AN ABSTRACT OF THE THESIS OF

Rama Krishna Sarangapani for the degree of Master of Science in Food Science and Technology presented on March 21, 2008.

Title: Impact of Weak and Strong Acids on the Destruction of Lactic Acid Bacteria During High Pressure Processing

Abstract approved:

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Thomas H. Shellhammer

High hydrostatic pressure (HHP) affects the pH of weak acids due to an increased degree of acid dissociation while under pressure. The temporary pH reduction by high pressure in such a case may influence the barotolerance of microorganisms. The objective of this study was to determine the impact of weak and strong acids on the barotolerance of lactic acid bacteria across a range of pH under high hydrostatic pressure conditions. The effect of two acid groups (weak and strong) was studied on *Lactobacillus plantarum* (strain MDOS 32) at different pH levels (3.5 to 5). All high pressure treatments were carried for 1 minute over a range of pressures (350 – 525 MPa) at 25°C (at pressure). Population reductions were assessed by serial dilution followed by spiral plating, and pressure dependency of these reductions was modeled using the Weibull equation. Microbial inactivation was significantly affected by the type of acid (weak vs. strong), pH and pressure. In general, high pressure lethality of weak acids was

greater compared to that of strong mineral acids. As the pH decreased from 5 to 3.5, high pressure lethality increased. The presence of whey protein isolate did not alter the lethality of the acid buffer systems.

Impact of Weak and Strong Acids on the Destruction of Lactic Acid Bacteria During High Pressure Processing

by

Rama Krishna Sarangapani

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APPROVED:

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Dean of the Graduate School

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Rama Krishna Sarangapani, Author

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Impact of Weak and Strong Acids on the Destruction of Lactic Acid Bacteria During High Pressure Processing

CHAPTER 1
LITERATURE REVIEW

1.1 High Pressure Processing

High pressure processing is a non-thermal processing technique used with a specific objective to reach up to the demands of consumer for fresh like properties and minimal processing of foods. This process retains most of the nutritional and organoleptic properties including flavor, texture and color in most of the foods. This process uses the minimal heat treatment of foods without the addition of chemical preservatives to achieve a microbiologically safer product. The first attempt to preserve the food using high pressure processing was made in 1899 by Hite and others where pressures as high as 650 MPa were used to treat different foods (Barbosa-Cánovas, 2005). The use of this processing technique has proven its commercial importance in the food industries in 1990's. Since then a variety of products such as guacamole, jams, jellies, fruit juices, oysters and meat products have been high pressure treated and made available in American, European and Japanese markets (Barbosa-Cánovas 2005, Tewari and others 2007 and Rastogi and others 2007). High pressure processing has also been in use by industries other than food industries such as ceramic, diamond, steel, alloy and plastics during 20^{th} century (Barbosa-Cánovas, 2005 & Tewari and others, 2007).

The mechanism of high pressure processing involves application of high pressures in the range of hundreds of megapascals (MPa) to compress the fluid. According to Pascal's law, this fluid in turn transmits the pressure change uniformly throughout the food. This uniform distribution of pressure to a food does not affect the shape or size of the food under consideration, provided the food does not contain high amounts of free and non dissolved gas content, making high pressure processing technique suitable for a variety of foods. During the pressure processing, adiabatic heating increases the temperature of the water (pressure transmitting fluid) by 3°C for every 100 MPa increase in pressure (Rasanayagam and others 2003).

High pressure processing has a significant effect on vegetative cells of microorganisms but does not have a significant effect on the spores. But this technology could be used in addition with various other processing techniques to successfully affect the microorganisms and to impact the pathogenic spores to a greater extent in foods (Picart and others 2004, Perrier-Cornett and others 2005, Marcos and others 2005).

1.2 Food Acidulants

1.2.1 Citric Acid

Citric acid is a principle organic acid found in berry fruits and citrus fruits such as oranges, grape fruit, lemon and pineapple. It is added to the foods for imparting a characteristic sour taste to the foods. Though it is found majorly as a fruit acid it has got much greater importance in Krebs metabolic cycle in human beings (Arnold, 1975). It is a tri-carboxylic acid with three available units of H^+ ion sites (Figure 1.1). It has three

pKa values of 3.1, 4.7 and 6.4 which are relatively closer to each other helping the second H^+ ion dissociate appreciably before the first H^+ ion dissociation is complete and similar explanation applies between second and third H^+ ion (Kristiansen and others, 1999).

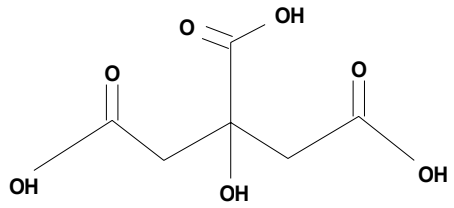

Figure 1.1: Structural formula of citric acid

Citric acid is a very useful acidulant as an antioxidant synergist with a powerful sequestering action on heavy metals and also helps in inhibiting the flavor and color deterioration in foods.

1.2.2 Malic Acid

Malic acid is similar to citric acid found as a major acid in fruits such as cherries, plum, water melon, apples and peaches. L-malic acid is found as a major component imparting sourness to apple juice (Jamin, 2000). It is another organic acid other than citric acid which plays a major role in Krebs cycle in human beings (Arnold, 1975). It is a di-carboxylic acid with two available units of H^+ ion sites (Figure 1.2) with pKa values of 3.4 and 5.2 (Dawson and others 1959).

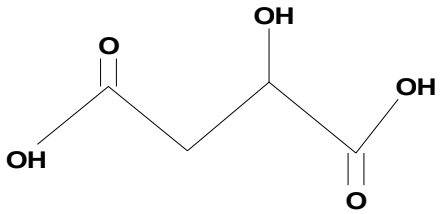

Figure 1.2: Structural formula of malic acid

Malic acid is found in anhydrous form in nature which makes it easier to handle and more attractive compared to citric acid while dealing with dry powdered mixtures (Arnold, 1975).

1.2.3 Tartaric Acid

Tartaric acid, a dihydroxy, dicarboxylic weak organic acid, is found mainly in grape juice, bananas and tamarind. This acid is found to impart sourness to a variety of foods. It has two available units of H^+ ion sites (Figure 1.3) with pKa values of 3.0 and 4.4 (Dawson and others 1959).

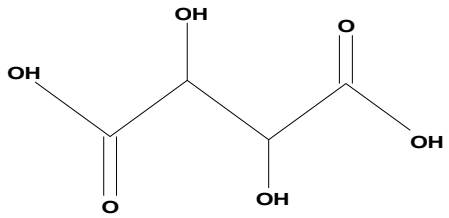

Figure 1.3: Structural formula of tartaric acid

Due to its higher astringency compared to citric and malic acids, tartaric acid is highly preferred as an acidulant in the foods for the astringent flavor attribution. However, tartaric acid has lower level of sourness attributes compared to citric and malic acids (Sortwell and others 1996).

1.2.4 Lactic Acid

Lactic acid is a dihydroxy carboxylic acid commonly found in dairy products (Figure 1.4) as a result of anaerobic carbohydrate metabolism. It has only one available unit of H^+ ion sites (figure 4) with pKa value of 3.86 (Dawson and others 1959).

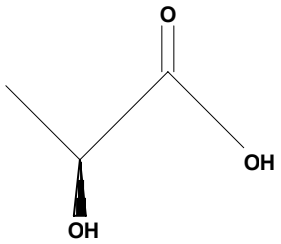

Figure 1.4: Structural formula of lactic acid

Lactic acid is used as an acidulant in the manufacture of hard candies made with isomalt to match the delayed sweetness of isomalt. However, if lactic acid is used as a primary acidulant, it might result in a very sour beverage (Sortwell 2004).

1.2.5 Phosphoric Acid

Phosphoric acid has three available units of H^+ ion sites (Figure 1.5) with pKa values of 1.97, 6.82 and 12.5. The first ionization level (pKa 1.97) resembles the properties as a stronger acid compared to most of the organic acidulants. But the levels of second and third ionization constants make it a weakly dissociating acid compared to other mineral acids which dissociate completely in aqueous solutions (Dawson and others 1959).

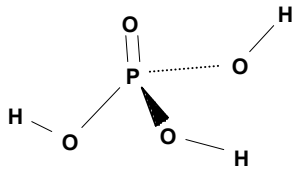

Figure 1.5: Structural formula of phosphoric acid

1.2.6 Sulfuric Acid

Sulfuric acid has two available units of H^+ ion sites (Figure 1.6) with pKa values of -3.0 and 1.99. Sulfuric acid is not a major food acidulant because of its corrosive and harmful nature when used at concentrated levels. However, sulfuric acid has a special use in the treatment of water for brewing (Arnold, 1975). According to US EPA act 2004, sulfuric acid was generally recognized as safe when used as a pH adjusting agents in pesticides.

