
	290	16.2	16.0	16.1	-0.2	24.2
	330	15.8	15.6	15.7	-0.2	24.9
pH 3 400 ppm acetic acid	240	18.3	17.9	18.1	-0.4	24.8
	275	17.2	17.8	17.5	0.6	25.2
	290	16.2	16.0	16.1	-0.2	24.2
	330	15.8	15.0	15.4	-0.8	24.6
pH 3 100 ppm sorbic acid	240	18.4	17.8	18.1	-0.6	24.8
	275	17.3	16.8	17.1	-0.5	24.8
	310	16.2	16.0	16.1	-0.2	24.8
pH 3 500 ppm sorbic acid	240	18.4	17.8	18.1	-0.6	24.8
	275	17.3	16.8	17.1	-0.5	24.8
	310	16.2	16.0	16.1	-0.2	24.8
pH 3 150 ppm propionic acid	240	18.3	16.8	17.6	-1.5	24.3
	275	17.4	17.0	17.2	-0.4	24.9
	310	16.2	15.2	15.7	-1.0	24.4
	330	15.8	15.0	15.4	-0.8	24.6
pH 3 1500 ppm propionic acid	240	18.3	17.6	18.0	-0.7	24.7
	275	17.4	18.0	17.7	0.6	25.4
	310	16.2	17.5	16.9	1.3	25.5
	330	15.8	15.0	15.4	-0.8	24.6
pH 3 150 ppm benzoic acid	240	18.4	17.8	18.1	-0.6	24.8
	275	17.3	16.8	17.1	-0.5	24.8
	310	16.2	16.0	16.1	-0.2	24.8
pH 3 1500 ppm benzoic acid	240	18.4	17.8	18.1	-0.6	24.8
	275	17.3	16.8	17.1	-0.5	24.8
	310	16.2	16.0	16.1	-0.2	24.8
pH 4.2 citrate	300	16.7	17.5	17.1	0.8	25.5
	350	15.1	17.2	16.2	2.1	26.0
	400	13.9	15.2	14.6	1.3	25.8
	450	12.5	12.8	12.7	0.3	25.3

Data Temperature and Estimated Temperature at Pressure for Antimicrobial Study (cont.)

pH 4.2 150 ppm benzoic acid	300	16.7	17.5	17.1	0.8	25.5
	350	15.1	17.2	16.2	2.1	26.0
	375	14.5	15.3	14.9	0.8	25.4
	400	13.8	15.8	14.8	2.0	26.0
	410	13.4	13.9	13.7	0.5	25.1
pH 4.2 1500 ppm benzoic acid	300	16.7	17.5	17.1	0.8	25.5
	350	15.1	17.2	16.2	2.1	26.0
	375	14.5	15.3	14.9	0.8	25.4
	400	13.8	15.8	14.8	2.0	26.0
	410	13.4	13.9	13.7	0.5	25.1
pH 4.8 citrate	300	17.5	18.2	17.9	0.7	26.3
	400	14.8	16.7	15.8	1.9	27.0
	450	12.3	15.2	13.8	2.9	26.4
	450	12.4	12.8	12.6	0.4	25.2
	500	11.0	11.9	11.5	0.9	25.5
	500	11.1	13.0	12.1	1.9	26.1
pH 4.8 100 ppm acetic acid	300	17.5	18.2	17.9	0.7	26.3
	400	14.8	16.7	15.8	1.9	27.0
	450	12.3	15.2	13.8	2.9	26.4
	470	11.0	12.9	12.0	1.9	25.1
pH 4.8 400 ppm acetic acid	400	13.7	14.7	14.2	1.0	25.4
	450	12.4	13.8	13.1	1.4	25.7
	470	11.0	12.9	12.0	1.9	25.1
pH 4.8 100 ppm sorbic acid	400	13.9	15.0	14.5	1.1	25.7
	450	12.3	13.6	13.0	1.3	25.6
	470	11.8	12.3	12.1	0.5	25.2
pH 4.8 500 ppm sorbic acid	400	13.9	15.0	14.5	1.1	25.7
	450	12.5	13.6	13.1	1.1	25.7
	470	11.8	12.3	12.1	0.5	25.2
pH 4.9 citrate	400	13.9	14.0	14.0	0.1	25.2
	450	12.4	12.8	12.6	0.4	25.2
	500	11.0	11.9	11.5	0.9	25.5
	550	9.5	11.3	10.4	1.8	25.8
pH 4.9 150 ppm propionic acid	400	13.9	15.0	14.5	1.1	25.7
	450	12.3	14.5	13.4	2.2	26.0
	500	11.0	13.4	12.2	2.4	26.2
pH 4.9 1500 ppm propionic acid	400	13.8	14.3	14.1	0.5	25.3
	450	12.5	13.6	13.1	1.1	25.7
	470	11.8	12.3	12.1	0.5	25.2
	470	11.8	13.0	12.4	1.2	25.6
					average	25.2
					SD	0.76
					CV (%)	3.02

APPENDIX B. Statistical Analysis - Pressure Sensitivity

TABLE B-3. Statistical Analysis of the Inactivation of *L. plantarum* ATCC 8014 by HPP in Buffers of Varying Sensitivity

pH	Buffer	Mean	Var.	Obs.	Pooled Var.	Hyp. Mean Diff.	df	t Stat	P (T<=t) one-tail	t Critical one-tail	P (T<=t) two-tail	t Critical two-tail
3	HEPES	2.453	0.0235	3	0.0210	0	4	-3.807	0.0095	2.1318	0.0190	2.7765
3	phosphate	2.903	0.0184	3								
3	HEPES	2.453	0.0235	3	0.0184	0	4	-8.996	0.0004	2.1318	0.0008	2.7765
3	citrate	3.450	0.0133	3								
3	phosphate	2.903	0.0184	3	0.0159	0	4	-5.316	0.0030	2.1318	0.0060	2.7765
3	citrate	3.450	0.0133	3								
4	HEPES	5.299	0.1574	3	0.4247	0	4	-5.900	0.0021	2.1318	0.0041	2.7765
4	citrate	8.439	0.6921	3								
5	MES	8.529	1.6493	6	1.2569	0	10	0.616	0.2757	1.8125	0.5514	2.2281
5	citrate	8.130	0.8646	6								
6	MES	4.107	0.0422	3	0.0281	0	4	-24.27	0.0000	2.1318	0.0000	2.7765
6	ACES	7.427	0.0140	3								
6	MES	4.107	0.0422	3	0.2159	0	4	-5.098	0.0035	2.1318	0.0070	2.7765
6	phosphate	6.041	0.3897	3								
6	MES	4.107	0.0422	3	0.0237	0	4	-22.89	0.0000	2.1318	0.0000	2.7765
6	citrate	6.985	0.0052	3								
6	ACES	7.427	0.0140	3	0.2018	0	4	3.779	0.0097	2.1318	0.0195	2.7765
6	phosphate	6.041	0.3897	3								
6	ACES	7.427	0.0140	3	0.0096	0	4	5.531	0.0026	2.1318	0.0052	2.7765
6	citrate	6.985	0.0052	3								
6	phosphate	6.041	0.3897	3	0.1974	0	4	-2.601	0.0300	2.1318	0.0600	2.7765
6	citrate	6.985	0.0052	3								
7	ACES	2.480	0.2496	3	0.1652	0	4	-0.157	0.4413	2.1318	0.8826	2.7765
7	phosphate	2.532	0.0809	3								

