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I am submitting herewith a thesis written by Paula J. Herald entitled "Antibacterial Activity of Selected Naturally Occurring Hydroxycinnamic Acids." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.

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ANTIBACTERIAL ACTIVITY OF SELECTED NATURALLY OCCURRING HYDROXYCINNAMIC ACIDS

A Thesis

Presented for the

Master of Science

Degree

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Paula J. Herald

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DEDICATION

This thesis is dedicated to Mrs. Jo Dunn, my grandmother, whose unselfish contributions, inspiration, and love have made my life, and others, more meaningful.

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ABSTRACT

The hydroxycinnamic acids are naturally occurring phenolic compounds commonly found in most plants. They have been shown to possess lipid antioxidant properties, in addition to antifungal, antiviral, and antibacterial activities. The reports on the antibacterial activity have been conflicting. The purpose of this study was to further investigate the antibacterial effects of caffeic, p-coumaric, and ferulic acids, the three most common hydroxycinnamic acids, against a variety of gram-positive and gram-negative organisms.

Bacillus cereus, Escherichia coli, Pseudomonas fluorescens, and Staphylococcus aureus were grown in Trypticase Soy Broth (TSB) at optimum incubation temperatures for 9 or 48 hours at pH's of 5.0, 6.0, 6.5, or 7.0 depending upon the organism. Growth was measured by plate counts in Trypticase Soy Agar (TSA).

Limited inhibition of P. fluorescens with the compounds was found at pH 7.0, 6.5, and 6.0. Growth of E. coli was inhibited by the hydroxycinnamic acids at pH 6.0 and 5.0, but not at 7.0. Results suggested that inhibition was inversely related to the pH. The hydroxycinnamic acids were inhibitory to S. aureus at pH 7.0, 6.0, and 5.0 with the level of inhibition increasing at pH 5.0. The most effective acid was p-coumaric acid which exhibited lethal activity at pH 5.0. Bacillus cereus was sensitive to all of the acids, and a lethal

effect was observed with p-coumaric acid. Higher concentrations of ferulic acid had increased activity while 1000 ppm of p-coumaric acid had no significant increase in activity over 500 ppm.

Injury of S. aureus with the test compounds was monitored in TSA and Staphylococcus #110 medium. The most injury occurred during the lag phase of growth with 500 ppm p-coumaric acid at pH 7.0 and 6.0. No significant injury over time was observed with the other compounds.

Escherichia coli was grown in a glucose salts medium containing a ^{14}C-labeled amino acid mixture. Leakage of incorporated ^{14}C-labeled compounds after exposure to 1000 ppm p-coumaric acid was detected with liquid scintillation counting. There was a maximum loss of 3.92% of incorporated ^{14}C-labeled compounds, expressed as a percentage of the counts per min (cpm) of the whole cells, which suggested inhibition of E. coli by p-coumaric acid was at least partially due to leakage.

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CHAPTER I

INTRODUCTION

The hydroxycinnamic acids are naturally occurring phenolic compounds found in most higher plants. The three most common hydroxycinnamic acids, generally found in fruits and vegetables, are caffeic, p-coumaric, and ferulic acids (Maga, 1978; Ribereau-Gayon, 1972). These compounds are present in the free form as well as glucose or tartaric acid esters, often at concentrations high enough to provide a natural defense mechanism against micro-organisms (Harborne and Corner, 1961). Numerous studies have shown that these compounds also possess lipid antioxidant activity (Daniels and Martin, 1967, 1968; Hudson and Mahgoub, 1980; Pratt and Birac, 1979).

The hydroxycinnamic acids have been shown to have antifungal activity against Saccharomyces cerevisiae (Baranowski et al., 1980), Fusarium nivale (Valle, 1957), and Aspergillus parasiticus (Swaminathan and Koehler, 1976). Caffeic acid has been shown to have antiviral activity (Grodzinska-Zachwieja et al., 1976). There have been conflicting reports as to the antibacterial action of the hydroxycinnamic acids. For example, Grodzinska-Zachwieja and Kahl (1966) demonstrated that chlorogenic acid had bacteriostatic activity, while Leifertova et al. (1975) found no appreciable antibacterial activity with the hydroxycinnamic acids against a variety of bacteria.

Therefore, the objectives of this study were to (1) to determine the various antibacterial effects of selected naturally occurring

hydroxycinnamic acids on a variety of gram-positive and gram-negative bacteria and (2) to investigate possible mechanisms of inhibitory action by these hydroxycinnamic acids.

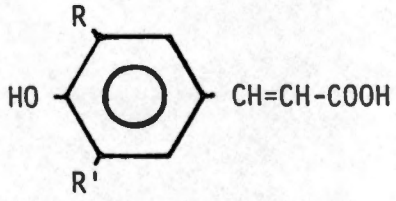
Future evaluations of food additives, such as antimicrobials, will be based on a balance between risks and benefits. Therefore, compounds with dual functions, such as antioxidant/antimicrobial properties, will be especially beneficial (Branen et al., 1980).

CHAPTER II

REVIEW OF LITERATURE

I. CHEMICAL AND PHYSICAL PROPERTIES OF HYDROXYCINNAMIC ACIDS

The most commonly occurring hydroxycinnamic acids are represented by the following structure:



where:

R = R' = H; p-coumaric acid

R = OH; R' = H; caffeic acid

R = OCH_3; R' = H; ferulic acid

R = R' = OCH_3; sinapic acid

Because these compounds possess a double bond, they may exist in either cis or trans isomeric forms. The trans isomers are more stable and are the predominant natural forms that occur in most higher plants (Ribereau-Gayon, 1972).

The reported melting points for caffeic, p-coumaric, and ferulic acids are 195-198°C, 210-213°C, and 89-90°C, respectively (Herrman, 1958). Ong and Nagel (1978) determined the pK_a values of the hydroxycinnamic acids to be in the range of 4.5.

The hydroxycinnamic acids have very similar ultraviolet absorption spectra maxima differing from one another by only 3-5 nm. Reported maxima in ethanol were 324 nm for caffeic acid, 310 nm for p-coumaric acid, and 322 nm for ferulic acid (Ong and Nagel, 1978).

II. BIOSYNTHESIS OF HYDROXYCINNAMIC ACIDS

The major biosynthetic pathway for cinnamic acid and its derivatives has been found to involve the transformation of shikimic acid (Ollis, 1961; Ribereau-Gayon, 1972). Shikimic acid is formed from a condensation of a 4-carbon sugar phosphate, erythose 4-phosphate, which comes from the pentose-phosphate pathway and phosphoenolpyruvic acid. The reaction yields a 7-carbon sugar acid which is then cyclized to shikimic acid. The resultant shikimic acid is transformed to either phenylalanine or tyrosine via several phosphorylated intermediates. Both amino acids are deaminated to form cinnamic acid derivatives. Cinnamic acid is formed from phenylalanine while tyrosine yields p-coumaric acid. Successive hydroxylation of p-coumaric acid yields caffeic acid; ferulic acid results from methoxylation of caffeic acid (Harborne, 1964; Ribereau-Gayon, 1972). Direct evidence for this sequence was shown by feeding ^{14}C-labeled phenylalanine and tyrosine to plants. Measurement of incorporated ^{14}C supported the previous findings. Some plants are unable to convert tyrosine to p-coumaric acid, but all plants can utilize phenylalanine to form cinnamic acid which is then converted to p-coumaric acid (Harborne, 1964).

III. OCCURRENCE OF HYDROXYCINNAMIC ACIDS

The three most common hydroxycinnamic acids in plants are p-coumaric, caffeic, and ferulic. At least one of these acids exists in practically all higher plants and, as a result, in many food

systems (Ribereau-Gayon, 1972; Harborne, 1964). Generally these acids are present as glucose esters which may be liberated by hydrolysis with acid or alkali. The frequent occurrence of these esters suggests that they may be formed during the metabolism of free hydroxycinnamic acids (Harborne and Corner, 1961). In addition to the sugar esters, many cinnamic acid derivatives are esterified with tartaric or quinic acids. These esters are especially common in grapes (Ribereau-Gayon, 1972).