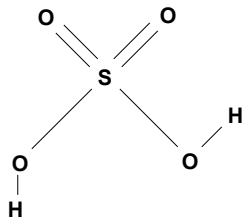

Figure 1.6: Structural formula of sulfuric acid

1.2.7 Hydrochloric Acid

Hydrochloric acid is a strong mineral acid used as a minor or marginal acidulant in food industries. It is a monobasic acid with pKa of -8.0 in aqueous solution. The most common form of usage of this acid in foods is its sodium and calcium salts (Arnold, 1975). Due to very low pKa, it readily dissociates in aqueous solutions under normal temperature and pressure conditions.

1.3 Ionization of Acids

The pH of an acid system can be determined using the Henderson-Hasselbalch equation

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

where, K_a is the acid dissociation constant

$$HA \rightleftharpoons H^+ + A^-$$

Under normal conditions of temperature and pressure, acids ionize releasing H^+ ions and the degree of ionization is based on the pH of the solution and pKa of the acid. The extent of ionization of an acid in an aqueous solution depends on the type of acid whether it is weak or strong acid. Strong mineral acids in general ionize completely in the aqueous solutions but weak organic acids have relatively lower degree of ionization. When the pH is greater than pK_a, acid dissociates under chemical equilibrium and once $pH = pK_a$ the concentrations of dissociated and undissociated forms are in equal proportion.

1.4 pH Shift Under High Pressures

According to Le-Chatelier's principle, whenever a chemical equilibrium is disturbed by changing the conditions like concentration, temperature or pressure, the equilibrium shifts in such a direction so as to counter act the change. When high pressures are applied to the weak acids, they tend to dissociate more which in turn results in a pH drop. When the pressure is applied it results in a change in molar volume (ΔV_o) of the ionic solution and this change is the difference between the volume of the acid in its pure state (V_o) and

its volume in an infinitely dilute solution (V_∞). Also, this difference is much larger in the case of dissociated acids compared to undissociated acids. This effect of change in molar volume and the dependence of pKa was presented by Hamman in the form of a classic thermodynamic equation which illustrates the relation between pKa, pressure (P) and change molar volumes (ΔV_o) in aqueous solutions and can be seen in the following equation:

$$\frac{\partial \ln \mathrm{K}a}{\partial P} = \frac{\Delta Vo}{RT} \tag{1.2}$$

Further integration of equation (2) resulted in the following equation

$$\ln(\mathrm{Ka}(P)) = \ln(\mathrm{Ka}) - \frac{P.\Delta Vo}{R.T(1+b.P)} \tag{1.3}$$

Where T is absolute temperature, R is the universal gas constant and b = 9.2 X 10^{-4} MPa^{-1}.

Further on Kitamura and others in 1987 have suggested a relation between pH and pKa when the pressure is raised from atmospheric pressure to a higher pressures P and the equation was represented as follows:

$$(pH)_P - (pH)_1 = (pK_a)_P - (pK_a)_1 + [\log(\frac{\gamma_A}{\gamma_{HA}})]_P - [\log(\frac{\gamma_A}{\gamma_{HA}})]_1 \tag{1.4}$$

Where, γ_A and γ_{HA} are the activity coefficients of A^- and HA and subscripts P and 1 refer to pressure P atm and atmospheric pressure of 1 atm.

Also this pressure dependency of pKa and pH drop under pressure can be related with the theory of Electrostriction. Electrostriction is defined as contraction in the volume of ions occurring due to the influence of electrical charge on the ions and density of the surrounding water. As the pressure increases, water becomes densely packed around the

ions compared to that of non-polar undissociated acids and causes a decrease in the molar volume of acids (LeNoble, 1988). Thus, according to equations (1.3) and (1.4) the reduction in molar volumes brings about a drop in pH.

Further, this equation was developed by Mathys and others (2007) has explained the dependence of pH and pKa which holds true for most of the buffers up to 1000 MPa. This relation can be seen in the following equation:

$$pH(P) = pK_A(P) + \log(\frac{\gamma_A}{\gamma_{HA}}) + \log(pH - pK_A) \quad (1.5)$$

1.5 Lactic Acid Bacteria (LAB)

1.5.1 General Physiology and Classification

The first pure culture of Lactic acid bacterium (LAB) was obtained as a "milk-souring bacteria" by Lister in 1873 and was termed as "*Bacterium lactis*". Most of the commonly found LAB are gram-positive, non-spore forming, strictly fermentative bacteria with lactic acid as the major end product of sugar fermentation (Salminen and others 2004). Depending on the type of strain, LAB could be classified as spoilage or probiotic (healthy) bacteria. Commonly found probiotic LAB are Bifidobacteria and Propionobacteria. Studies have been carried out using various analytical techniques to isolate spoilage LAB and recently found spoilage LAB were mostly Lactobacillus species and to name some of them are *L. plantarum, L. brevis, L. fermentum, L. rhamnosus* and *L. casei* (Haakensen and others 2007, Motoharu and others 2007, Fujii and others 2005).

1.5.2 Resistance of LAB

As it applies for most of the bacteria, LAB developed resistance to various conditions and the resistance is different for different LAB species and the source it is isolated from. Depending on the type of growth conditions, LAB might show resistance towards pH, temperature and pressure. Various methods have been used to identify and study the resistance of different LAB species. Recent investigations have identified low pH and high bile resistant *L. plantarum* species in fermented dairy products (Fujii and others 2004, Brink and others 2006, Harutoshi and others 2007). Similarly, some of the LAB strains were shown to be resistant towards heat. Also, the heat resistance among the LAB is not the same and varies depending on the species (Bidan and others 1983). Some of the meat spoilage LAB were shown to have varied heat resistance with few of them being sensitive and some of them being highly resistant where the D-values for the highly resistant strains were shown to have as high as 53 min at 63°C (Franz and others 1995).

1.6 Acid Inactivation of LAB

The cytoplasmic membrane of the bacteria including LAB, has a restricted ion-permeability which allows to establish certain electrochemical ion gradients between internal and external medium, such as a pH gradient, across the cell membrane in order to control the depletion of energy sources for its survival (Konings and others 2002). In general, lactic acid bacteria have ability to maintain a fairly constant intracellular pH with the decreasing external pH. But at a certain level of external pH, the difference in pH across the membrane reduces to zero and thus loses its cell viability (Adraiana and others

2002). Also, it was observed that undissociated form of acids are more diffusible through the cell membrane compared to dissociated form of acid and after entering the cell these undissociated acids tend to dissociate as the pH inside the cell is usually around neutral (Padan and others 1981, Slonczewski and others 1981). Thus, it is quite evident that, greater the amount of undissociated acid more is the destruction of bacterial cells. Most of the rod shaped lactic acid bacteria were observed to grow at a pH as low as 4.4 depending on the type of strain (Salminen and others 2004). However, studies conducted to date have revealed few LAB strains which are highly tolerant towards acidic environment and the growth of these LAB strains were observed at a pH as low as 2 and 2.5 in the intestines of birds (Hong Liu and others 2006).

1.7 High Pressure Inactivation of LAB

High pressure inactivation of LAB depends on various factors like, pressure range applied, time duration of pressure applied, type of strain and its resistance towards pressure (Fonberg-Broczek and others 2005). Various factors like time of processing, temperature and pH have been used along with high pressures to inactivate different LAB. However, these lactic acid bacteria were shown to have varied resistance towards high pressures depending on the type of strain and the source from which it is isolated (Hong and others 1999, Ulmer and others 2000, Park and others 2001, Mallidis and others 2002). The ability to maintain a better pH gradient and an increased ATPase activity varies between different LAB and helps in developing a resistance towards high pressure. However, this ability is observed to be lost due to a failure of cell membrane functionality

with an increase in pressure and duration of application of pressure (Wouters and others 1998). Although, the pressure application might result in a failure in cell membrane functionality, the cell wall which is more resistant to pressure compared to cell membrane might be left with an injury and no significant morphological changes in cell wall can be expected. These pressure injured cells could be recovered depending on the storage conditions after the pressure treatment of the cells (Patterson, 2005). High pressure damage of the microbial cells is also associated with a decrease in intracellular pH of the cells and various methods, such as GFP flourocence spectrophotometry has been employed for the prediction of intracellular pH drop under high pressures (Molina-Gutierrez and others 2002, Kilimann 2005). In the above methods, an optical fiber was connected between the acid media and the spectrophotometer and as the pressure is varied, the fluorescent intensities were determined in terms of absorbance units at different wave lengths.

1.8 Baroprotective Effects of Food Matrix on LAB

High pressure inactivation of LAB is highly influenced by various environmental factors such as presence of various food components which could demonstrate protective effects. In general, various probiotic LAB have been shown to be protected against low pH environment using various food materials such as starch, alginates and whey protein gels (Sultana and others 2000; Gunasekharan and others 2007 and Reid and others 2007). Sugars and salts in the medium act as osmotic balancer which helps in increasing the osmotic pressure of the medium. This increase in osmotic pressure enhances the tolerance

of microorganisms towards high hydrostatic pressures (Molina-Höppner and others 2003). Studies conducted by Gervilla and others have indicated that the fat component has baroprotective effect on LAB and other pathogens. But the protective effect did not increase with the increase in the % fat content. Rather, increase in fat content has shown a barodestructive effect on other microorganisms due to the interchange of triglycerides of the milk with the cell membrane lipoproteins and forming the fat crystals. Thus, a large variation in the baroprotective and barodestructive effects of different food components might influence the HHP lethality of microorganisms and it becomes important to investigate the high pressure inactivation of microorganisms in real food systems.