TABLE B-4. Statistical Analysis of the Inactivation of *E. coli* K12 by HPP in Buffers of Varying Sensitivity

pH	Buffer	Mean	Var.	Obs.	Pooled Var.	Hyp. Mean Diff.	df	t Stat	(T<=t) one-tail	Critical one-tail	P (T<=t) two-tail	t Critical two-tail
3	HEPES	2.430	0.0853	3	0.0952	0	4	-2.589	0.0304	2.1318	0.0608	2.7765
3	phosphate	3.082	0.1051	3								
3	HEPES	2.430	0.0853	3	0.0540	0	4	-5.383	0.0029	2.1318	0.0058	2.7765
3	citrate	3.452	0.0227	3								
3	phosphate	3.082	0.1051	3	0.0639	0	4	-1.789	0.0741	2.1318	0.1481	2.7765
3	citrate	3.452	0.0227	3								
4	HEPES	1.354	0.0045	3	0.0133	0	4	-3.005	0.0199	2.1318	0.0398	2.7765
4	citrate	1.638	0.0222	3								
5	MES	2.543	0.0897	6	0.6177	0	13	-0.491	0.3159	1.7709	0.6319	2.1604
5	citrate	2.746	0.9476	9								
6	MES	4.583	0.0057	3	0.0050	0	4	-1.08	0.1698	2.1318	0.3395	2.7765
6	ACES	4.646	0.0044	3								
6	MES	4.583	0.0057	3	0.0223	0	4	22.90	0.0000	2.1318	0.0000	2.7765
6	phosphate	1.789	0.0390	3								
6	MES	4.583	0.0057	3	0.0037	0	4	57.08	0.0000	2.1318	0.0000	2.7765
6	citrate	1.740	0.0018	3								
6	ACES	4.646	0.0044	3	0.0217	0	4	23.75	0.0000	2.1318	0.0000	2.7765
6	phosphate	1.789	0.0390	3								
6	ACES	4.646	0.0044	3	0.0031	0	4	64.06	0.0000	2.1318	0.0000	2.7765
6	citrate	1.740	0.0018	3								
6	phosphate	1.789	0.0390	3	0.0204	0	4	0.418	0.3487	2.1318	0.6973	2.7765
6	citrate	1.740	0.0018	3								
7	ACES	4.243	0.0192	3	0.0148	0	4	18.33	0.0000	2.1318	0.0001	2.7765
7	phosphate	2.421	0.0104	3								

APPENDIX C. Sample SAS Code for PROC NLMIXED Analyses

```
SAS Code for nonlinear regression, fixed effects model.
Statistical comparisons made by examining the significance of the
difference parameterization (c12 and d12 coefficients).


/* This is the code for dose-response curve comparison

   Drop in the pair of data sets   */

data trial;
   input sample pressure S;
   if pressure=0 then delete;
   if sample=1 then z1=1; else z1=0;
   if sample=2 then z2=1; else z2=0;
   datalines;

1       300     -0.09
1       350     -0.45
1       400     -2.22
1       450     -5.80
2       300     -0.04
2       350     -0.74
2       375     -1.79
2       400     -4.01
2       410     -4.21
;
proc print data=trial; run;

/* Getting separate parameters rather than differences */
proc nlmixed data=trial;
  parms a11=368 a12=353 b11=8.8 b12=10.11 s2e=0.1;
  a = (a11*z1+a12*z2);
  b = (b11*z1+b12*z2);
  c = (1.0/a)**b;
  predv = -1.0*c*(pressure**b);
  model   s ~ normal(predv,s2e);
  title 'Getting separate parameters rather than differences';
  run;

/* Difference parameterization  */
proc nlmixed data=trial;
  parms c11=368 c12=-8 d11=8.8 d12=1.5 s2e=0.1;
  a = (c11+c12*z2);
  b = (d11+d12*z2);
  c = (1.0/a)**b;
  predv = -1.0*c*(pressure**b);
  model   s ~ normal(predv,s2e);
  title 'Difference parameterization';
  run;
```

APPENDIX D. Statistical Analysis - SAS Output

pH 3.0 Control vs 100ppm acetic acid 1
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	240	-0.74	1	0
2	1	275	-0.76	1	0
3	1	290	-2.54	1	0
4	1	310	-5.02	1	0
5	1	320	-4.90	1	0
6	2	200	-1.06	0	1
7	2	240	-0.24	0	1
8	2	275	-1.12	0	1
9	2	290	-4.06	0	1
10	2	310	-6.07	0	1

Parameters

a11	a12	b11	b12	s2e	NegLogLike
261	258	8.3	9.9	0.1	18.0309955

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	261.04	10.7048	10	24.39	<.0001	0.05	237.19	284.90	-0.00013
a12	257.57	9.1850	10	28.04	<.0001	0.05	237.11	278.04	-0.00001
b11	8.3012	1.8764	10	4.42	0.0013	0.05	4.1205	12.4820	-0.00002
b12	9.8906	2.0992	10	4.71	0.0008	0.05	5.2133	14.5679	-0.00002
s2e	0.4059	0.1815	10	2.24	0.0493	0.05	0.001438	0.8104	0.000029

pH 3.0 Control vs 100ppm acetic acid 4
Difference parameterization 23:54 Monday, March 7, 2005