One or more of the common hydroxycinnamic acids has been observed in coffee, tea, molasses, grains, fruits, and vegetables (Maga, 1978). Wheat contains up to 22 ppm p-coumaric acid and lesser amounts of ferulic and caffeic acid. Oats, barley, corn, peanut flour, and soy flour have been shown to contain various hydroxycinnamic acids at levels between 5 to 40 ppm. Total phenolic acid composition of these products ranges from 100 to over 200 ppm (Maga, 1978). The hydroxycinnamic acids are present in both the free and bound forms (Daniels and Martin, 1967, 1968; Maga, 1978; Pratt and Birac, 1979).

Hydroxycinnamic acids have been isolated from vegetables and fruits at levels as high as 1000 ppm (e.g., caffeic acid in apples) (Maga, 1978). Chicory, potatoes, and radishes, contain the higher levels of caffeic acid while spinach contains over 100 ppm p-coumaric acid (Table 1). These hydroxycinnamic acids are also found in various fruits such as apples, grapes, grapefruit, lemons, oranges, pears, and strawberries. Studies have shown that

TABLE 1. Hydroxycinnamic Acids Isolated From Vegetables

Vegetable	Compound	Amount (ppm)
Broccoli	Caffeic	8
	p-Coumaric	13
	Ferulic	13
	Sinapic	40
Cauliflower	Caffeic	9
	p-Coumaric	35
	Ferulic	2
	Sinapic	20
Chicory	Caffeic	767
	Ferulic	<0.5
	Sinapic	<0.5
Garlic	Caffeic	20
	p-Coumaric	58
	Ferulic	27
	Sinapic	27
Potatoes	Caffeic	280
	p-Coumaric	4
	Ferulic	28
	Sinapic	3
Radish	Caffeic	163
	p-Coumaric	91
	Ferulic	16
	Sinapic	<0.5
Spinach	p-Coumaric	133
	Ferulic	16

Maga (1978).

high concentrations of the hydroxycinnamic acids may be found in the peel of citrus fruits, as shown in Table 2, suggesting that phenolics may function to increase resistance to pests or pathogens (Maga, 1978). There was also evidence that the levels of the hydroxycinnamic acids were influenced by maturity and storage. In general, all concentrations decreased with time (Maga, 1978).

IV. ANALYSIS OF HYDROXYCINNAMIC ACIDS

The hydroxycinnamic acids have been solublized and extracted in polar solvents such as ethanol (Ong and Nagel, 1978), methanol (Pratt and Birac, 1979), and ethyl acetate (Pratt et al., 1981; Swaminathan and Koehler, 1976). Separation and identification methods for these compounds have varied. Ong and Nagel developed a high pressure liquid chromatographic technique to identify and quantitate hydroxycinnamic acid-tartaric acid esters in grapes. Pratt and Birac (1979) and Pratt et al., (1981) used thin layer chromatography (TLC), UV spectral analysis, and gas liquid chromatography (GLC) to study phenolic compounds in soy products. A fungitoxic compound was isolated from potatoes and identified based on its behavior on TLC systems and UV spectrum (Swaminathan and Koehler, 1976).

V. TOXIC AND BENEFICIAL SIGNIFICANCE OF HYDROXYCINNAMIC ACIDS

The hydroxycinnamic acids are present in most higher plant tissues, including plants used as foods, at levels up to 100 ppm.

TABLE 2. Hydroxycinnamic Acid Distribution in Citrus Fruits

	Grapefruit (ppm)		Lemon (ppm)		Orange (ppm)	
Compound	pulp	peel	pulp	peel	pulp	peel
Caffeic	40	51	21	35	50	36
p-Coumaric	0	53	6	102	5	17
Ferulic	30	34	14	40	10	19
Sinapic	5	4	14	10	7	19

Maga (1978).

Both their toxic and beneficial effects were recently summarized by Singleton (1981). He reported that chlorogenic acid, an ester of caffeic and quinic acids, slowed digestion by pepsin at 1 g/l. However, a mixture of hydroxycinnamic acids and phenolic acids, at levels similar to those found in 5% distiller's dried solubles, significantly increased the growth rate of chickens. p-Coumaric acid, as well as o-coumaric acid, butylated hydroxyanisole, and butylated hydroxytoluene, were inhibitory to the activity of some chemical carcinogens when present before or in combination with the chemical. Caffeic acid also has demonstrated the ability to inhibit toxic reactions by tying up free radical intermediates.

Stomach acid secretion and bile flow increased when caffeic acid was administered to laboratory animals at 5-100 ppm. Caffeoyl tartrate, among others, has been recommended as a bile flow stimulant to reduce blood cholesterol. Caffeic, ferulic, and p-coumaric acids have been found as animal excretory products.

Singleton concluded that man has adapted and evolved detoxification mechanisms for the plant phenols due to his diversified diet and regular exposure to these plants constituents.

VI. PROPERTIES OF HYDROXYCINNAMIC ACIDS IN FOODS

Sensory Properties

Phenolic acids have been reported to cause astringent or bitter sensations (Maga, 1978). Taste threshold levels for various hydroxycinnamic acids compared to p-hydroxybenzoic acid are shown

in Table 3. Based upon the amounts present and threshold values, it has been concluded that p-coumaric and ferulic acids may be organoleptically detectable in wheat flour. The combination of various equal portions of phenolic acids had a synergistic effect. For example, p-coumaric and ferulic acids have a combination threshold of 25 ppm (Maga, 1978).

TABLE 3. Taste Thresholds for Various Phenolic Acids

Compound	Threshold (ppm)
p-Hydroxybenzoic	40
Caffeic	90
p-Coumaric	40
Ferulic	90

Maga (1978).

Color

Darkening and discoloration is a common problem with some foods, including sunflower and soy products. This darkening is due to the oxidation of phenolic compounds into dark green or brown colors and is directly proportional to the phenolic content (Maga, 1978). Swaminathan and Koehler (1976) demonstrated this by eliminating the browning reaction in potatoes, which contain caffeic and chlorogenic acids, by immersing sliced potatoes in ethyl acetate which extracted the acids.

Antioxidant Properties

Various hydroxycinnamic acids have been shown to possess some degree of antioxidant activity. Pratt and Birac (1979) found that the hydroxycinnamic acids had antioxidant activity in soybean and other soy products. The major cinnamic acid derivatives found were chlorogenic acid (2.8×10^{-2} moles/kg) and caffeic acid (1.0×10^{-4} moles/kg) with only trace amounts of p-coumaric and ferulic acids. They determined that chlorogenic and caffeic acids possessed appreciable antioxidant activity, indicating that ortho - dihydroxylation is important for antioxidant activity. Daniels and Martin (1967, 1968) extracted caffeic and ferulic acids from oats and measured their antioxidant activities. Their studies showed that caffeic acid has stronger antioxidant activity than ferulic acid. Thumann and Herrman (1980) investigated the antioxidant activity of hydroxycinnamic acids in lard. Caffeic acid was determined to be a strong inhibitor while p-coumaric and ferulic possessed only slight activity.

The naturally occurring phenolic antioxidants have also been compared to artificial antioxidants (Hudson and Mahgoub, 1980; Porter et al., 1977). Phenolic compounds, including caffeic, p-coumaric, and ferulic acids, were extracted from the lipids of ryegrass, broad beans, and lucerne leaves. They were shown to have antioxidant activity, however, the naturally occurring plant phenols were inferior to synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Hudson and Mahgoub, 1980).