1.9 Hypothesis

In the current research it is hypothesized that, under high pressure conditions degree of acid dissociation (pKa) will influence microbial lethality. Under atmospheric pressure conditions, strong mineral acids dissociate to a greater extent in aqueous solutions, and will have no further significant amount of H^+ ions to release under high pressures. Thus, the microbial destruction under high pressures for strong acids is solely dependant on the initial H^+ ion concentration. In contrast, weak acids have poor dissociation in aqueous solutions and the undissociated acid is further capable of releasing H^+ ions when high pressures are applied. This additional release of H^+ ions from the undissociated acid, under high pressure, results in a pH drop. Hence, the microbial destruction under high pressures for weak acids, depends on initial H^+ ion concentration as well as amount of its undissociated acid. Thus, weak acids are capable of resulting in greater microbial destruction compared to strong acids under high pressures.

In the following sections, the above mentioned hypothesis is studied in three phases. The first phase focuses on testing the hypothesis by comparing the lethality of weak and strong acids towards lactic acid bacteria under high pressures. The second phase tests the hypothesis over a range of pH levels and examines the influence of pH on weak acid lethality. The third phase deals with testing the hypothesis in a model food system containing whey protein isolates, and examining the effect of buffering action of whey protein isolate on the microbial lethality of weak acids under high pressures.

CHAPTER 2

JOURNAL MANUSCRIPT

Impact of Weak and Strong Acids on the Microbial Destruction via High Pressures

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Short title: Lethality of weak Vs strong acids under high hydrostatic pressure

Journal section: Food Microbiology and Safety

2.1 Abstract

High hydrostatic pressure (HHP) affects the pH of weak acids due to an increased degree of acid dissociation while under pressure. The temporary pH reduction by high pressure in such a case may influence the barotolerance of microorganisms. The objective of this study was to determine the impact of weak and strong acids on the barotolerance of lactic acid bacteria across a range of pH under high hydrostatic pressure conditions. The effect of two acid groups (weak and strong) was studied on *Lactobacillus plantarum* (strain MDOS 32) at different pH levels (3.5 to 5). All high pressure treatments were carried out for 1 minute over a range of pressures (350 – 525 MPa) at 25°C (at pressure). Population reductions were assessed by serial dilution followed by spiral plating, and pressure dependency of these reductions was modeled using the Weibull equation. Microbial inactivation was significantly affected by the type of acid (weak vs. strong), pH and pressure. In general, high pressure lethality of weak acids was greater compared to that of strong mineral acids. As the pH decreased from 5 to 3.5, high pressure lethality increased. The presence of whey protein isolate did not alter the lethality of the acid buffer systems.

2.2 Introduction

High pressure processing is a non-thermal processing technique used to extend the shelf life of foods while retaining their fresh like properties. This process can inactivate enzymes and microorganisms which is useful in preserving a variety of foods. It can produce high quality foods which are microbiologically safe because it efficiently destroys the vegetative cells of most of the bacteria by physically damaging the cell membranes of these bacteria (Knorr, 1993). In addition, elevated temperature, time of pressure treatment and acidity contributes to microbial destruction in pressure processed foods (Begonya and others 2005). In terms of acidity, weak acids, under normal atmospheric pressure conditions, have shown a higher microbial lethality due to the presence of higher amount of undissociated acid. The undissociated form of a weak acid was shown to have a higher diffusibility through the cell membrane of the bacteria compared to its dissociated form and thus proved to be more effective on the microbial destruction (Padan and others 1981, Slonczewski and others 1981 and Eklund, 1983). Also, some pressure sensitive weak acids were observed to under go a temporary pH shift under high pressure (Hayert and others 1999; Molina-Gutierrez and others 2002). This phenomenon is useful in the case of ionization of pressure sensitive weak acid buffers under high pressures which results in an intracellular pH drop in the microorganisms and results in a microbial death (Hamann and others 1982 and Molina-Gutierrez and others 2002). But this phenomenon of pH shift of the acids under high pressure may not be significant for strong acids as they are completely dissociated in aqueous solutions under ambient pressure conditions.

The pH of an acid system can be determined using the Henderson- Hasselbalch equation

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

Furthermore, the pressure dependency of the equilibrium constant (Ka) is given by:

$$\frac{d \ln K_a}{dP} = \frac{\Delta V^\circ(P)}{RT}$$

In the current research it is hypothesized that weak acids result in higher lethality compared to strong acids towards lactic acid bacteria under high hydrostatic pressure (HHP) conditions. This is the case because weak acids in aqueous solution contain considerable amount of undissociated acid which in turn contains more H^+ ions to release. But strong acids in comparison to weak acids, dissociate completely in aqueous solutions hence no further release of H^+ ions is possible. Thus, under HHP conditions, weak acids will have more scope of releasing H^+ ions resulting in a pH drop. This pH drop due to weak acid dissociation helps in destroying the microorganism more efficiently compared to strong acids.

Extensive research on the destruction of LAB under high pressures was conducted and the dissociation of weak acid buffers under high pressure conditions was also studied in the past (Ulmer and others 2000; Mallidis and others 2002 and Cornet and others 2004). But the phenomenon in which weak acids can predominantly show higher HHP lethality against LAB in comparison to strong mineral acids was not tested. Also, it was shown that various food components such as starch, protein and different salts protected microorganisms against acidic and high pressure conditions (Sulatana and others 2000; Molina-Höppner and others 2003 and Bjornsdottir and others 2006).

Therefore, there is a need to understand whether weak acid dissociation under high pressures can prove lethal towards microorganisms and observe if the presence of the food components such as proteins would influence the weak acid lethality under high pressures. This understanding may prove its importance while choosing weak organic acids as potential acidulants in foods. The two main objectives of the current research were 1) to test if the weak acids can be more efficient in destroying the food spoilage lactic acid bacteria when compared to strong acids and 2) to test if the efficiency of weak acid lethality will be altered due to the presence of whey protein isolates.

2.3 Materials and Methods

2.3.1 Preparation of Bacterial Cultures

A strain of *L. plantarum* (MDOS 32) was obtained from Department of Food Science and Technology, Ohio State University (Courtesy of Dr. Ahmed Yousef). Stock cultures of each organism were stored in 1:1 MRS broth and 80% glycerol at -80°C prior to each experimental run and a loop of frozen culture was inoculated into 500 mL solution of freshly prepared MRS broth and incubated at 32°C for 24 hrs. After 24 hrs, cultured MRS solutions were centrifuged at 8000 g for 5 min and the supernatant was discarded. The remaining bacterial culture was washed using 0.09 M NaCl solution and re-centrifuged at 8000 g for 5 min. The supernatant was discarded and the remaining bacterial culture was ready to be suspended in either weak acid buffers or strong mineral acid solutions. An aliquot of 3 mL of acidified bacterial cultures were transferred to polyethylene bags, heat sealed and stored under ice until pressure treatment.

2.3.2 Buffer Preparation

Two types of acid systems were used for the experiments: weak organic acids (citric, phosphoric, malic, tartaric) and strong mineral acid solutions (hydrochloric and sulfuric). All buffer solutions and mineral acid solutions were prepared by adding 0.09 M of each acid in 1000 mL of milli-Q water and then titrating with 5N NaOH solution and adjusted the pH to desired levels (3.5, 4, 4.5 and 5). All reagents were purchased from Sigma-Aldrich Corporation, USA.

2.3.3 Acidified Whey Protein Isolate Solutions

6% whey protein isolate solution (BiPro WPI, Davis Co. Foods) at pH 7 was acidified by adding 0.09 M of weak acid buffer or strong acid solution and then adjusted the pH to 4 with 5N NaOH. Cloudiness was observed with all the acids, therefore the solutions were centrifuged at 12000 g for 10 min. The supernatant was decanted and analyzed for the % protein using LECO protein analyzer and determined to be 4.5% (Table 2.1).