Parameters

c11	c12	d11	d12	s2e	NegLogLike
261	-3	8.3	1.6	0.1	18.0309955

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	260.84	10.7636	10	24.23	<.0001	0.05	236.86	284.82	-0.00084
c12	-3.2017	14.1381	10	-0.23	0.8254	0.05	-34.7033	28.2998	0.000974
d11	8.2661	1.8738	10	4.41	0.0013	0.05	4.0910	2.4412	-0.00171
d12	1.6384	2.8143	10	0.58	0.5733	0.05	-4.6322	7.9090	-0.00104
s2e	0.4061	0.1817	10	2.24	0.0494	0.05	0.001304	0.8108	0.003521

SAS output (cont.)

pH 3.0 Control vs 400ppm acetic acid 6
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	240	-0.74	1	0
2	1	275	-0.76	1	0
3	1	290	-2.54	1	0
4	1	310	-5.02	1	0
5	1	320	-4.90	1	0
6	2	240	-0.12	0	1
7	2	275	-0.80	0	1
8	2	290	-3.63	0	1
9	2	330	-4.63	0	1

pH 3.0 Control vs 400ppm acetic acid 7
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005
Parameters

a11	a12	b11	b12	s2e	NegLogLike
261	253	8.3	6	0.1	21.5337852

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	261.00	12.1751	9	21.44	<.0001	0.05	233.46	288.54	-0.00003
a12	253.00	15.7968	9	16.02	<.0001	0.05	217.27	288.74	0.000827
b11	8.2934	2.1309	9	3.89	0.0037	0.05	3.4729	13.1139	-0.00209
b12	5.9695	1.5583	9	3.83	0.0040	0.05	2.4443	9.4947	0.000307
s2e	0.5248	0.2474	9	2.12	0.0629	0.05	-0.03482	1.0844	-0.00046

pH 3.0 Control vs 400ppm acetic acid 9
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	260.98	12.1914	9	21.41	<.0001	0.05	233.40	288.56	0.000389
c12	-7.9702	19.9516	9	-0.40	0.6989	0.05	-53.1039	37.1635	0.000888
d11	8.2899	2.1322	9	3.89	0.0037	0.05	3.4664	13.1133	-0.00031
d12	-2.3198	2.6410	9	-0.88	0.4026	0.05	-8.2941	3.6545	-0.00003
s2e	0.5248	0.2474	9	2.12	0.0629	0.05	-0.03480	1.0843	-0.00079

SAS output (cont.)

pH 3.0 Control vs 100ppm sorbic acid 11
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	240	-0.74	1	0
2	1	275	-0.76	1	0
3	1	290	-2.54	1	0
4	1	310	-5.02	1	0
5	1	320	-4.90	1	0
6	2	240	-0.29	0	1
7	2	275	-2.15	0	1
8	2	310	-6.28	0	1

pH 3.0 Control vs 100ppm sorbic acid 12
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
261	256	8.3	9.5	0.1	7.14263865

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	261.07	7.9176	8	32.97	<.0001	0.05	242.81	279.32	0.000114
a12	255.76	8.4936	8	30.11	<.0001	0.05	236.17	275.35	0.000179
b11	8.3050	1.3888	8	5.98	0.0003	0.05	5.1024	11.5075	-4.28E-7
b12	9.5736	1.7244	8	5.55	0.0005	0.05	5.5971	13.5502	-0.00073
s2e	0.2223	0.1111	8	2.00	0.0805	0.05	-0.03398	0.4786	-0.0021

pH 3.0 Control vs 100ppm sorbic acid 14
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	261.06	7.9195	8	32.96	<.0001	0.05	242.80	279.32	2.729E-8
c12	-5.3018	11.6147	8	-0.46	0.6602	0.05	-32.0854	21.4818	4.256E-8
d11	8.3037	1.3888	8	5.98	0.0003	0.05	5.1011	11.5064	-2.3E-7
d12	1.2695	2.2145	8	0.57	0.5822	0.05	-3.8370	6.3761	-1.53E-7
s2e	0.2223	0.1112	8	2.00	0.0805	0.05	-0.03402	0.4787	1.084E-6

SAS output (cont.)

pH 3.0 Control vs 500ppm sorbic acid 16
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	240	-0.74	1	0
2	1	275	-0.76	1	0
3	1	290	-2.54	1	0
4	1	310	-5.02	1	0
5	1	320	-4.90	1	0
6	2	240	-0.19	0	1
7	2	275	-2.09	0	1
8	2	310	-5.84	0	1

pH 3.0 Control vs 500ppm sorbic acid 17
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
261	256	8.3	9.5	0.1	7.80151655

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	261.07	8.0581	8	32.40	<.0001	0.05	242.49	279.66	0.000196
a12	257.04	8.9203	8	28.81	<.0001	0.05	236.47	277.61	1.49E-6
b11	8.3061	1.4138	8	5.88	0.0004	0.05	5.0459	11.5662	0.000089
b12	9.4501	1.8280	8	5.17	0.0009	0.05	5.2348	13.6654	0.000078
s2e	0.2303	0.1152	8	2.00	0.0805	0.05	-0.03528	0.4960	0.002209

pH 3.0 Control vs 500ppm sorbic acid 19
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	261.06	8.0604	8	32.39	<.0001	0.05	242.47	279.65	-9.23E-7
c12	-4.0240	12.0222	8	-0.33	0.7464	0.05	-31.74732	3.6993	-1.88E-6
d11	8.3037	1.4135	8	5.87	0.0004	0.05	5.0442	11.5633	3.836E-6
d12	1.1460	2.3106	8	0.50	0.6332	0.05	-4.1822	6.4743	8.632E-6
s2e	0.2303	0.1152	8	2.00	0.0805	0.05	-0.03524	0.4959	-0.00004

SAS output (cont.)

pH 3.0 Control vs 150ppm benzoic acid 21
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	240	-0.74	1	0
2	1	275	-0.76	1	0
3	1	290	-2.54	1	0
4	1	310	-5.02	1	0
5	1	320	-4.90	1	0
6	2	240	-0.51	0	1
7	2	275	-2.41	0	1
8	2	310	-7.19	0	1

pH 3.0 Control vs 150ppm benzoic acid 22
Getting separate parameters rather than differences