Porter and coworkers (1977) evaluated naturally occurring and synthetic antioxidants in linolenic acid monolayers on silica to simulate lipid exposure in freeze-dried membranes of whole tissue foods. Contrary to the study previously cited, it was found that caffeic acid, while less active than BHA, was considerably more active than tertiary butylhydroquinone (TBHQ), BHT, and tocopherol.

Hudson and Mahgoub (1980) suggested that the hydroxycinnamic acids they tested as antioxidants acted in the same manner as BHA and tocopherols, i.e. as free radical inhibitors. They also may have ability to chelate metals due to the interaction of the -CO- group and either the 3- or 4-hydroxy groups.

VII. HYDROXYCINNAMIC ACIDS AS ANTIMICROBIALS

Antibacterial Activity

Only limited studies have been made on the antibacterial activity of the hydroxycinnamic acids, and varying results have been reported. Grodzinska-Zachwieja and Kahl (1966) studied the bacteriostatic action of an ethyl acetate extract of chicory roots containing chlorogenic acid, (an ester of caffeic acid with quinic acid) and isochlorogenic acid. A conventional disc method with sterile filter paper discs containing 10, 25, 50, and 75 mg of the extracted acids was used to evaluate their effect on 24 bacterial strains. Inhibition zones of the microorganisms grown on agar plates were recorded. It was found that the chicory extracts had their strongest bacteriostatic against the gram-positive organisms, including

some Bacillus species and Staphylococcus aureus. Brucella abortus and Salmonella species were the most inhibited of the gram-negatives tested. Leifertova and coworkers (1975) used an agar diffusion method to evaluate several hydroxycinnamic acids as antibacterial agents against a variety of gram-positive and gram-negative bacteria. In contrast to the previous study by Grodzinska-Zachwieja and Kahl, it was concluded that a 1% solution of the hydroxycinnamic acids had no appreciable biological activity against the organisms tested. It has been suggested that differences in these studies may be related to the antimicrobial assay method used, concentration of the compounds, and strain of the organism tested (Davidson and Branen, 1981).

Obtusastyrene (4-cinnamylphenol) and other cinnamylphenol derivatives, compounds structurally analogous to the hydroxycinnamic acids, have been investigated for antimicrobial activity (Jurd et al., 1971). Obtusastyrene and other related compounds were found to be inhibitory to gram-positive bacteria such as B. cereus, S. aureus, and Streptococcus lactis at the 500 ppm level. Lewis and Jurd (1972) found sporostatic action of cinnamylphenols on B. megaterium at 32 ppm. Reversibility of this sporostatic action was observed upon removal of the compound from the nutrient broth.

Antifungal Activity

Most studies on the hydroxycinnamic acids have been investigations of their antifungal activity. Valle (1957) studied an antifungal

factor present in potato leaves, namely chlorogenic acid. He found that Fusarium nivale was totally inhibited by 1000 ppm of chlorogenic acid. Since chlorogenic acid is an ester of caffeic and quinic acids, the antifungal effect of caffeic acid was tested by Valle. The test organism was inhibited with 500 ppm caffeic acid, and he then concluded that the chlorogenic acid in potato leaves may function as a defense mechanism. Conversely, Leifertova et al. (1975) found only slight, if any, inhibition with 300 ppm of either caffeic, p-coumaric, or ferulic acids in a disc assay system. Penicillium notatum was weakly inhibited by caffeic acid. Due to the low solubility of the hydroxycinnamic acids in polar solvents, there may have been little diffusion of the compounds into the medium (Davidson and Branen, 1981).

Swaminathan and Koehler (1976) found that 0.01 M caffeic acid in yeast extract-sucrose (YES) medium caused a 25% reduction in the growth of Aspergillus parasiticus and 50% reduction in the production of aflatoxin. They also isolated a hydroxycinnamic acid derivative with fungitoxic properties that lacks the ortho-dihydroxy structure of caffeic acid.

A peculiar phenomenon in grape must fermentations is the occasional cessation of yeast growth (Lafon-Lafourcade and Ribereau-Gayon, 1979). It has been suggested that these failures may be influenced by the presence of hydroxycinnamic acids in the plant tissue (Baranowski et al., 1980). Ong and Nagel (1978) used high pressure liquid chromatography to show that grapes contain 323 ppm caffeoyl-, 64 ppm

p-coumaroyl-, and 16 ppm feruloyl-tartaric acid esters of the corresponding hydroxycinnamic acids. Baranowski and others (1980) studied the antifungal effect of caffeic, p-coumaric, and ferulic acids against Saccharomyces cerevisiae, a yeast commonly used in grape must fermentation. The yeast was grown in Trypticase Soy Broth (TSB) in the presence of the hydroxycinnamic acids, and growth was monitored by turbidity measurements. They found that 1000 ppm caffeic acid only slowed the growth of the yeast, however p-coumaric and ferulic acids exhibited greater inhibitory action. An increase in the lag phase of the growth of S. cerevesiae was observed with as little as 100 ppm of p-coumaric acid while 1000 ppm caused total inhibition. Ferulic acid had greater activity as total inhibition occurred at levels of 250 ppm or above. The researchers concluded that the activity of these acids was related inversely to their polarity.

Antiviral Activity

The hydroxycinnamic acids also have been examined for antiviral activity. Grodzinska-Zachwieja and co-workers (1976) studied the effect of caffeic acid on multiplication of the DNA (vaccinia and adenovirus) and RNA (poliomyelitis type 1 and parainfluenza type 3) virus strains in cell cultures. They showed that 100 ppm of caffeic acid prevented replication of the DNA viruses, however, 300 ppm was required to inhibit the RNA virus, parainfluenza. No inhibition of poliomyelitis virus was observed. They concluded that caffeic acid could be regarded as a potential antiviral agent, particularly for DNA virus strains.

Numerous studies have been made on the antiviral activity of fruit extracts which consequently may be related to the phenolic compounds present in the fruit tissues. Konowalchuk and Speirs (1976a) studied the inactivation of a variety of viruses with extracts from several fruits including strawberries and grapes. They found that the strawberry extract inactivated poliovirus, herpes simplex virus and coxsackievirus, however inactivation was directly related to the extract concentration and length of exposure. This effect varied with virus type; poliovirus and herpes simplex were the most sensitive. Further studies by Konowalchuk and Speirs (1976b) found that the antiviral activity of grape skin was greater than that of grape pulp leading to the conclusion that compounds responsible for the activity were located in the skin.

These researchers postulated that opposite charges on the phenolic compounds and viruses resulted in coating of the viruses causing inactivation. The active groups are believed to be hydroxyl groups which participate in hydrogen bonding on protein molecules (Konowalchuk and Speirs, 1976a).

VIII. MICROBIAL METABOLISM OF CINNAMIC ACID AND ITS DERIVATIVES

In general, oxidative metabolism of phenolic compounds by microorganism involves hydroxylation of the ring to form diphenolic compounds that may be used as energy sources (Blakley and Simpson, 1964; Harborne, 1964). Whiting and Carr (1959) showed that

Lactobacillus pastorianus var. quinicus, metabolized caffeic acid to dihydrocaffeic acid and ethyl catechol, reduced cinnamic acid to dihydrocinnamic acid, and completely metabolized p-coumaric acid to p-hydroxyphenyl propionic acid and p-ethyl phenol. They concluded that the organism reduced the side chains of the compounds, and then the compounds were subject to decarboxylation.

Blakley and Simpson (1964) investigated the degradation of cinnamic acid by a Pseudomonas species isolated from soil. The oxygen consumption of cells grown in the presence of cinnamic acid was measured, and chromatography was used to identify metabolic intermediates. They found that o-hydroxyphenlypropionic and 2,3-dihydroxyphenylpropionic acids were produced. They concluded that the metabolism of cinnamic acid involved reduction of the double bond on the side chain producing derivatives of hydroxyphenylpropionic acid and then ring cleavage to carbon dioxide and other products. Harborne (1964) reports that ring cleavage involves low energy release reactions while subsequent oxidation of intermediates to carbon dioxide are high energy releasing reactions catalyzed by the organisms' enzymes.