Table 2.1: Protein % values measured using LECO FP-528 protein analyzer. The nitrogen – protein conversion factor used for whey protein isolate solution was 6.38 (Onwulata and others 2006)

Acid	% Nitrogen	Average % Nitrogen	% Protein
Citric acid	0.663	0.686	4.4
	0.708		
Phosphoric acid	0.698	0.697	4.4
	0.696		
Malic acid	0.712	0.701	4.5
	0.689		
Sulfuric acid	0.698	0.721	4.6
	0.743		
Hydrochloric acid	0.687	0.703	4.5
	0.719		

2.3.4 High Pressure Runs

All pressure runs were carried out using a 22L EPSI (Haverhill, MA) press in conjunction with a Flow International (South Kent, WA) 40hp intensifier. The polyethylene bags consisting of bacterial suspensions were placed in a 1 L plastic screw top bottle filled with water at the appropriate temperature and this bottle was then placed in to the water-filled 22 L high pressure vessel. All treatments lasted for 1 min at 25°C (at pressure) and over a range of pressures (350 MPa to 525 MPa). Since the temperature of water increases at 3°C per 100 MPa increase in pressure (Rasanayagam and others 2003), the initial temperature of the water was adjusted so as to maintain the temperature of 25°C at pressure. The temperature of the water inside 1 L plastic bottle was measured before and after each pressure run and the temperatures varied less than 3°C for all the pressure runs. After pressure treatments samples were placed under ice until enumeration.

2.3.5 Microbial Enumeration

Once the samples were pressure treated, the bacterial cultures were serial diluted and plated on freshly prepared MRS agar. The plating was performed using an AUTOPLATE 4000 spiral plater (Spiral Biotech Inc, USA). The plates were incubated at 32°C for 48 hrs before counting.

2.3.6 Data Analysis

The results for log reductions in microbial population for pressure treated cultures were compared with that of non-pressure treated cultures in presence of different acid

media. The data was collected in three replications and the comparisons were statistically analyzed. For this purpose the Weibull equation was fitted to the pressure dose-response survival data using the PROC NLMIXED function in SAS software:

$$\log S = -(1/a).P^b \qquad (1)$$

Where, *S* is the survivor ratio (N_t/N_o), P is pressure (MPa) and *a* & *b* are fitted parameters describing the onset of inactivation and steepness of the curvature, respectively. Setting *log S* to a desired reduction level, one log reduction for instance, and solving for pressure (*P*) yields $(a)^{1/b}$ which can be used as a point comparison among treatments. The Weibull model has been used in the past due to simplicity and flexibility for use with microbial inactivation studies (Virto and others 2006). It was also suggested that Weibull model has a better fit compared to other models particularly when the steepness of the curvature (*b*) varies largely from 1 (Rodrigo and others 2002). Statistical analysis, using Weibull model, conducted by Giron (2005) for studying high pressure inactivation of *L. plantarum* was adapted and conducted in the current research. Statistically significant differences were determined by pairwise comparisons based on $a^{1/b}$ & *b* values among different acid treatments at similar conditions of pressure, time and at a particular pH. For all the pair wise comparisons between different acids, we used Bonferroni correction for significance factor (α)

$$\alpha_{corrected} = \alpha / k$$

Where k = number of pair wise comparisons

2.3.7 Bonferroni Correction

Bonferroni Correction is the adjustment of the significance factor (α) for multiple pairwise comparisons taking into account all possible pairwise comparisons in order to effectively reduce the error in drawing the statistical conclusions. In the current research, pairwise comparisons were made between two different acids under similar pH, temperature and pressure conditions. Significant differences for all of the pairwise comparisons were made by adjusting the α value taking into account the total number of pairwise comparisons. For instance, in the case of experiments with four different acids, the total number of pairwise comparisons among acids is 6. Therefore, the corrected value of traditional significance factor ($\alpha = 0.05$) now becomes

$$\alpha_{corrected} = \alpha / k = 0.05/6 = 0.008$$

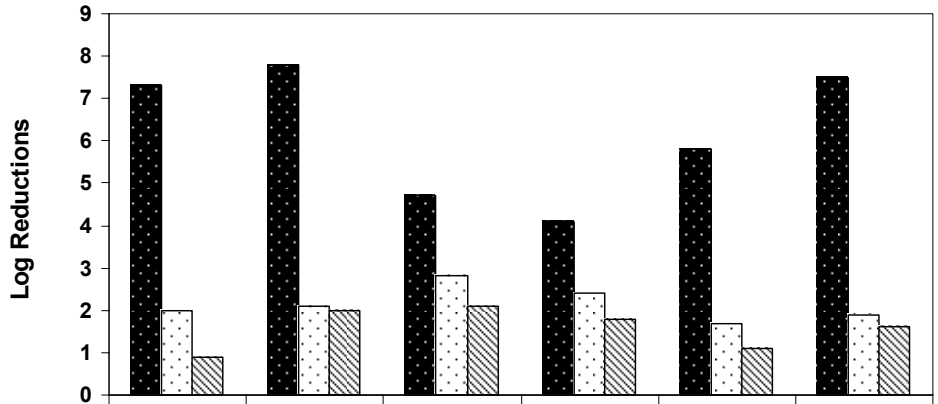
Thus, instead of testing each pairwise comparison at $\alpha = 0.05$, we now test with reference to $\alpha_{corrected} = 0.008$. This testing of each pairwise comparison at the adjusted level of $\alpha = 0.008$ would ensure the overall chance of making error is still less than 0.05.

2.4 Results and Discussion

2.4.1 Impact of Acids on HHP Inactivation of *L. plantarum* MDOS 32 at pH 4

Experiments were conducted at pH 4 with different acid systems (weak and strong) with three different strains of *L. plantarum,* strain MDOS 32 and strain ATCC 8014 and *L. fermentum,* strain NF 85. The pressure sensitivity of these strains was compared with each other at 525 MPa (maximum pressure applied for the experiments). Figure 2.1

clearly indicates that the maximum number of log reductions obtained for strain 32 and strain NF 85 for various acid treatments, are very low when compared to that of strain 8014. This shows that strain 32 and 85 are highly pressure resistant compared to strain 8014. These experimental results are in close agreement with the literature for pressure sensitivity of strain 32 and 85 (Waite and others 2006). Figure 2.2 represents the pressures required for 5 log reductions for strain 32 and strain 85 for different acid treatments. Having observed that 32 & 85 are highly pressure resistant (Figure 2.1), relatively lower amounts of pressure for 5 log reductions of strain 32 compared to that of strain 85 (Figure 2.2), can be attributed to the acid sensitivity of strain 32.



	Citric	Phosphoric	Malic	Tartaric	Hydrochloric	Sulfuric
ATCC 8014	7.3	7.8	4.7	4.1	5.8	7.5
MDOS 32	2	2.1	2.8	2.4	1.7	1.9
NF 85	0.9	2	2.1	1.8	1.1	1.6

Figure 2.1: Pressure sensitivity of strains ATCC 8014, MDOS 32 and NF 85 for weak and strong acids at pH 4 and 525 MPa pressure. Pressure delivered for 1 min at 25°C.

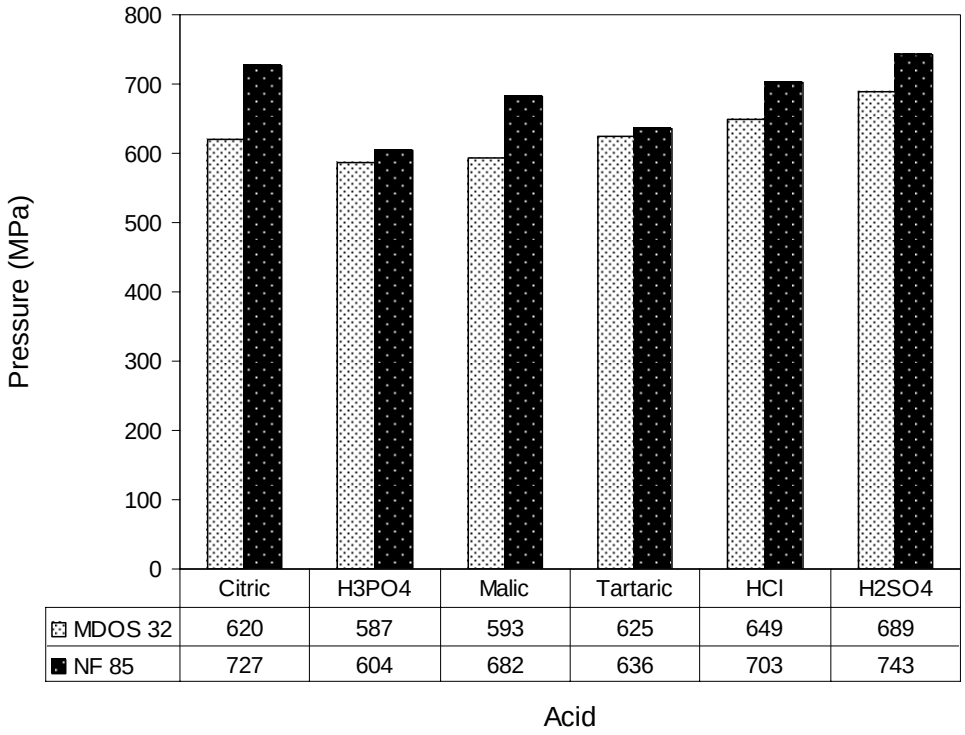


Figure 2.2: Acid sensitivity of strain MDOS 32 Vs NF 85 towards weak and strong acids at pH 4. Pressure delivered for 1 min at 25°C.