Parameters

a11	a12	b11	b12	s2e	NegLogLike
261	257	8.3	10	0.1	9.81888009

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	261.06	7.7809	8	33.55	<.0001	0.05	243.12	279.00	-8.33E-6
a12	251.40	7.9103	8	31.78	<.0001	0.05	233.16	269.64	-1.11E-7
b11	8.3037	1.3645	8	6.09	0.0003	0.05	5.1571	11.4502	9.452E-7
b12	9.4235	1.4772	8	6.38	0.0002	0.05	6.0170	12.8300	1.406E-7
s2e	0.2146	0.1073	8	2.00	0.0805	0.05	-0.03284	0.4621	5.059E-6

pH 3.0 Control vs 150ppm benzoic acid 24
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	261.06	7.7809	8	33.55	<.0001	0.05	243.12	279.00	-3.79E-6
c12	-9.6633	11.0958	8	-0.87	0.4092	0.05	-35.2504	15.9238	3.687E-6
d11	8.3037	1.3645	8	6.09	0.0003	0.05	5.1572	11.4503	0.000023
d12	1.1198	2.0110	8	0.56	0.5929	0.05	-3.5177	5.7572	-0.00002
s2e	0.2146	0.1073	8	2.00	0.0805	0.05	-0.03284	0.4621	0.000011

SAS output (cont.)

pH 3.0 Control vs 150ppm propionic acid 31
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	240	-0.74	1	0
2	1	275	-0.76	1	0
3	1	290	-2.54	1	0
4	1	310	-5.02	1	0
5	1	320	-4.90	1	0
6	2	240	-0.49	0	1
7	2	275	-0.91	0	1
8	2	310	-4.10	0	1
9	2	330	-7.47	0	1

pH 3.0 Control vs 150ppm propionic acid 32
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
261	271	8.3	10	0.1	7.66303726

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	261.06	7.5473	9	34.59	<.0001	0.05	243.98	278.13	-0.00006
a12	271.02	7.0824	9	38.27	<.0001	0.05	255.00	287.04	0.000046
b11	8.3031	1.3234	9	6.27	0.0001	0.05	5.3094	11.2968	-0.00004
b12	10.2421	1.4593	9	7.02	<.0001	0.05	6.9410	13.5432	0.000059
s2e	0.2019	0.09517	9	2.12	0.0629	0.05	-0.01340	0.4172	-0.00014

pH 3.0 Control vs 150ppm propionic acid 34
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	261.05	7.5467	9	34.59	<.0001	0.05	243.98	278.12	0.000167
c12	9.9781	10.3483	9	0.96	0.3601	0.05	-13.4313	33.3876	0.000209
d11	8.3016	1.3229	9	6.28	0.0001	0.05	5.3090	11.2942	-0.00137
d12	1.9413	1.9695	9	0.99	0.3501	0.05	-2.5141	6.3966	-0.00037
s2e	0.2018	0.09511	9	2.12	0.0628	0.05	-0.01333	0.4170	-0.00698

SAS output (cont.)

pH 3.0 Control vs 150ppm propionic acid 36
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	240	-0.74	1	0
2	1	275	-0.76	1	0
3	1	290	-2.54	1	0
4	1	310	-5.02	1	0
5	1	320	-4.90	1	0
6	2	240	-0.49	0	1
7	2	275	-0.91	0	1
8	2	310	-4.10	0	1
9	2	330	-7.47	0	1

pH 3.0 Control vs 150ppm propionic acid 37
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
261	271	8.3	10	0.1	7.66303726

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	261.06	7.5473	9	34.59	<.0001	0.05	243.98	278.13	-0.00006
a12	271.02	7.0824	9	38.27	<.0001	0.05	255.00	287.04	0.000046
b11	8.3031	1.3234	9	6.27	0.0001	0.05	5.3094	11.2968	-0.00004
b12	10.2421	1.4593	9	7.02	<.0001	0.05	6.9410	13.5432	0.000059
s2e	0.2019	0.09517	9	2.12	0.0629	0.05	-0.01340	0.4172	-0.00014

pH 3.0 Control vs 150ppm propionic acid 39
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	261.05	7.5467	9	34.59	<.0001	0.05	243.98	278.12	0.000167
c12	9.9781	10.3483	9	0.96	0.3601	0.05	-13.4313	33.3876	0.000209
d11	8.3016	1.3229	9	6.28	0.0001	0.05	5.3090	11.2942	-0.00137
d12	1.9413	1.9695	9	0.99	0.3501	0.05	-2.5141	6.3966	-0.00037
s2e	0.2018	0.09511	9	2.12	0.0628	0.05	-0.01333	0.4170	-0.00698

SAS output (cont.)

pH 4.2 150ppm vs 1500ppm benzoic acid 41
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	300	-0.04	1	0
2	1	350	-0.74	1	0
3	1	375	-1.79	1	0
4	1	400	-4.01	1	0
5	1	410	-4.21	1	0
6	2	300	-0.16	0	1
7	2	350	-0.88	0	1
8	2	375	-1.36	0	1
9	2	400	-5.10	0	1
10	2	410	-4.24	0	1

pH 4.2 150ppm vs 1500ppm benzoic acid 42
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
368	353	8.8	10.11	0.1	45.2007034

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	353.09	11.2126	10	31.49	<.0001	0.05	328.11	378.08	-0.00003
a12	351.57	10.4217	10	33.73	<.0001	0.05	328.35	374.79	0.001389
b11	10.1040	2.3887	10	4.23	0.0017	0.05	4.7816	15.4263	-0.00036
b12	10.4186	2.2207	10	4.69	0.0009	0.05	5.4706	15.3666	-0.00042
s2e	0.2872	0.1284	10	2.24	0.0493	0.05	0.001056	0.5733	-0.00267

pH 4.2 150ppm vs 1500ppm benzoic acid 44
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	353.11	11.2119	10	31.49	<.0001	0.05	328.12	378.09	-4.41E-6
c12	-1.6787	15.3332	10	-0.11	0.9150	0.05	-35.8431	32.4858	-1.84E-6
d11	10.1068	2.3896	10	4.23	0.0017	0.05	4.7823	15.4312	0.000022
d12	0.2828	3.2606	10	0.09	0.9326	0.05	-6.9822	7.5478	4.097E-6
s2e	0.2872	0.1284	10	2.24	0.0493	0.05	0.001015	0.5734	0.000184

SAS output (cont.)