IX. MECHANISM OF INHIBITION OF PHENOLIC COMPOUNDS

For many years, researchers have defined the possible mechanism of all antimicrobials into three areas: (a) destruction or loss of functionality of genetic mechanism, (b) inactivation

of essential enzyme systems, and (c) perturbation of the cell membrane causing permeability changes resulting in loss of cellular contents (Davidson and Branen, 1981; Wyss, 1948). In general, researchers are in agreement that inhibition by phenolic compounds is a result of reaction with the cell membrane, inactivation of essential enzymes, or a combination of these two effects (Davidson and Branen, 1981).

It has been postulated that the free hydroxyl group on many antimicrobial phenolic compounds is the reactive portion since modification or substitution of these side groups will alter the activity (Davidson and Branen, 1981). The exact mechanism of inhibition by phenolic compounds has not been determined, and investigation of some naturally occurring phenolic compounds, such as the hydroxycinnamates, has not been reported. Therefore, more research is needed in this area (Davidson and Branen, 1981).

CHAPTER III

MATERIALS AND METHODS

I. SOURCE OF MATERIALS

Microorganisms

Bacillus cereus ATCC 14579, *Escherichia coli* ATCC 11776, *Psuedomonas fluorescens* ATCC 15456, and *Staphylococcus aureus* ATCC 12600 were obtained from the culture collection of the Food Technology and Science Department of the University of Tennessee, Knoxville. The cultures were grown for 24 hours on slants of Trypticase Soy Broth (TSB, BBL) with 1.5% agar added and stored at 4°C. Optimum incubation temperatures were used for each organism: *B. cereus* (32°C), *E. coli* (35°C), *P. fluorescens* (26°C), *S. aureus* (35°C). In order to maintain viability, cultures were transferred at two week intervals.

Hydroxycinnamic Acids

Caffeic acid (3,4-dihydroxycinnamic acid), *p*-coumaric acid (*p*-hydroxycinnamic acid), and ferulic acid (3-methoxy, 4-hydroxycinnamic acid) were purchased from Sigma Chemical Company, St. Louis, MO. The compounds were stored in their original containers at 4°C until used. Stock solutions of these acids were prepared by dissolving the compounds in 95% ethanol. Solutions of 50,000 ppm (5%) and 100,000 ppm (10%) of each acid were placed in sterile volumetric flasks and stored at room temperature (21°C) to avoid crystallization.

Media

Trypticase Soy Broth (TSB), agar, and peptone were obtained from BBL, Cockeysville, MD. and stored at room temperature until used. The TSB was rehydrated with distilled water as directed, and the pH was adjusted to 7.0 $\pm$ 0.1, or to other pHs as desired, with 1N HCl using a Fisher Accumet Model 230A pH/Ion meter (Fisher Scientific, Pittsburgh, PA.). The TSB was then dispensed in 99 ml quantities in screw cap bottles and autoclaved at 121°C for 15 minutes. Trypticase Soy Agar (TSA) was prepared by adding 1.5% agar to TSB, dispensing into media bottles, and autoclaving at the conditions stated previously. A 0.1% peptone buffer was prepared for use as a diluent, dispensed in 99 ml quantities and autoclaved as before.

II. PREPARATION OF GROWTH MEDIA

One ml of each of the hydroxycinnamic acid stock solutions (50,000 and 100,000 ppm) was added aseptically to bottles of 99 ml sterile TSB, resulting in concentrations of 500 and 1000 ppm of each of the compounds. A control bottle was prepared by adding 1 ml of 95% ethanol to 99 ml of TSB.

III. ENUMERATION OF CELL GROWTH

Prior to inoculation of the growth media, the stock culture (1 loop) was grown for 24 hours in 10 ml TSB at the pH desired for study. The 24 hour culture was then transferred (0.1 ml) into 10 ml of TSB and grown for the length of time required to reach mid-log phase.

Then 1.0 ml of the culture was added to 99 ml of TSB containing either 500 or 1000 ppm of caffeic acid, p-coumaric acid, ferulic acid, or 1 ml of 95% ethanol (control). These solutions were then incubated at optimum temperatures for the particular organism used.

Growth of the organism was monitored at periodic intervals according to the pH at which the organism was grown. Cell growth was determined by plate counts (A.P.H.A., 1972) in TSA. The plates were incubated at the appropriate temperatures for each organism and counted after 48 hours.

Table 4 lists the precise conditions of pregrowth times, incubation temperatures, pH levels, and time intervals used to evaluate each organism.

IV. STATISTICAL ANALYSIS

A split-plot design over time was used to analyze log counts from the growth of each bacteria in the presence of the hydroxycinnamic acids at each pH level. Two replications were performed. Analysis of variance (ANOVA) using the General Linear Models Procedures (GLM) was employed to determine the effects of acid, levels, and pH on the growth of the organism over time (Sokal and Rohlf, 1969; Barr and Goodnight, 1979). Significant effects ($p<0.05$) were analyzed with multiple regression analysis, and significant differences in mean values were identified by estimate statements (Barr and Goodnight, 1979). Studies at pH 5.0 with S. aureus and E. coli were evaluated separately due to the different time intervals required to monitor

TABLE 4. Conditions for Growth of Organisms in Presence of the Hydroxycinnamic Acids

Organism	pH	Pre-growth time (hrs)	Incubation temp.(°C)	Enumeration interval (hrs)
B. cereus ATCC 14579	7.0	2.5	32	0, 3, 6, 9
	6.5	3.0	32	0, 3, 6, 9
	6.0	3.5	32	0, 3, 6, 9
E. coli ATCC 11776	7.0	2.0	35	0, 3, 6, 9
	6.0	2.0	35	0, 3, 6, 9
	5.0	12.0	35	0, 12, 24, 36, 48
P. fluorescens ATCC 15456	7.0	3.0	26	0, 3, 6, 9
	6.5	3.5	26	0, 3, 6, 9
	6.0	4.0	26	0, 3, 6, 9
S. aureus ATCC 12600	7.0	2.0	35	0, 3, 6, 9
	6.0	2.5	35	0, 3, 6, 9
	5.0	10.0	35	0, 12, 24, 36, 48

growth. A t-test (Barr and Goodnight, 1979) was used to further separate and identify differences among concentrations of the individual acids.

The percent (%) inhibition of the acids was calculated according to the following formula:

$$\% \text{ Inhibition} = \frac{\text{control cell count - sample cell count}}{\text{control cell count}} \times 100.$$

VI. DETERMINATION OF INJURY TO *STAPHYLOCOCCUS AUREUS* BY THE HYDROXYCINNAMIC ACIDS

Material and Methods

S. aureus was grown at the identical conditions and pH levels as previously described in Table 4. Enumeration of cell growth was performed at the intervals noted in Table 4, however, plate counts were made simultaneously in both TSA and Staphylococcus #110 Medium (Staph 110, Difco, Detroit, MI.). The Staph 110 medium contained 7% NaCl which would be inhibitory to structurally injured cells (Gray et al., 1974). The plates were incubated at 35°C and counted after 48 hours.

Statistical Analysis

Statistical analysis followed the same procedure as stated in Section IV with differences between the two media due to the hydroxycinnamic acids evaluated over time. A t-test was used to find significant differences between the two media over time for each concentration of the hydroxycinnamic acids.