Figure 2.1 indicates that weak acids such as malic and tartaric produced 2.8 and 2.4 log reductions, respectively, at 525 MPa with strain 32. While strong acids such as hydrochloric and sulfuric have produced 1.7 and 1.9 log reductions at 525 MPa. Also, from Figure 2.3, it can be clearly observed that weak acids in comparison to strong acids required lower amounts of pressure to achieve 1 and 5 log reductions in the microbial population. These results show a clear predominance of weak acid lethality over strong acid lethality. Results presented thus far for the sensitivity of strain 32 towards type of acid system and the lethality of different acid systems (weak Vs strong) towards strain 32, indicate that weak acids have greater lethality compared to strong acids.

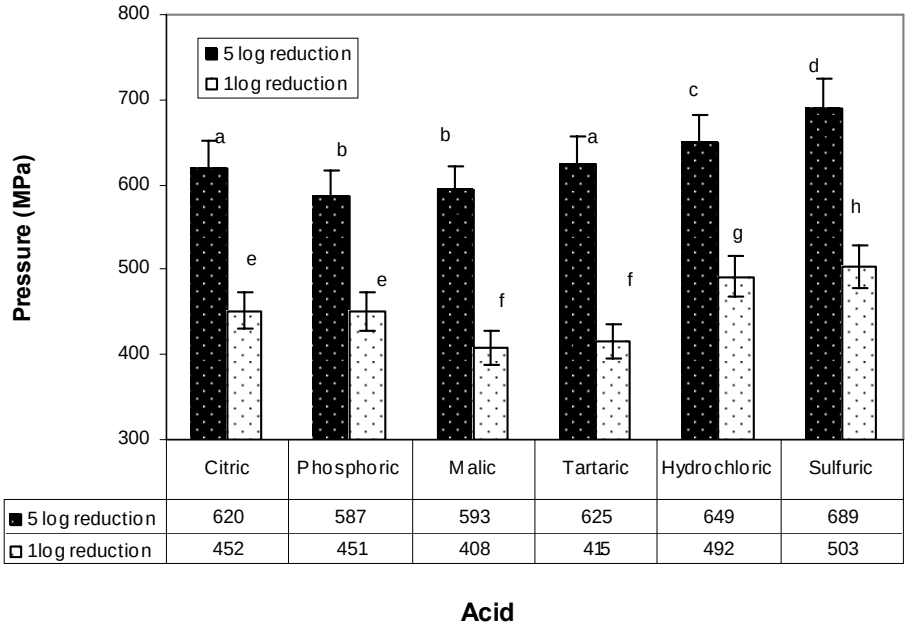


Figure 2.3: Pressure required for 1 and 5 log reductions in the population of *L. plantarum* MDOS 32 at pH 4. Pressure delivered for 1 min at 25°C. Means with same superscripts are not significantly different ($\alpha = 0.05$). Error bars represent ± 5% error, n = 5.

2.4.2 pH Induced HHP Inactivation of *L. plantarum* MDOS 32

A total of four acids (malic, phosphoric, citric and sulfuric acids) were chosen and were further examined for their HHP lethality at different pH levels of 3.5, 4.5, and 5 and over the pressure range of 425 MPa to 525 MPa. The choice of the acids was based on the lethality of these acids towards strain 8014 and strain 32 at pH 4 (Figure 2.1). Acid sensitive and pressure tolerant strain *L. plantarum* MDOS 32 was chosen for these experiments. In most of the cases, weak acids, in comparison with strong acids, were predominantly more lethal as the pH decreased from 5 to 3.5. The Weibull coefficients (a & b) for different acid treatments at pH 3.5, 4.5 and 5 are presented in Table 2.2. In Table 2.2, lethality of acids was compared based on the values of $(2a)^{1/b}$ (pressure required for

two log reductions) and b (gradient of the pressure-log reduction curve). Lower the value of $(2a)^{1/b}$ and/or higher the value of b, more lethal the acid is. From Table 2.2, it can be observed that, as pH decreases from pH 5 to 3.5, for both weak and strong acids, either value of $(2a)^{1/b}$ is decreasing and/or value of b is increasing. This trend clearly indicates that the HHP lethality of both weak and strong acids decreased as pH increased. Figure 2.4 demonstrates this trend. Also, it can be observed from Table 2.2 that weak acids, overall, had either significantly lower value of $(2a)^{1/b}$ and/or significantly higher value of b compared to those of strong acids. Thus, the results from Table 2.2 indicate that weak acids were predominantly more lethal compared to strong acids across the range of pH (3.5 to 5). Literature published by Conner and others (1995), Bjornsdottir and others (2006), indicated that, under normal atmospheric pressure conditions, malic and citric acids proved to be highly lethal with *E. coli* at pH 3.2 and have prevented the growth of *E. coli* at pH 4.5. Results from Table 2.2 for malic and citric acids, which have resulted in greater lethality in comparison to strong acids at pH 3.5 and 4.5, are in close agreement with above mentioned literature. Our results, presented so far in this section, from Figure 2.4 and Table 2.2 for both weak and strong acids and the published literature validate our hypothesis over a range of pH (3.5 to 5).

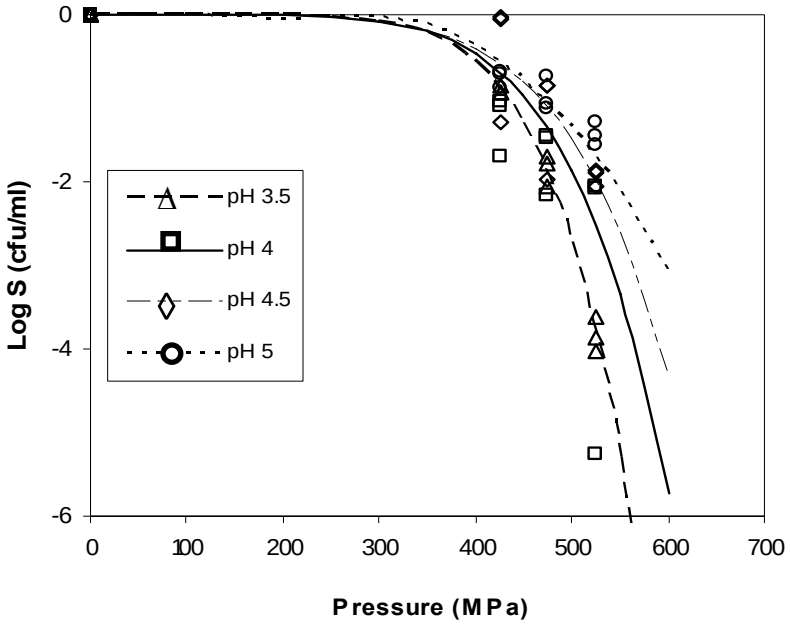


Figure 2.4: Impact of phosphoric acid at pH 3.5, 4, 4.5 & 5 on the pressure-death dose-response behavior of *L. plantarum* MDOS 32. Pressure delivered for 1 min at 25°C.

Table 2.2: Statistical comparisons among various acid treatments using Weibull coefficients (a & b), for *L. plantarum* strain MDOS 32 at pH 3.5, 4.5 & 5. Columns with $(2a)^{1/b}$ represents pressure required for 2 log reductions in microbial population. Significant differences among various acid treatments, within each column, at each pH level are represented with different superscripts ($\alpha = 0.05$).

Acid	pH 3.5		pH 4.5		pH 5	
	$(2a)^{1/b}$	B	$(2a)^{1/b}$	b	$(2a)^{1/b}$	b
Phosphoric	479.3^{a}	7^{a}	525.4^{a}	5.9^{a}	525.2^{a}	3.2^{a}
Malic	408.3^{b}	2.8^{b}	472.4^{b}	2.5^{b}	$592.1^{a,b}$	3.5^{b}
Citric	468.2^{c}	5.3^{c}	$442.4^{a,c}$	$2.7^{b,c}$	$558.6^{b,c}$	4.6^{c}
Sulfuric	480.8^{a}	3.5^{d}	551.7^{c}	7.5^{d}	693.4^{b}	2.3^{d}

2.4.3 Acid Based HHP Lethality in Model Food System

Experimental results for acidified WPI solution and simple acidic buffers at pH 4 were compared with each other. Acid sensitive and pressure tolerant strain *L. plantarum* MDOS 32 was chosen for these experiments. HHP lethality of weak acids did not change

significantly with the addition of WPI to buffer solutions. Figure 2.5 shows a trend in weak and strong acid lethality, with and without the presence of WPI.