pH 4.2 Control vs 150ppm benzoic acid 46
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	300	-0.09	1	0
2	1	350	-0.45	1	0
3	1	400	-2.22	1	0
4	1	450	-5.80	1	0
5	2	300	-0.04	0	1
6	2	350	-0.74	0	1
7	2	375	-1.79	0	1
8	2	400	-4.01	0	1
9	2	410	-4.21	0	1

pH 4.2 Control vs 150ppm benzoic acid 47
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
368	353	8.8	10.11	0.1	0.19475838

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	368.14	6.1363	9	59.99	<.0001	0.05	354.26	382.02	1.06E-10
a12	353.11	4.7027	9	75.09	<.0001	0.05	342.47	363.74	-342E-12
b11	8.7799	0.7676	9	11.44	<.0001	0.05	7.0435	10.5163	7.7E-10
b12	10.1067	1.0023	9	10.08	<.0001	0.05	7.8394	12.3741	3.492E-9
s2e	0.05053	0.02382	9	2.12	0.0629	0.05	-0.00336	0.1044	-7.71E-8

pH 4.2 Control vs 150ppm benzoic acid 49
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	368.14	6.1363	9	59.99	<.0001	0.05	354.26	382.02	9.046E-8
c12	-15.0341	7.7312	9	-1.94	0.0837	0.05	-32.5233	2.4550	8.557E-8
d11	8.7799	0.7676	9	11.44	<.0001	0.05	7.0435	10.5163	-5.32E-7
d12	1.3268	1.2625	9	1.05	0.3207	0.05	-1.5291	4.1828	-4.26E-7
s2e	0.05053	0.02382	9	2.12	0.0629	0.05	-0.00336	0.1044	5.706E-6

SAS output (cont.)

pH 4.2 Control vs 1500ppm benzoic acid 51
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	300	-0.09	1	0
2	1	350	-0.45	1	0
3	1	400	-2.22	1	0
4	1	450	-5.80	1	0
5	2	300	-0.16	0	1
6	2	350	-0.88	0	1
7	2	375	-1.36	0	1
8	2	400	-5.10	0	1
9	2	410	-4.24	0	1

pH 4.2 Control vs 1500ppm benzoic acid 52
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
368	353	8.8	10.11	0.1	12.0682359

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	368.07	14.5309	9	25.33	<.0001	0.05	335.20	400.95	-0.00031
a12	351.43	10.3841	9	33.84	<.0001	0.05	327.94	374.92	-0.00003
b11	8.7719	1.8150	9	4.83	0.0009	0.05	4.6661	12.8778	4.893E-9
b12	10.3893	2.2022	9	4.72	0.0011	0.05	5.4075	15.3711	0.00008
s2e	0.2831	0.1335	9	2.12	0.0629	0.05	-0.01881	0.5850	0.000841

pH 4.2 Control vs 1500ppm benzoic acid 54
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	368.14	14.5235	9	25.35	<.0001	0.05	335.29	400.99	-1.16E-6
c12	-16.7125	17.8536	9	-0.94	0.3737	0.05	-57.1002	23.6752	-1.41E-6
d11	8.7799	1.8167	9	4.83	0.0009	0.05	4.6702	12.8897	4.918E-6
d12	1.6097	2.8549	9	0.56	0.5866	0.05	-4.8485	8.0679	7.359E-6
s2e	0.2831	0.1334	9	2.12	0.0629	0.05	-0.01879	0.5849	0.000032

SAS output (cont.)

pH 4.2 vs pH 4.8, no antimicrobials 56
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	300	-0.09	1	0
2	1	350	-0.45	1	0
3	1	400	-2.22	1	0
4	1	450	-5.80	1	0
5	2	300	-0.18	0	1
6	2	400	-0.32	0	1
7	2	450	-3.62	0	1
8	2	450	-3.63	0	1
9	2	500	-6.60	0	1
10	2	500	-6.92	0	1

pH 4.2 vs pH 4.8, no antimicrobials 57
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
368	384	9	7	0.1	8.97994786

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	368.11	10.8722	10	33.86	<.0001	0.05	343.89	392.34	-0.00023
a12	384.44	9.3970	10	40.91	<.0001	0.05	363.50	405.38	0.000045
b11	8.7766	1.3592	10	6.46	<.0001	0.05	5.7482	11.8050	0.000031
b12	7.3327	0.7276	10	10.08	<.0001	0.05	5.7114	8.9540	-0.00003
s2e	0.1586	0.07091	10	2.24	0.0493	0.05	0.000564	0.3166	-0.0004

pH 4.2 vs pH 4.8, no antimicrobials 59
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	368.14	10.8700	10	33.87	<.0001	0.05	343.92	392.36	4.663E-9
c12	16.2975	14.3692	10	1.13	0.2832	0.05	-15.7190	48.3140	1.397E-8
d11	8.7799	1.3597	10	6.46	<.0001	0.05	5.7503	11.8096	1.267E-8
d12	-1.4475	1.5422	10	-0.94	0.3700	0.05	-4.8837	1.9887	-1.37E-7
s2e	0.1586	0.07091	10	2.24	0.0493	0.05	0.000562	0.3166	-4.11E-7

SAS output (cont.)

pH 4.2 vs pH 4.9, no antimicrobials 61
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	300	-0.09	1	0
2	1	350	-0.45	1	0
3	1	400	-2.22	1	0
4	1	450	-5.80	1	0
5	2	400	-0.88	0	1
6	2	450	-3.94	0	1
7	2	500	-6.22	0	1

pH 4.2 vs pH 4.9, no antimicrobials 62
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
368	374	9	6	0.1	6.42228574

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	368.14	10.2432	7	35.94	<.0001	0.05	343.92	392.36	0.000279
a12	373.53	12.7726	7	29.24	<.0001	0.05	343.32	403.73	0.000408
b11	8.7801	1.2814	7	6.85	0.0002	0.05	5.7501	11.8101	-0.00207
b12	6.3654	0.8111	7	7.85	0.0001	0.05	4.4474	8.2833	-0.00557
s2e	0.1409	0.07527	7	1.87	0.1034	0.05	-0.03710	0.3189	-0.01198

pH 4.2 vs pH 4.9, no antimicrobials 64
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	368.14	10.2489	7	35.92	<.0001	0.05	343.90	392.37	-5.62E-8
c12	5.3764	16.3840	7	0.33	0.7524	0.05	-33.3656	44.1184	-7.73E-8
d11	8.7799	1.2820	7	6.85	0.0002	0.05	5.7484	11.8115	1.186E-6
d12	-2.4150	1.5174	7	-1.59	0.1555	0.05	-6.0030	1.1730	1.358E-6
s2e	0.1410	0.07535	7	1.87	0.1036	0.05	-0.03721	0.3191	-8.38E-7