Percent (%) injury was determined by the following formula:

$$\% \text{ Injury} = \frac{\text{TSA cell count - Staph 110 cell count}}{\text{TSA cell count}} \times 100.$$

V. LEAKAGE OF ^{14}C-LABELED AMINO ACIDS FROM ESCHERICHIA COLI

A glucose salts (GS) medium, first desribed by Kay and Gronlund (1969), was modified to contain the following ingredients: dipotassium phosphate, 3g/l; ferric sulfate, 0.5mg/l; magnesium sulfate, 0.5g/l; glucose, 4g/l; mannitol, 1g/l; and NaCl, 2g/l. All of the ingredients except glucose and magnesium sulfate were dissolved in distilled water and adjusted to pH 6.0 with 1N HCl prior to autoclaving at 121°C for 15 min. Appropriate quantities of filter sterilized stock solutions of glucose and magnesium were added to complete the medium. Ammonium phosphate (3g/l), normally present in GS medium, was omitted to avoid a nitrogen source other than those to be added later. Mannitol was added as an additional carbon source (Buchanan and Gibbons, 1974). Sodium chloride was also added since it was present in most defined media for E. coli (Guirard and Snell, 1981).

E. coli ATCC 11776 was grown in TSB at pH 6.0 for 24 hours at 35°C and then transferred (10 ml) to 250 ml of the modified GS medium containing 0.48 mg/ml of a 30 mg/l amino acid stock solution containing the following amino acids: L-alanine, L-arginine, L-asparti c

acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, and L-valine. Twenty five ml of the cells were then transferred to 250 ml of GS medium containing 12.5 µci of (^{14}C(U))-L-amino acid mixture (New England Nuclear, Boston, MA) in addition to 0.24 µg/ml of the cold amino acid mixture required to insure adequate growth. The final concentration of the ^{14}C-amino acids was 0.38 µg/ml of GS medium. The same amino acids used in the cold sample were present in the radioactive mixture (Table 5).

The medium was incubated for 24 hrs. at 35°C. Centrifugation at 8000 xg and 25°C in a IEC B-20A centrifuge (International Equipment Co., Needham Heights, MA.) was used to remove the cells. The cells were washed three times with a 0.05 M citrate buffer, pH 6.0. The cells were then resuspended in the buffer to an optical density of 0.4 measured at 625 nm on a Shimadzu UV-190 spectrophotometer (Kyoto, Japan), and then distributed in 20 ml aliquots in centrifuge tubes. Cells were exposed to either 95% ethanol or 1000 ppm p-coumaric acid for 0, 10, 20, or 30 minutes. The tubes were then centrifuged at 12,000 xg to remove the cells. Samples (2ml) of the resultant supernatant liquid were added to vials of 15 ml of Scintiverse (Fisher Scientific Co., Fairlawn, NJ) scintillation fluid. A 2 ml aliquot of the cell suspension was also added to the scintillation vials. Counts per min (cpm) were determined on a Searle Tracor Analytic liquid scintillation spectrometer (Searle, Chicago, IL.).

Similar methods were used in attempt to measure the leakage

TABLE 5. (^{14}C(U))-L-amino Acids Present in Mixture and Their Relative Concentration

(^{14}C(U))-L-amino acid	μCi amino acid/ mCi mixture
L-Alanine	80
L-Arginine	70
L-Aspartic acid	80
L-Glutamic acid	125
Glycine	40
L-Histidine	15
L-Isoleucine	50
L-Leucine	40
L-Lysine	60
L-Phenylalanine	80
L-Proline	50
L-Serine	40
L-Threonine	50
L-Tyrosine	40
L-Valine	80

of ^{14}C-labeled amino acids from S. aureus or B. cereus. However, little or no growth of the organisms in the modified GS medium occurred.

CHAPTER IV

RESULTS AND DISCUSSION

I. INHIBITION OF PSEUDOMONAS FLUORESCENS

Pseudomonas fluorescens was not inhibited significantly with any of the hydroxycinnamic acids tested. Table 6 shows the percent inhibition of the spoilage organism at 9 hours where inhibition was the greatest. Because the concentrations (500 and 1000 ppm) of the compounds used were not a significant factor influencing inhibition, all subsequent data was averaged in Table 6. Growth and inhibition at pH 6.0 was significantly less than that at pH 7.0 and 6.5. This pH level (6.0) was found to be at the lower end of a pH range tested for P. fluorescens growth in preliminary studies. The stress of the organism at pH 6.0 prevented a growth rate comparable to those at pH 7.0 and 6.5 which further indicated that the compounds alone did not affect the growth rate. Figures 1, 2, and 3 are growth curves of P. fluorescens in the presence of the hydroxycinnamic acids, without distinction between concentrations. The figures show the rates of growth for all of the compounds closely followed that of the control. It may be observed that a pH 6.5 and 7.0 p-coumaric acid slowed the growth of the organism to a greater extent, but this was not found to be significant ($p<0.05$).

As stated in the Review of Literature, a Pseudomonas species was able to metabolize cinnamic acid (Blakley and Simpson, 1964),

TABLE 6. Percent Inhibition of *P. fluorescens* with the Hydroxycinnamic Acids at 9 Hours[1]

Hydroxycinnamic acid	Percent Inhibition		
	pH 7.0	pH 6.5	pH 6.0
Caffeic	73.7	73.4	0.0
p-Coumaric	90.9	91.9	74.0
Ferulic	64.9	66.8	89.6

[1]Average percent inhibition for 500 and 1000 ppm.

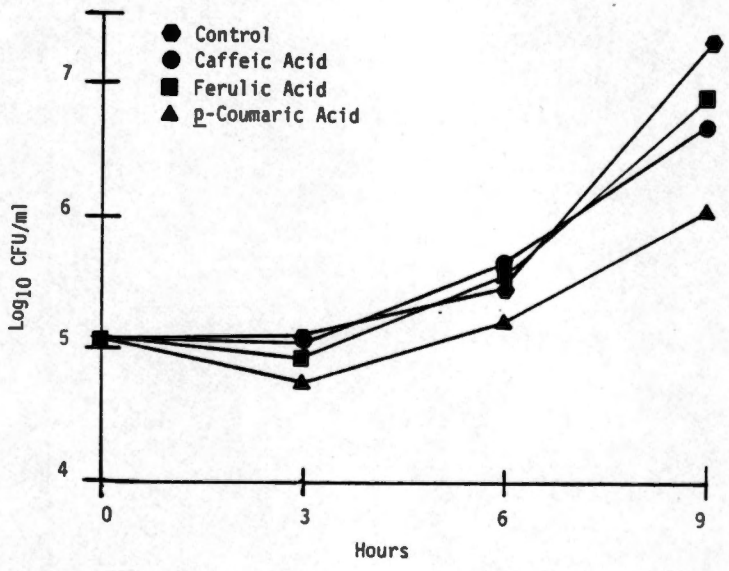


FIGURE 1. Growth of P. fluorescens in the presence of the hydroxycinnamic acids at pH 7.0. (Concentrations of 500 and 1000 ppm were not significantly different and were pooled).

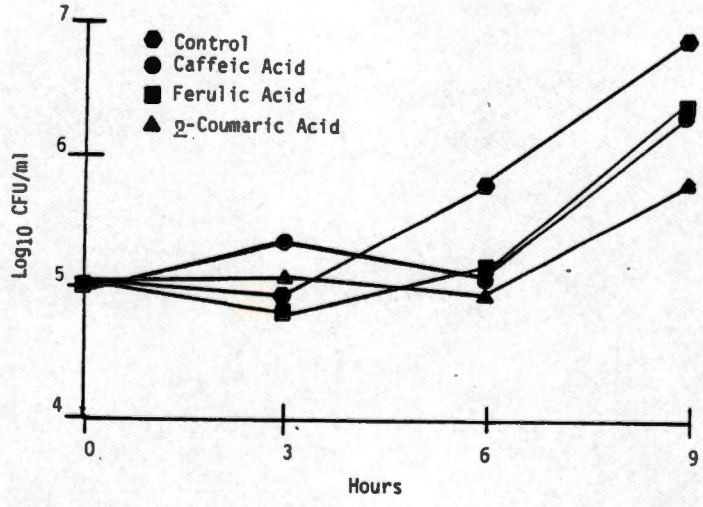


FIGURE 2. Growth of P. fluorescens in the presence of the hydroxycinnamic acids at pH 6.5. (Concentrations of 500 and 1000 ppm were not significantly different and were pooled).