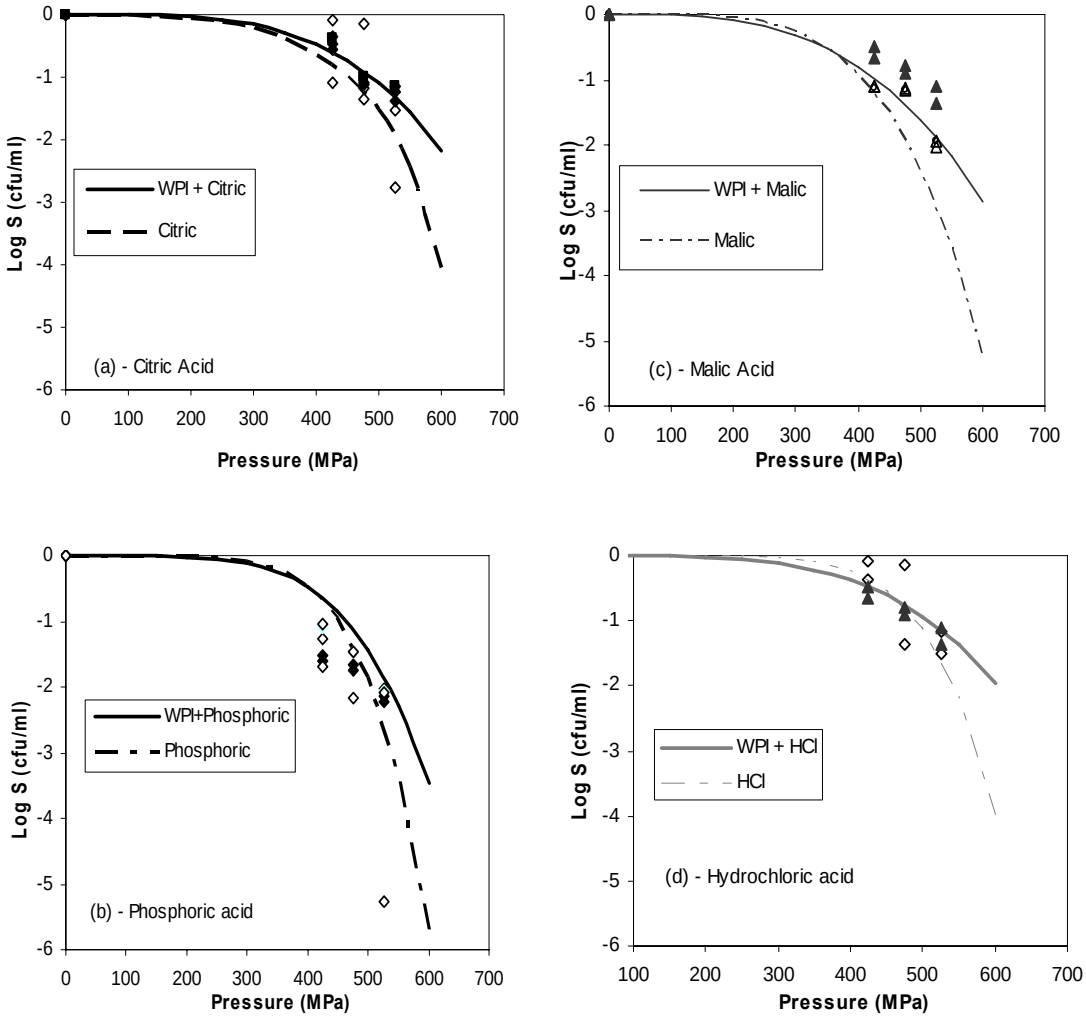


Figure 2.5: Impact of acidified whey proteins and normal buffer systems on *L.plantarum* MDOS 32. All buffers prepared at 0.09M, pH 4. Pressure delivered for 1 min at 25°C.

WPI being a buffering agent, in general, is expected to show a buffering action towards acid dissociation under high pressure. As a result of this WPI buffering action towards acid dissociation, the weak acid lethality on microorganisms should be altered.

But this is not the case as observed from Figure 2.6, where the pressures required to achieve 1 log reduction in the microbial population with and without the presence of WPI are not significantly different. Thus, it could be understood that the buffering action of WPI was not effective against acid dissociation under high pressure and hence did not alter the microbial lethality. A similar trend was observed across each acid under comparison. Also, it can be observed from Figure 2.6 that, in the presence of WPI, weak acids in general required lower pressures compared to strong acids to achieve a 1 log reduction in the microbial population. Thus, the two observations mentioned above, regarding the effect of WPI on microbial lethality validate our hypothesis on the predominance of weak acid lethality compared to that of strong acids in our model food system.

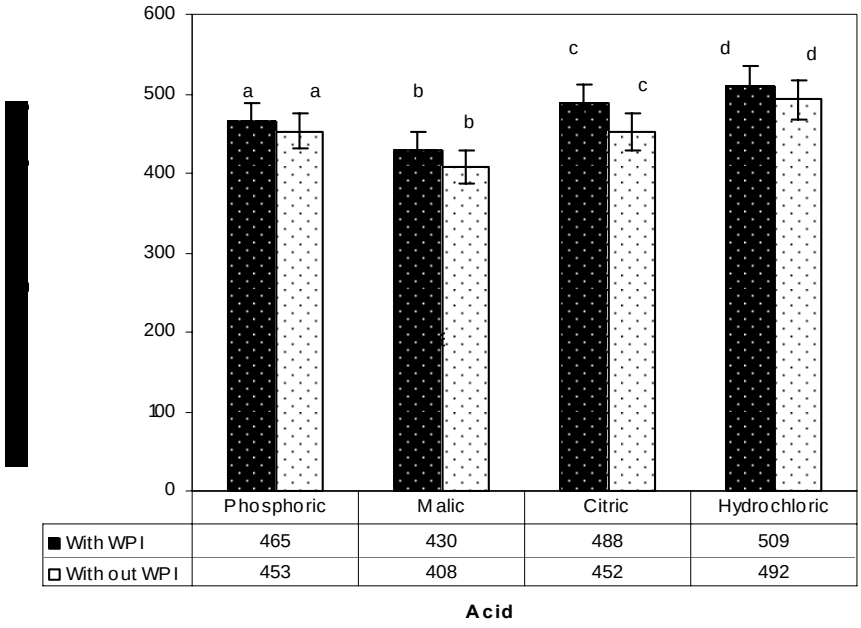


Figure 2.6: Pressure required for 1 log reduction in the population of *L. plantarum* MDOS 32 at pH 4 with and with out WPI. Means with same superscripts are not significantly different ($\alpha = 0.05$). Error bars represent ± 5% error, n = 3.

2.5 Conclusions

The hypothesis of the current research, predominance of weak acid lethality over strong acid lethality under high pressures, was tested and validated from the experimental results at pH 4. The results from Table 2.2 and Figure 2.1 have shown that under high pressures weak acids demonstrate a higher microbial lethality compared to strong mineral acids. In particular, citric, malic, and phosphoric acids have shown highest lethality among all acids tested for the hypothesis. Lower amount of log reductions with strain 32 indicated its pressure resistance while the variation in its response to different pressure – acid treatments shows its acid sensitivity under pressure. Further, the experimental results at other pH levels 3.5, 4.5 and, 5 demonstrated that weak acids were increasingly lethal with a decrease in pH from 5 to 3.5. In particular, citric and malic acid proved to be highly lethal and these results have been confirmed with the previous findings where these acids have shown highest lethality against other microorganisms. Experimental results with WPI solutions as model food system have shown that the buffering action of WPI was not effective against weak acid lethality under high pressures. This was clearly evident when no significant difference, between the control and WPI added buffers, was observed in terms of pressures required for 1 log reduction in microbial population. Also, the predominance of weak acid lethality over strong acid lethality both in presence and absence of WPI validated our hypothesis in our model food system.

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APPENDIX

DATA ANALYSIS

SAS SAMPLE CODE AND COMPUTER OUTPUTS

FOR PROC NLMIXED ANALYSIS

Statistical pair wise comparisons were conducted by examining the significance of the difference in the parameters c12 and d12.