SAS output (cont.)

pH 4.8 100 vs 400ppm acetic acid 66
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	300	-0.36	1	0
2	1	400	-0.55	1	0
3	1	450	-3.90	1	0
4	1	470	-5.49	1	0
5	2	400	-0.23	0	1
6	2	450	-4.28	0	1
7	2	470	-5.76	0	1

pH 4.8 100 vs 400ppm acetic acid 67
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
401	401	10.9	10.9	0.1	6.40498989

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	401.20	11.6706	7	34.38	<.0001	0.05	373.60	428.80	-0.00023
a12	402.95	10.0648	7	40.04	<.0001	0.05	379.15	426.75	0.000152
b11	10.9525	2.1773	7	5.03	0.0015	0.05	5.8039	16.1010	-0.00096
b12	11.6868	2.0371	7	5.74	0.0007	0.05	6.8698	16.5039	-0.0003
s2e	0.2031	0.1085	7	1.87	0.1035	0.05	-0.05359	0.4597	-0.00079

pH 4.8 100 vs 400ppm acetic acid 69
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	400.91	11.7560	7	34.10	<.0001	0.05	373.11	428.71	-0.00101
c12	2.2288	15.4586	7	0.14	0.8894	0.05	-34.3250	38.7826	0.001584
d11	10.8993	2.1750	7	5.01	0.0015	0.05	5.7562	16.0424	0.002171
d12	0.8254	2.9840	7	0.28	0.7901	0.05	-6.2307	7.8815	0.00191
s2e	0.2032	0.1087	7	1.87	0.1038	0.05	-0.05386	0.4603	0.011429

SAS output (cont.)

pH 4.8 100 vs 500ppm sorbic acid 71
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	400	-0.11	1	0
2	1	450	-3.28	1	0
3	1	470	-6.24	1	0
4	2	400	-0.22	0	1
5	2	450	-3.68	0	1
6	2	470	-6.51	0	1

pH 4.8 100 vs 500ppm sorbic acid 72
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
420	414	16	15	0.1	0.82324972

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	419.96	4.4293	6	94.82	<.0001	0.05	409.13	430.80	-2.74E-9
a12	414.30	4.6170	6	89.73	<.0001	0.05	403.00	425.60	-3.54E-9
b11	16.3595	1.6333	6	10.02	<.0001	0.05	12.3630	20.3560	6.779E-9
b12	14.9604	1.4109	6	10.60	<.0001	0.05	11.5081	18.4127	1.093E-8
s2e	0.05936	0.03427	6	1.73	0.1340	0.05	-0.02450	0.1432	7.515E-9

pH 4.8 100 vs 500ppm sorbic acid 74
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	419.96	4.4293	6	94.82	<.0001	0.05	409.13	430.80	-815E-12
c12	-5.6620	6.3983	6	-0.88	0.4103	0.05	-21.3181	9.9940	3.943E-9
d11	16.3595	1.6333	6	10.02	<.0001	0.05	12.3630	20.3560	2.836E-8
d12	-1.3991	2.1584	6	-0.65	0.5408	0.05	-6.6805	3.8822	2.313E-8
s2e	0.05936	0.03427	6	1.73	0.1340	0.05	-0.02450	0.1432	-1.25E-6

SAS output (cont.)

pH 4.8 Control vs 100ppm acetic acid 76
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	300	-0.18	1	0
2	1	400	-0.32	1	0
3	1	450	-3.62	1	0
4	1	450	-3.63	1	0
5	1	500	-6.60	1	0
6	1	500	-6.92	1	0
7	2	300	-0.36	0	1
8	2	400	-0.55	0	1
9	2	450	-3.90	0	1
10	2	470	-5.49	0	1

pH 4.8 Control vs 100ppm acetic acid 77
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
384	401	7.3	10.9	0.1	7.54624051

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	384.43	10.4826	10	36.67	<.0001	0.05	361.08	407.79	-0.00007
a12	401.25	11.4977	10	34.90	<.0001	0.05	375.63	426.87	-0.00018
b11	7.3322	0.8116	10	9.03	<.0001	0.05	5.5238	9.1406	0.000621
b12	10.9612	2.1480	10	5.10	0.0005	0.05	6.1753	15.7472	0.000619
s2e	0.1973	0.08819	10	2.24	0.0493	0.05	0.000763	0.3938	-0.0083

pH 4.8 Control vs 100ppm acetic acid 79
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	384.44	10.4835	10	36.67	<.0001	0.05	361.08	407.80	-7.5E-7
c12	16.8200	15.5578	10	1.08	0.3050	0.05	-17.8450	51.4849	-7.91E-7
d11	7.3324	0.8117	10	9.03	<.0001	0.05	5.5238	9.1411	-7.97E-7
d12	3.6304	2.2963	10	1.58	0.1450	0.05	-1.4861	8.7470	3.621E-6
s2e	0.1973	0.08825	10	2.24	0.0493	0.05	0.000699	0.3940	5.867E-6

SAS output (cont.)

pH 4.8 Control vs 100ppm sorbic acid 81
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	300	-0.18	1	0
2	1	400	-0.32	1	0
3	1	450	-3.62	1	0
4	1	450	-3.63	1	0
5	1	500	-6.60	1	0
6	1	500	-6.92	1	0
7	2	400	-0.11	0	1
8	2	450	-3.28	0	1
9	2	470	-6.24	0	1

pH 4.8 Control vs 100ppm sorbic acid 82
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
384	420	7.3	16	0.1	6.65312195

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	384.44	10.1808	9	37.76	<.0001	0.05	361.41	407.47	-2.1E-8
a12	419.96	7.8427	9	53.55	<.0001	0.05	402.22	437.70	-5.51E-8
b11	7.3324	0.7883	9	9.30	<.0001	0.05	5.5492	9.1157	2.539E-7
b12	16.3595	2.8920	9	5.66	0.0003	0.05	9.8174	22.9016	1.354E-7
s2e	0.1861	0.08773	9	2.12	0.0629	0.05	-0.01236	0.3845	4.68E-8

pH 4.8 Control vs 100ppm sorbic acid 84
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	384.44	10.1808	9	37.76	<.0001	0.05	361.41	407.47	-1.35E-8
c12	35.5264	12.8518	9	2.76	0.0220	0.05	6.4536	64.5991	-2.25E-8
d11	7.3324	0.7883	9	9.30	<.0001	0.05	5.5492	9.1157	-9.37E-9
d12	9.0271	2.9976	9	3.01	0.0147	0.05	2.2459	15.8082	7.967E-8
s2e	0.1861	0.08773	9	2.12	0.0629	0.05	-0.01236	0.3845	1.65E-6