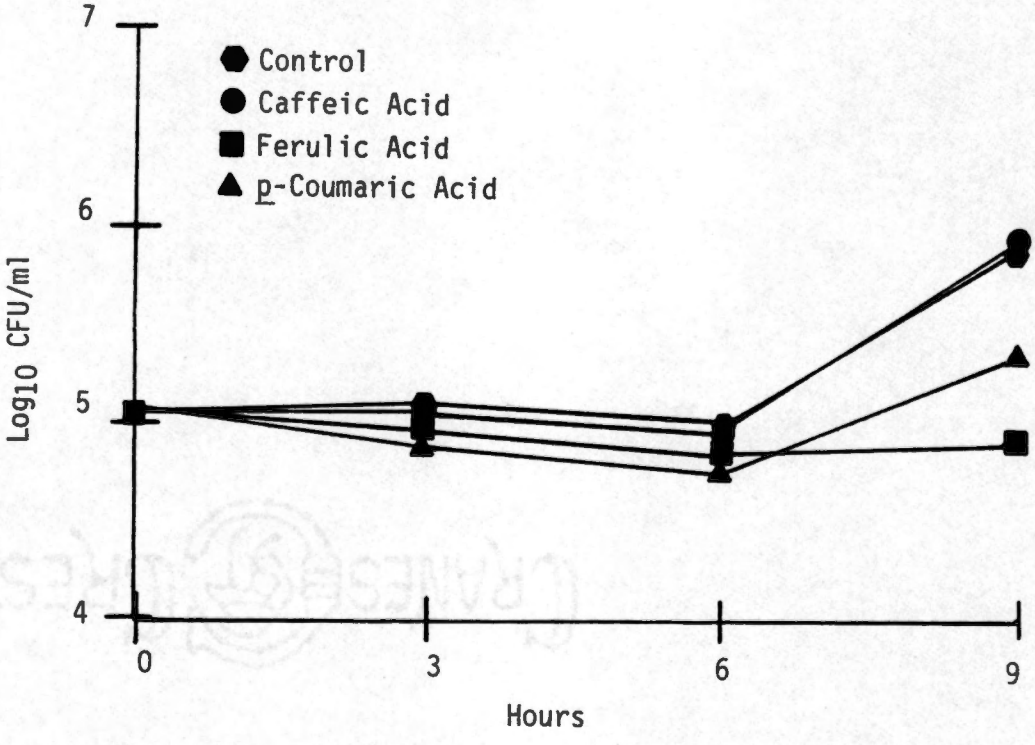


FIGURE 3. Growth of P. fluorescens in the presence of the hydroxycinnamic acids at pH 6.0. (Concentrations of 500 and 1000 ppm were not significantly different and were pooled).

and Frazier and Westhoff (1978) also report that many Pseudomonas species are able to utilize noncarbohydrate carbon sources for energy. This suggested that P. fluorescens may have partially incorporated the hydroxycinnamic acids. Also, the gram-negative cell wall, which contains a high level of lipid, may have provided a barrier to the compounds tested (Lamanna, 1973). Slime formation, characteristic of Pseuomonas spp., also may have been a factor (Frazier and Westhoff, 1978). However, another study with the same P. fluorescens ATCC 15456, (Davidson and Branen, 1980) found it to be susceptible to phenolic antioxidants (butylated hydroxyanisole and butylated hydroxytoluene).

II. INHIBITION OF ESCHERICHIA COLI

The hydroxycinnamic acids provided significant inhibitory action on E. coli over time at pH 6.0 and 5.0 ($p<0.05$), but the compounds were not different from the control at pH 7.0. The concentrations of the acids (500 and 1000 ppm) did not influence inhibition at pH 7.0 or 6.0, but comparison of means of concentrations averaged over time at pH 5.0 showed that 1000 ppm was significantly more inhibitory than 500 ppm ($p<0.01$). (Equations of the regression lines for the growth curves are presented in Appendix A).

Figures 4, 5, and 6 are growth curves for E coli depicting the inhibition trends of the hydroxycinnamic acids. As observed in Figure 4, the growth rate of E. coli was not affected by the presence of the hydroxycinnamic acids at pH 7.0. However, p-coumaric

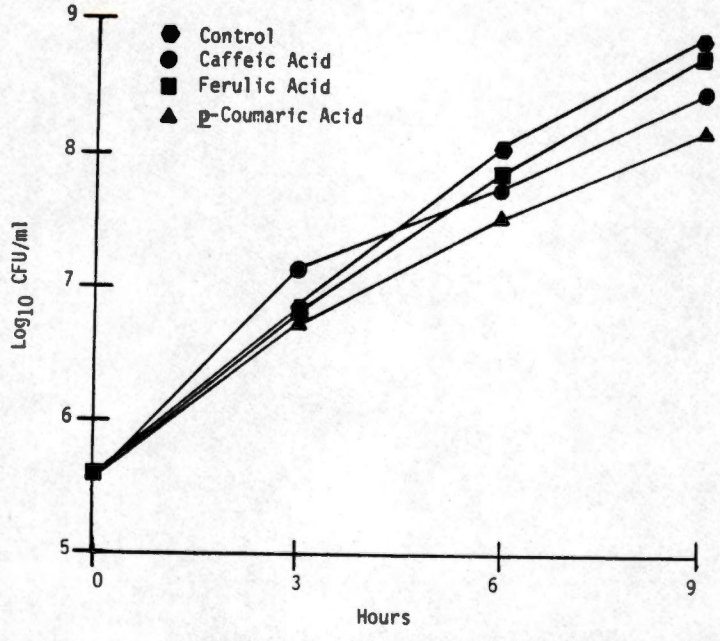


FIGURE 4. Growth of E. coli in the presence of the hydroxycinnamic acids at pH 7.0. (Concentrations of 500 and 1000 ppm were not significantly different and were pooled).

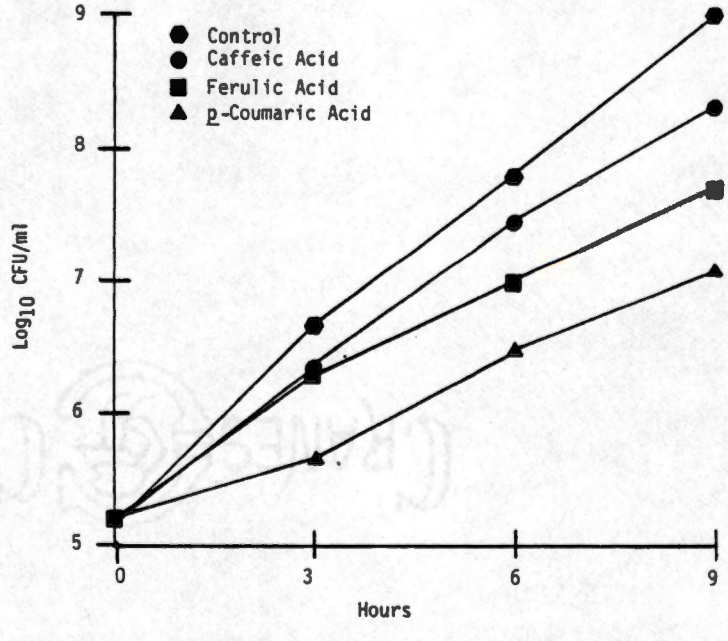


FIGURE 5. Growth of E. coli in the presence of the hydroxycinnamic acids at pH 6.0. (Concentrations of 500 and 1000 ppm were not significantly different and were pooled).