Sample SAS Code:

```
 /* 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     400   -0.11
1     450   -3.28
1     470   -6.24
2     400   -0.22
2     450   -3.68
2     470   -6.51
;
proc print data=trial;
title 'Printing data array and coded variables';
run;

/* Getting separate parameters rather than differences */
proc nlmixed data=trial;
  parms a11=420 a12=414 b11=16 b12=15 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=420 c12=-6 d11=16 d12=-1 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;
```

Note: a_{11} and a_{12} signifies the pressures required by acid1 and acid 2 under comparison, to bring one log reduction in the microbial count during high pressure processing. c_{12} is

the difference between a_{11} and a_{12} and determines which acid is more lethal (acid 1 or acid 2). b_{11} and b_{12} are the gradients of the microbial destruction curves for acid 1 and acid 2 and higher the gradient, more lethal the acid is. d_{12} is the difference between b_{11} and b_{12} and determines which acid is more lethal (acid 1 or acid 2).

pH 3.5 Citric Vs Sulfuric, MDOS 32

Printing data array and coded variables

Obs	sample	pressure	S	z1	z2
1	1	425	-1.304	1	0
2	1	475	-2.213	1	0
3	1	525	-3.710	1	0
4	1	425	-1.189	1	0
5	1	475	-2.099	1	0

Obs	sample	pressure	S	z1	z2
6	1	525	-3.595	1	0
7	1	425	-1.164	1	0
8	1	475	-2.098	1	0
9	1	525	-3.759	1	0
10	2	425	-1.487	0	1
11	2	475	-3.232	0	1
12	2	525	-2.692	0	1
13	2	425	-0.665	0	1
14	2	475	-1.710	0	1
15	2	525	-2.276	0	1
16	2	425	-1.138	0	1
17	2	475	-1.704	0	1
18	2	525	-2.917	0	1

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	410.84	12.8199	18	32.05	<.0001	0.05	383.91	437.78	-1.76E-6
a12	394.40	21.1874	18	18.61	<.0001	0.05	349.88	438.91	-5.41E-7
b11	5.3142	0.7691	18	6.91	<.0001	0.05	3.6985	6.9299	0.000018
b12	3.5190	0.7802	18	4.51	0.0003	0.05	1.8799	5.1580	0.000025
s2e	0.1419	0.04731	18	3.00	0.0077	0.05	0.04253	0.2413	0.000202

Difference parameterization

Parameters

c11	c12	d11	d12	s2e	NegLogLike
420	-6	16	-1	0.1	32052.0489

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	410.84	12.8198	18	32.05	<.0001	0.05	383.91	437.78	5.626E-7
c12	-16.4478	24.7640	18	-0.66	0.5150	0.05	-68.4751	35.5794	4.699E-7
d11	5.3142	0.7691	18	6.91	<.0001	0.05	3.6985	6.9299	-0.00001
d12	-1.7952	1.0955	18	-1.64	0.1186	0.05	-4.0968	0.5063	-0.00002
s2e	0.1419	0.04731	18	3.00	0.0077	0.05	0.04253	0.2413	6.021E-6

pH 4 Citric Vs Sulfuric, MDOS 32

Printing data array and coded variables

Obs	sample	pressure	S	z1	z2
1	1	425	-0.383	1	0
2	1	475	-0.763	1	0
3	1	525	-1.161	1	0
4	1	425	-0.320	1	0
5	1	475	-0.760	1	0
6	1	525	-1.362	1	0
7	2	350	-0.362	0	1
8	2	400	-1.362	0	1
9	2	450	-1.518	0	1
10	2	425	-0.097	0	1
11	2	475	-0.155	0	1
12	2	525	-1.155	0	1
13	2	425	-1.079	0	1
14	2	475	-1.176	0	1
15	2	525	-2.778	0	1

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	503.05	21.3609	15	23.55	<.0001	0.05	457.52	548.58	-1.01E-7
a12	451.35	24.2011	15	18.65	<.0001	0.05	399.77	502.93	2.587E-8
b11	5.6516	3.7851	15	1.49	0.1561	0.05	-2.4161	13.7193	3.297E-7
b12	3.7909	1.7962	15	2.11	0.0520	0.05	-0.03753	7.6193	-5.38E-7
s2e	0.2615	0.09549	15	2.74	0.0152	0.05	0.05798	0.4650	-5.97E-6

Difference parameterization

Parameters

c11	c12	d11	d12	s2e	NegLogLike
420	-6	16	-1	0.1	23805.1881

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	503.05	21.3610	15	23.55	<.0001	0.05	457.52	548.58	4.018E-7
c12	-51.6990	32.2798	15	-1.60	0.1301	0.05	-120.50	17.1039	2.652E-8
d11	5.6516	3.7851	15	1.49	0.1561	0.05	-2.4161	13.7193	-1.19E-6
d12	-1.8607	4.1896	15	-0.44	0.6633	0.05	-10.7907	7.0693	-2.75E-7
s2e	0.2615	0.09549	15	2.74	0.0152	0.05	0.05798	0.4650	0.000032

pH 3.5 Sulfuric Vs Phosphoric, MDOS 32

Printing data array and coded variables

Obs	sample	pressure	S	z1	z2
1	1	425	-0.920	1	0
2	1	475	-1.691	1	0
3	1	525	-3.608	1	0
4	1	425	-0.863	1	0
5	1	475	-1.782	1	0
6	1	525	-3.858	1	0
7	1	425	-0.925	1	0
8	1	475	-2.066	1	0
9	1	525	-4.018	1	0
10	2	425	-1.487	0	1
11	2	475	-3.232	0	1
12	2	525	-2.692	0	1
13	2	425	-0.665	0	1
14	2	475	-1.710	0	1
15	2	525	-2.276	0	1
16	2	425	-1.138	0	1
17	2	475	-1.704	0	1
18	2	525	-2.917	0	1

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	434.09	10.1530	18	42.76	<.0001	0.05	412.76	455.42	6.485E-8
a12	394.40	21.7474	18	18.14	<.0001	0.05	348.71	440.09	-2.2E-7
b11	7.0455	0.9607	18	7.33	<.0001	0.05	5.0271	9.0639	-2.98E-6
b12	3.5190	0.8008	18	4.39	0.0003	0.05	1.8366	5.2013	4.049E-6
s2e	0.1495	0.04984	18	3.00	0.0077	0.05	0.04481	0.2542	-0.0001

Difference parameterization

Parameters

c11	c12	d11	d12	s2e	NegLogLike
420	-6	16	-1	0.1	31965.7164

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	434.09	10.1530	18	42.76	<.0001	0.05	412.76	455.42	3.06E-7
c12	-39.6955	24.0008	18	-1.65	0.1155	0.05	-90.1192	10.7283	3.11E-7
d11	7.0455	0.9607	18	7.33	<.0001	0.05	5.0271	9.0639	-0.00001
d12	-3.5265	1.2507	18	-2.82	0.0113	0.05	-6.1542	-0.8989	-8.56E-6
s2e	0.1495	0.04984	18	3.00	0.0077	0.05	0.04481	0.2542	0.00003

pH 4 Sulfuric Vs Phosphoric, MDOS 32

Printing data array and coded variables

Obs	sample	pressure	S	z1	z2
1	1	425	-0.383	1	0
2	1	475	-0.763	1	0
3	1	525	-1.161	1	0
4	1	425	-0.320	1	0
5	1	475	-0.760	1	0
6	1	525	-1.362	1	0
7	1	475	-0.880	1	0
8	1	525	-1.542	1	0
9	1	525	-5.103	1	0
10	2	425	-1.103	0	1
11	2	475	-1.455	0	1
12	2	525	-2.057	0	1
13	2	425	-1.038	0	1
14	2	475	-1.477	0	1
15	2	525	-2.079	0	1
16	2	475	-1.687	0	1
17	2	525	-2.156	0	1
18	2	525	-5.258	0	1

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	503.43	6.2258	18	80.86	<.0001	0.05	490.35	516.51	-1.96E-8
a12	452.67	8.6215	18	52.50	<.0001	0.05	434.56	470.78	-1.82E-6
b11	10.7176	1.0145	18	10.56	<.0001	0.05	8.5862	12.8490	-8.33E-7
b12	6.1962	0.5918	18	10.47	<.0001	0.05	4.9529	7.4396	0.000016
s2e	0.08900	0.02967	18	3.00	0.0077	0.05	0.02667	0.1513	0.000074

Difference parameterization

Parameters

c11	c12	d11	d12	s2e	NegLogLike
420	-6	16	-1	0.1	377044.684

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient

c11	503.43	6.2258	18	80.86	<.0001	0.05	490.35	516.51	1.41E-6
c12	-50.7577	10.6344	18	-4.77	0.0002	0.05	-73.0997	-28.4156	9.674E-7
d11	10.7176	1.0145	18	10.56	<.0001	0.05	8.5862	12.8490	-0.00002
d12	-4.5213	1.1745	18	-3.85	0.0012	0.05	-6.9889	-2.0538	-0.00002
s2e	0.08900	0.02967	18	3.00	0.0077	0.05	0.02667	0.1513	0.000117

pH 3.5 Malic Vs Sulfuric, MDOS 32

Printing data array and coded variables

Obs	sample	pressure	S	z1	z2
1	1	425	-1.61688	1	0
2	1	475	-2.12689	1	0
3	1	525	-2.67834	1	0
4	1	425	-1.20579	1	0
5	1	475	-2.25979	1	0
6	1	525	-2.30943	1	0
7	2	425	-0.15895	0	1
8	2	475	-0.65308	0	1
9	2	525	-1.11827	0	1
10	2	425	-0.13429	0	1
11	2	475	-0.89899	0	1
12	2	525	-1.57515	0	1

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	357.96	22.4709	12	15.93	<.0001	0.05	309.00	406.92	-1.12E-8
a12	503.01	6.4051	12	78.53	<.0001	0.05	489.06	516.97	9.348E-9
b11	2.4647	0.4770	12	5.17	0.0002	0.05	1.4254	3.5041	6.3E-7
b12	7.4918	1.6484	12	4.54	0.0007	0.05	3.9003	11.0832	-4.48E-8
s2e	0.03945	0.01611	12	2.45	0.0306	0.05	0.004359	0.07454	9.73E-7