SAS output (cont.)

pH 4.8 Control vs 100ppm sorbic acid 86
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	300	-0.18	1	0
2	1	400	-0.32	1	0
3	1	450	-3.62	1	0
4	1	450	-3.63	1	0
5	1	500	-6.60	1	0
6	1	500	-6.92	1	0
7	2	400	-0.11	0	1
8	2	450	-3.28	0	1
9	2	470	-6.24	0	1

pH 4.8 Control vs 100ppm sorbic acid 87
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
430	420	13	16	0.1	33.7553979

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	384.44	10.1808	9	37.76	<.0001	0.05	361.41	407.47	5.874E-7
a12	419.96	7.8427	9	53.55	<.0001	0.05	402.22	437.70	1.156E-6
b11	7.3324	0.7883	9	9.30	<.0001	0.05	5.5492	9.1157	-8.59E-6
b12	16.3595	2.8920	9	5.66	0.0003	0.05	9.8174	22.9016	-3.53E-6
s2e	0.1861	0.08773	9	2.12	0.0629	0.05	-0.01236	0.3845	-2.55E-6

pH 4.8 Control vs 100ppm sorbic acid 89
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	384.44	10.1808	9	37.76	<.0001	0.05	361.41	407.47	-1.35E-8
c12	35.5264	12.8518	9	2.76	0.0220	0.05	6.4536	64.5991	-2.25E-8
d11	7.3324	0.7883	9	9.30	<.0001	0.05	5.5492	9.1157	-9.37E-9
d12	9.0271	2.9976	9	3.01	0.0147	0.05	2.2459	15.8082	7.967E-8
s2e	0.1861	0.08773	9	2.12	0.0629	0.05	-0.01236	0.3845	1.65E-6

SAS output (cont.)

pH 4.8 Control vs 400ppm acetic acid 91
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	300	-0.18	1	0
2	1	400	-0.32	1	0
3	1	450	-3.62	1	0
4	1	450	-3.63	1	0
5	1	500	-6.60	1	0
6	1	500	-6.92	1	0
7	2	400	-0.23	0	1
8	2	450	-4.28	0	1
9	2	470	-5.76	0	1

pH 4.8 Control vs 400ppm acetic acid 92
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
384	401	7.3	10.9	0.1	11.2791182

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	384.42	12.4143	9	30.97	<.0001	0.05	356.34	412.50	-0.00012
a12	402.94	11.7490	9	34.30	<.0001	0.05	376.36	429.52	0.000022
b11	7.3311	0.9610	9	7.63	<.0001	0.05	5.1573	9.5049	0.000016
b12	11.6857	2.3776	9	4.91	0.0008	0.05	6.3071	17.0643	8.727E-6
s2e	0.2766	0.1304	9	2.12	0.0629	0.05	-0.01836	0.5715	-6.3E-6

pH 4.8 Control vs 400ppm acetic acid 94
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	384.44	12.4115	9	30.97	<.0001	0.05	356.36	412.51	-2.84E-7
c12	18.5005	17.0915	9	1.08	0.3072	0.05	-20.1630	57.1641	-1.39E-6
d11	7.3324	0.9610	9	7.63	<.0001	0.05	5.1584	9.5064	-6.81E-6
d12	4.3526	2.5645	9	1.70	0.1239	0.05	-1.4487	10.1540	6.638E-6
s2e	0.2766	0.1304	9	2.12	0.0629	0.05	-0.01836	0.5715	-9.16E-6

SAS output (cont.)

pH 4.8 Control vs 500ppm sorbic acid 96
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	300	-0.18	1	0
2	1	400	-0.32	1	0
3	1	470	-6.24	1	0
4	1	450	-3.62	1	0
5	1	450	-3.63	1	0
6	1	500	-6.60	1	0
7	1	500	-6.92	1	0
8	2	400	-0.22	0	1
9	2	450	-3.68	0	1
10	2	470	-6.51	0	1

pH 4.8 Control vs 500ppm sorbic acid 97
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
384	414	7.3	15	0.1	23.8022028

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	371.29	15.3324	10	24.22	<.0001	0.05	337.12	405.45	-5.02E-6
a12	414.34	12.7314	10	32.54	<.0001	0.05	385.97	442.70	0.000191
b11	6.6035	1.0012	10	6.60	<.0001	0.05	4.3727	8.8344	0.000186
b12	14.9708	3.8953	10	3.84	0.0032	0.05	6.2916	23.6499	0.000071
s2e	0.4520	0.2022	10	2.24	0.0493	0.05	0.001601	0.9025	0.000026

pH 4.8 Control vs 500ppm sorbic acid 99
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	371.29	15.3324	10	24.22	<.0001	0.05	337.12	405.45	-2.9E-8
c12	43.0162	19.9362	10	2.16	0.0563	0.05	-1.4044	87.4367	4.569E-8
d11	6.6034	1.0012	10	6.60	<.0001	0.05	4.3726	8.8342	1.249E-6
d12	8.3570	4.0204	10	2.08	0.0643	0.05	-0.6011	17.3150	-3.08E-7
s2e	0.4520	0.2022	10	2.24	0.0493	0.05	0.001602	0.9025	5.877E-6

SAS output (cont.)

pH 4.8 vs pH 4.9, no antimicrobials 101
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	300	-0.18	1	0
2	1	400	-0.32	1	0
3	1	450	-3.62	1	0
4	1	450	-3.63	1	0
5	1	500	-6.60	1	0
6	1	500	-6.92	1	0
7	2	400	-0.88	0	1
8	2	450	-3.94	0	1
9	2	500	-6.22	0	1

pH 4.8 vs pH 4.9, no antimicrobials 102
Getting separate parameters rather than differences
23:54 Monday, March 7, 200