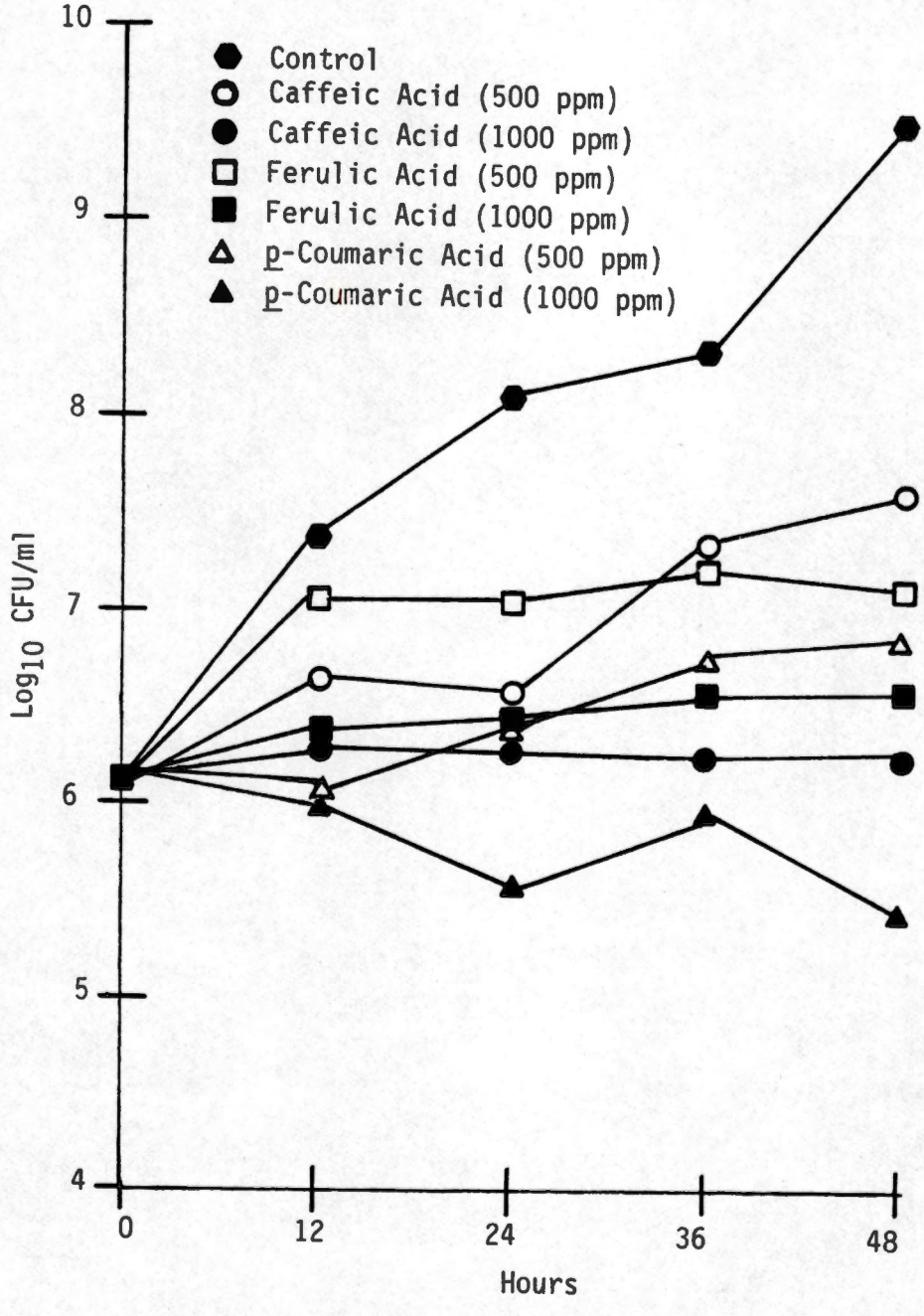


FIGURE 6. Growth of E. coli in the presence of the hydroxycinnamic acids at pH 5.0.

acid influenced the growth rate more than the other compounds even though this was not significant. Figure 5 shows how the growth rates were slowed significantly over time with p-coumaric and ferulic acids ($p<0.01$) with no difference between the two acids at pH 6.0. The growth rate with caffeic acid was not significantly different from the control or ferulic acid. All of the curves followed the same growth pattern, however, there was a difference in the growth rates. p-Coumaric acid was the most effective compound.

The growth rates were slowed to a greater degree at pH 5.0 (Figure 6). There was a marked increase in the lag period with a bacteriostatic effect continuing throughout the test period. Analysis showed that all of the acids were significantly more inhibitory than the control, ($p<0.01$) but no differences between the individual acids were found. It was also observed that p-coumaric acid was the most effective compound.

The inhibition of E. coli was inversely related to pH level in this study. This suggests a combination effect of the hydroxycinnamic acids and pH. The pK_a values of the hydroxycinnamic acids are reported to be in the range of 4.5 (Ong and Nagel, 1978); at lower pH levels, the acids would be more undissociated, and the effectiveness of inhibitors, such as sodium benzoate, increases as the compound becomes more undissociated (Frazier and Westhoff, 1978). This modification of the side chain (-CH=CH-COOH) may have

aided the penetration of the cell membrane allowing the free hydroxyl group to react (Davidson and Branen, 1981).

III. INHIBITION OF STAPHYLOCOCCUS AUREUS

Staphylococcus aureus was significantly inhibited over time by the hydroxycinnamic acids at each pH level tested. There was no effect of pH over time between pH 6.0 and 7.0, and the concentrations of the acids (500 and 1000 ppm) did not differ significantly in their inhibitory ability. (Equations of the regression lines for the growth curve are presented in Appendix A). Figures 7 and 8 show the growth of S. aureus in the presence of the hydroxycinnamic acids at pH 7.0 and 6.0, respectively. The log counts were averaged for concentrations because no significant differences were found between them. Analysis showed that p-coumaric acid was significantly more inhibitory than ferulic acid ($p<0.01$), caffeic acid ($p<0.01$), and the control ($p<0.01$) over time at both pH levels. At pH 7.0, the growth of S. aureus in the presence of p-coumaric acid never approached that of the control or other acids (Figure 7). Ferulic acid also slowed the growth rate significantly more than caffeic acid ($p<0.01$), while growth of S. aureus in the presence of caffeic acid closely followed that of the control. A similar inhibition trend was found at pH 6.0 as was seen with pH 7.0 (Figure 8). Again, p-coumaric acid slowed the growth rate to nearly zero, and ferulic acid significantly decreased the growth rate of S. aureus. The caffeic acid and control cultures had approximately the same rates of growth.

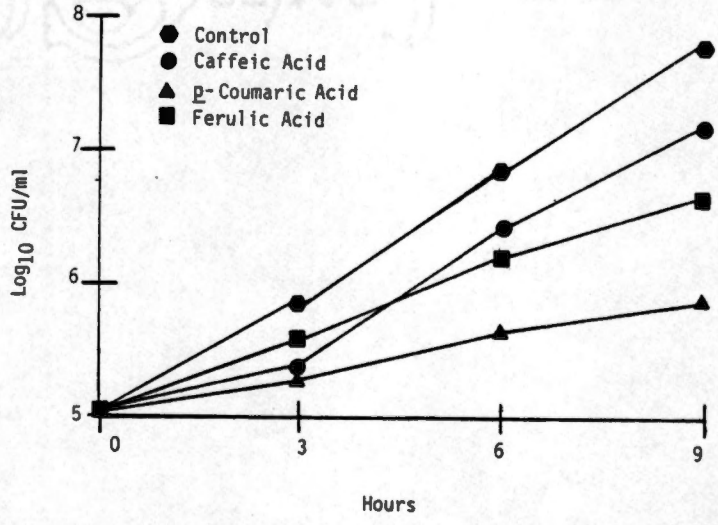


FIGURE 7. Growth of S. aureus in the presence of the hydroxycinnamic acids at pH 7.0. (Concentrations of 500 and 1000 ppm were not significantly different and were pooled).