Difference parameterization

Parameters

c11	c12	d11	d12	s2e	NegLogLike
420	-6	16	-1	0.1	23184.3955

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	358.04	0.09839	12	3638.80	<.0001	0.05	357.82	358.25	2.2711E9
c12	-358.04	0.09839	12	-3638.8	<.0001	0.05	-358.25	-357.82	2.2711E9
d11	2.4661	0.1562	12	15.78	<.0001	0.05	2.1257	2.8065	-0.05093
d12	-2.4738	0.1562	12	-15.83	<.0001	0.05	-2.8142	-2.1334	-0.04852
s2e	0.1540	0.06287	12	2.45	0.0306	0.05	0.01700	0.2909	0.005442

pH 4 Malic Vs Sulfuric, MDOS 32

Printing data array and coded variables

Obs	sample	pressure	S	z1	z2
1	1	425	-1.038	1	0
2	1	475	-2.266	1	0
3	1	525	-3.380	1	0
4	1	425	-1.292	1	0
5	1	475	-1.856	1	0
6	1	525	-2.735	1	0
7	1	425	-1.359	1	0
8	1	475	-1.469	1	0
9	1	525	-2.800	1	0
10	2	425	-0.383	0	1
11	2	475	-0.763	0	1
12	2	525	-1.161	0	1
13	2	425	-0.320	0	1
14	2	475	-0.760	0	1
15	2	525	-1.362	0	1
16	2	475	-0.880	0	1
17	2	525	-1.542	0	1
18	2	525	-5.103	0	1

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	408.36	11.5218	18	35.44	<.0001	0.05	384.15	432.57	1.126E-8
a12	503.43	5.2847	18	95.26	<.0001	0.05	492.32	514.53	-7.35E-8
b11	4.3097	0.5668	18	7.60	<.0001	0.05	3.1189	5.5006	-1.94E-7
b12	10.7176	0.8612	18	12.45	<.0001	0.05	8.9083	12.5268	5.146E-7
s2e	0.06413	0.02138	18	3.00	0.0077	0.05	0.01922	0.1090	-3.12E-7

Difference parameterization

Parameters

c11	c12	d11	d12	s2e	NegLogLike
420	-6	16	-1	0.1	185158.813

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	408.36	11.5218	18	35.44	<.0001	0.05	384.15	432.57	5.513E-8
c12	95.0665	12.6760	18	7.50	<.0001	0.05	68.4352	121.70	-1.31E-6
d11	4.3097	0.5668	18	7.60	<.0001	0.05	3.1189	5.5006	-0.00002
d12	6.4078	1.0310	18	6.22	<.0001	0.05	4.2418	8.5739	5.484E-6
s2e	0.06413	0.02138	18	3.00	0.0077	0.05	0.01922	0.1090	-0.00015

pH 4 Citric (WPI) Vs Citric MDOS 32

Printing data array and coded variables

Obs	sample	pressure	S	z1	z2
1	1	425	-0.571	1	0
2	1	475	-1.108	1	0
3	1	525	-1.389	1	0
4	1	425	-0.383	1	0
5	1	475	-0.991	1	0
6	1	525	-1.134	1	0
7	1	425	-0.466	1	0
8	1	475	-1.087	1	0
9	1	525	-1.249	1	0
10	2	350	-0.362	0	1
11	2	400	-1.362	0	1
12	2	450	-1.518	0	1
13	2	425	-0.097	0	1

14	2	475	-0.155	0	1
15	2	525	-1.155	0	1
16	2	425	-1.079	0	1
17	2	475	-1.176	0	1
18	2	525	-2.778	0	1

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	488.37	21.4436	18	22.77	<.0001	0.05	443.32	533.42	-1.14E-6
a12	451.35	22.5392	18	20.03	<.0001	0.05	404.00	498.70	6.689E-7
b11	3.7928	2.1082	18	1.80	0.0888	0.05	-0.6362	8.2219	-0.00002
b12	3.7909	1.6728	18	2.27	0.0360	0.05	0.2764	7.3054	-1.06E-6
s2e	0.2268	0.07561	18	3.00	0.0077	0.05	0.06798	0.3857	4.252E-6

Difference parameterization

Parameters

c11	c12	d11	d12	s2e	NegLogLike
420	-6	16	-1	0.1	29828.9398

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	488.37	21.4431	18	22.78	<.0001	0.05	443.32	533.42	2.081E-7
c12	-37.0183	31.1100	18	-1.19	0.2495	0.05	-102.38	28.3414	1.893E-7
d11	3.7929	2.1082	18	1.80	0.0888	0.05	-0.6362	8.2221	-6.53E-7
d12	-0.00205	2.6912	18	-0.00	0.9994	0.05	-5.6562	5.6521	-4.31E-7
s2e	0.2268	0.07561	18	3.00	0.0077	0.05	0.06798	0.3857	0.00001

pH 4 Phosphoric (WPI) Vs Phosphoric, MDOS 32

Printing data array and coded variables

Obs	sample	pressure	S	z1	z2
1	1	425	-1.103	1	0
2	1	475	-1.455	1	0
3	1	525	-2.057	1	0
4	1	425	-1.038	1	0
5	1	475	-1.477	1	0
6	1	525	-2.079	1	0
7	2	425	-1.267	0	1
8	2	475	-1.456	0	1
9	2	525	-2.034	0	1
10	2	425	-1.599	0	1
11	2	475	-1.743	0	1
12	2	525	-2.225	0	1
13	2	425	-1.528	0	1
14	2	475	-1.666	0	1
15	2	525	-2.133	0	1

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	418.49	8.9476	15	46.77	<.0001	0.05	399.42	437.56	7.887E-8
a12	354.79	15.8243	15	22.42	<.0001	0.05	321.06	388.52	7.717E-8
b11	3.1799	0.3718	15	8.55	<.0001	0.05	2.3874	3.9725	1.76E-6

b12	1.8742	0.2570	15	7.29	<.0001	0.05	1.3264	2.4220	-7.84E-6
s2e	0.01205	0.004400	15	2.74	0.0152	0.05	0.002671	0.02143	-0.00002

Difference parameterization

Parameters

c11	c12	d11	d12	s2e	NegLogLike
420	-6	16	-1	0.1	28572.2692

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	418.49	8.9477	15	46.77	<.0001	0.05	399.42	437.56	-2.54E-7
c12	-63.7023	18.1789	15	-3.50	0.0032	0.05	-102.45	-24.9549	1.766E-7
d11	3.1799	0.3718	15	8.55	<.0001	0.05	2.3874	3.9725	-4.98E-6
d12	-1.3057	0.4520	15	-2.89	0.0113	0.05	-2.2692	-0.3423	-0.00002
s2e	0.01205	0.004400	15	2.74	0.0152	0.05	0.002671	0.02143	

pH 4 Malic (WPI) Vs Malic, MDOS 32

Printing data array and coded variables

Obs	sample	pressure	S	z1	z2
1	1	425	-1.038	1	0
2	1	475	-2.266	1	0
3	1	525	-3.380	1	0
4	1	425	-1.292	1	0
5	1	475	-1.856	1	0
6	1	525	-2.735	1	0
7	1	425	-1.359	1	0
8	1	475	-1.469	1	0
9	1	525	-2.800	1	0
10	2	425	-1.138	0	1
11	2	475	-1.150	0	1
12	2	525	-1.939	0	1
13	2	425	-1.109	0	1
14	2	475	-1.130	0	1
15	2	525	-2.030	0	1
16	2	425	-1.114	0	1
17	2	475	-1.154	0	1
18	2	525	-1.947	0	1

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
a11	408.36	10.1712	18	40.15	<.0001	0.05	386.99	429.73	3.036E-8
a12	429.62	15.0714	18	28.51	<.0001	0.05	397.96	461.29	5.742E-9
b11	4.3097	0.5004	18	8.61	<.0001	0.05	3.2525	5.3610	-3.64E-7
b12	3.1599	0.7031	18	4.49	0.0003	0.05	1.6827	4.6372	-1.61E-7
s2e	0.04998	0.01666	18	3.00	0.0077	0.05	0.01498	0.08497	-3.62E-6

Difference parameterization

Parameters

c11	c12	d11	d12	s2e	NegLogLike
420	-6	16	-1	0.1	33626.1083

Parameter Estimates

Parameter	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper	Gradient
c11	408.36	10.1712	18	40.15	<.0001	0.05	386.99	429.73	7.152E-7
c12	21.2629	18.1825	18	1.17	0.2575	0.05	-16.9371	59.4629	1.395E-7
d11	4.3097	0.5004	18	8.61	<.0001	0.05	3.2525	5.3610	-0.00001
d12	-1.1498	0.8630	18	-1.33	0.1994	0.05	-2.9629	0.6633	-2.97E-6
s2e	0.04998	0.01666	18	3.00	0.0077	0.05			