Parameters

a11	a12	b11	b12	s2e	NegLogLike
368	374	9	6	0.1	897.114124

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	384.44	12.2930	9	31.27	<.0001	0.05	356.63	412.25	0.000028
a12	373.71	17.6943	9	21.12	<.0001	0.05	333.68	413.73	0.000606
b11	7.3326	0.9519	9	7.70	<.0001	0.05	5.1793	9.4858	-0.00019
b12	6.3766	1.1266	9	5.66	0.0003	0.05	3.8280	8.9252	-0.00004
s2e	0.2713	0.1279	9	2.12	0.0629	0.05	-0.01802	0.5607	-0.00019

pH 4.8 vs pH 4.9, no antimicrobials 104
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	384.44	12.2936	9	31.27	<.0001	0.05	356.63	412.25	1.341E-6
c12	-10.9211	21.5793	9	-0.51	0.6250	0.05	-59.7368	37.8946	9.954E-7
d11	7.3324	0.9519	9	7.70	<.0001	0.05	5.1791	9.4857	-0.00002
d12	-0.9675	1.4745	9	-0.66	0.5281	0.05	-4.3030	2.3681	-0.00002
s2e	0.2713	0.1279	9	2.12	0.0629	0.05	-0.01802	0.5607	0.000038

SAS output (cont.)

pH 4.9 150ppm vs 1500ppm propionic acid 106
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	400	-0.88	1	0
2	1	450	-3.94	1	0
3	1	500	-6.22	1	0
4	2	400	-0.35	0	1
5	2	450	-2.38	0	1
6	2	470	-3.79	0	1
7	2	470	-3.55	0	1

pH 4.9 150ppm vs 1500ppm propionic acid 107
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
416	421	6.7	12	0.1	63.1412907

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	373.52	12.9957	7	28.74	<.0001	0.05	342.79	404.25	0.000041
a12	421.22	10.8421	7	38.85	<.0001	0.05	395.58	446.86	0.00071
b11	6.3649	0.8252	7	7.71	0.0001	0.05	4.4137	8.3161	-0.00059
b12	11.9593	2.9554	7	4.05	0.0049	0.05	4.9709	18.9476	0.00011
s2e	0.1457	0.07790	7	1.87	0.1036	0.05	-0.03849	0.3299	0.003856

pH 4.9 150ppm vs 1500ppm propionic acid 109
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	373.52	12.9953	7	28.74	<.0001	0.05	342.79	404.25	-2.31E-8
c12	47.6160	16.9396	7	2.81	0.0261	0.05	7.5602	87.6718	-2.86E-8
d11	6.3649	0.8251	7	7.71	0.0001	0.05	4.4138	8.3160	-3.17E-8
d12	5.5712	3.0647	7	1.82	0.1119	0.05	-1.6757	12.8180	1.166E-7
s2e	0.1457	0.07788	7	1.87	0.1036	0.05	-0.03846	0.3298	-4.56E-8

SAS output (cont.)

pH 4.9 Control vs 150ppm propionic acid 111
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	400	-0.07	1	0
2	1	450	-1.51	1	0
3	1	500	-4.16	1	0
4	1	550	-6.28	1	0
5	2	400	-0.88	0	1
6	2	450	-3.94	0	1
7	2	500	-6.22	0	1

pH 4.9 Control vs 150ppm propionic acid 112
Getting separate parameters rather than differences
23:54 Monday, March 7, 200

Parameters

a11	a12	b11	b12	s2e	NegLogLike
416	374	6.7	6.3	0.1	8.66146814

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	415.87	16.5061	7	25.19	<.0001	0.05	376.84	454.90	-0.00008
a12	373.52	18.2976	7	20.41	<.0001	0.05	330.26	416.79	0.000021
b11	6.7108	1.0406	7	6.45	0.0004	0.05	4.2501	9.1715	-6.12E-6
b12	6.3654	1.1619	7	5.48	0.0009	0.05	3.6179	9.1129	5.655E-6
s2e	0.2889	0.1544	7	1.87	0.1036	0.05	-0.07625	0.6540	-0.00002

pH 4.9 Control vs 150ppm propionic acid 114
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	415.82	16.5108	7	25.18	<.0001	0.05	376.77	454.86	-0.00017
c12	-42.2511	24.6161	7	-1.72	0.1298	0.05	-100.46	15.9569	0.000484
d11	6.7079	1.0402	7	6.45	0.0004	0.05	4.2481	9.1676	0.000596
d12	-0.3404	1.5581	7	-0.22	0.8333	0.05	-4.0247	3.3440	-0.00548
s2e	0.2884	0.1539	7	1.87	0.1031	0.05	-0.07548	0.6523	-0.02126

SAS output (cont.)

pH 4.9 Control vs 1500ppm propionic acid 116
Printing data array and coded variables 23:54 Monday, March 7, 2005

Obs	sample	pressure	S	z1	z2
1	1	400	-0.07	1	0
2	1	450	-1.51	1	0
3	1	500	-4.16	1	0
4	1	550	-6.28	1	0
5	2	400	-0.35	0	1
6	2	450	-2.38	0	1
7	2	470	-3.79	0	1
8	2	470	-3.55	0	1

pH 4.9 Control vs 1500ppm propionic acid 117
Getting separate parameters rather than differences
23:54 Monday, March 7, 2005

Parameters

a11	a12	b11	b12	s2e	NegLogLike
416	421	6.7	12	0.1	4.1596396

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	415.90	11.8854	8	34.99	<.0001	0.05	388.49	443.31	0.000035
a12	421.14	11.0185	8	38.22	<.0001	0.05	395.73	446.54	0.000024
b11	6.7124	0.7496	8	8.95	<.0001	0.05	4.9838	8.4410	1.475E-6
b12	11.9371	2.9934	8	3.99	0.0040	0.05	5.0342	18.8400	0.000026
s2e	0.1499	0.07493	8	2.00	0.0805	0.05	-0.02293	0.3226	-0.00008

pH 4.9 Control vs 1500ppm propionic acid 119
Difference parameterization 23:54 Monday, March 7, 2005

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	415.89	11.8859	8	34.99	<.0001	0.05	388.48	443.30	-2.22E-7
c12	5.2390	16.2087	8	0.32	0.7548	0.05	-32.1382	42.6162	1.261E-7
d11	6.7121	0.7496	8	8.95	<.0001	0.05	4.9836	8.4406	3.615E-6
d12	5.2240	3.0858	8	1.69	0.1289	0.05	-1.8919	12.3399	-1.26E-6
s2e	0.1499	0.07493	8	2.00	0.0805	0.05	-0.02293	0.3226	6.569E-6