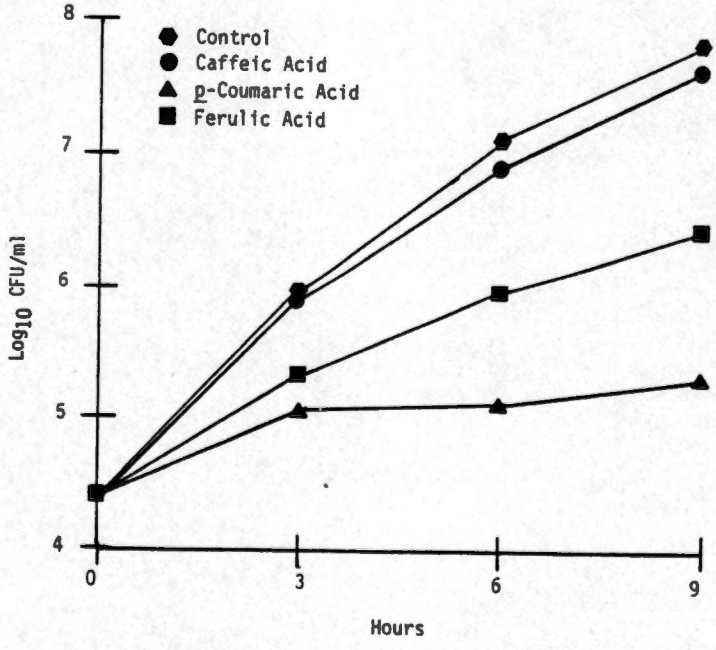


FIGURE 8. Growth of S. aureus in the presence of the hydroxycinnamic acids at pH 6.0. (Concentrations of 500 and 1000 ppm were not significantly different and were pooled).

A dramatic increase in inhibition of S. aureus was found at pH 5.0 (Figure 9). The concentrations of the acids used, 500 and 1000 ppm, were found to differ significantly in their effect on the growth of the microorganism over time ($p<0.01$). All of the hydroxycinnamic acids were significantly different from the control ($p<0.05$) at 500 ppm. p-Coumaric acid was the most effective compound while ferulic acid was intermediate in activity between caffeic and p-coumaric acids, having no significant difference from either compound. At 1000 ppm, it was evident that the compounds had a lethal activity up to 24 hours with log counts continuing to decline through 48 hours with p-coumaric and ferulic acids. Analysis showed that p-coumaric and ferulic acids had approximately the same activity over time, and that ferulic acid was different from caffeic acid at the 9% significant level.

The differences between 500 and 1000 ppm of the individual acids was examined further and it was found that there was no difference between the two concentrations of caffeic acid. This indicated that the inhibitory effectiveness of the compound was not dependent upon the concentration when evaluated over time. In contrast, it was shown that significant differences did exist between 500 and 1000 ppm ferulic acid ($p<0.05$) and p-coumaric acid ($p<0.05$). Therefore inhibition of S. aureus by these compounds was directly related to concentration. It also suggested that more inhibition might have been obtained with higher concentrations.

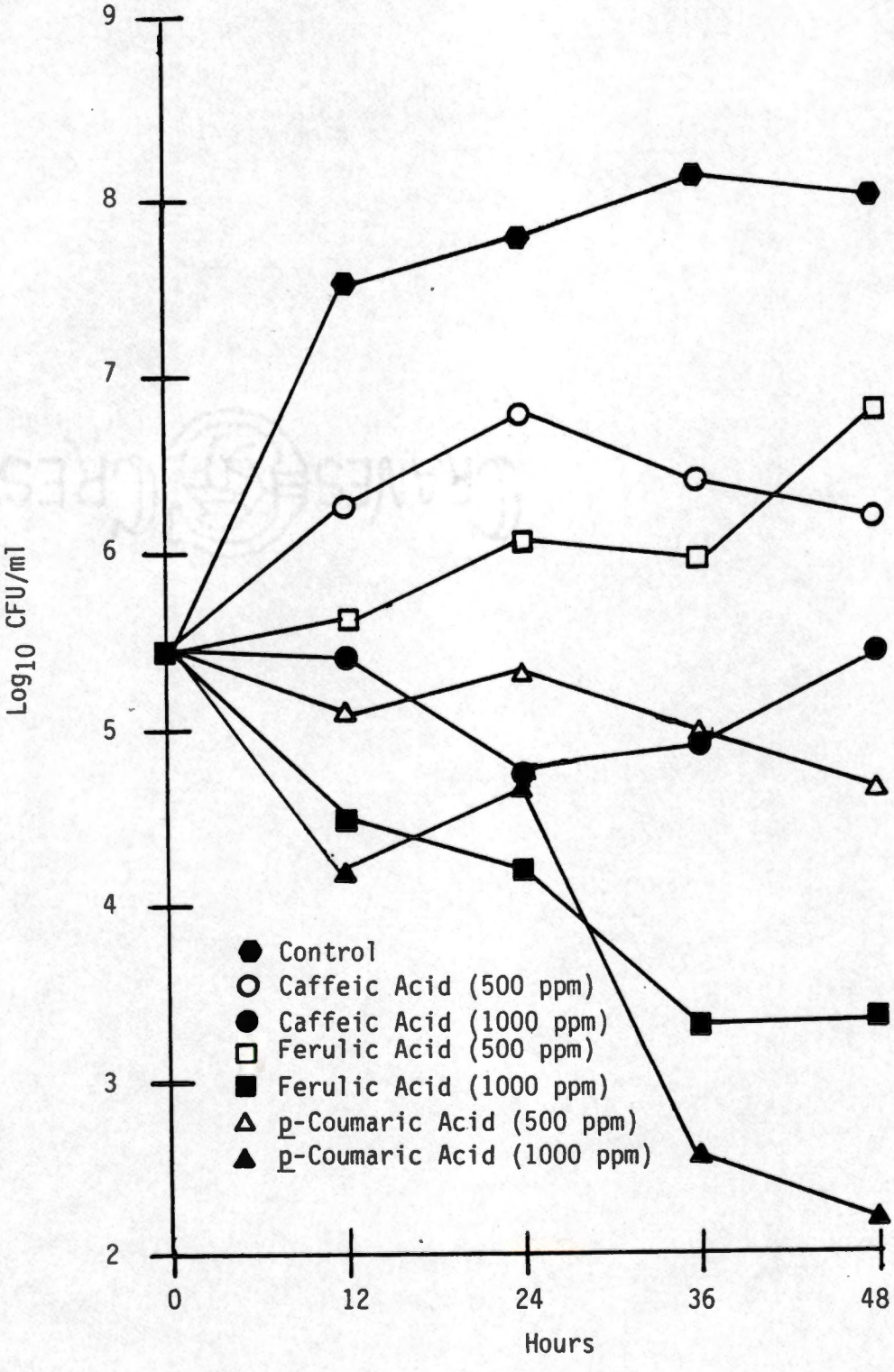


FIGURE 9. Growth of S. aureus in the presence of the hydroxycinnamic acids at pH 5.0.

As with E. coli, the effectiveness of the hydroxycinnamic acids against S. aureus increased as pH decreased from 6.0 to 5.0. In contrast to E. coli where none of the acids were lethal, both p-coumaric and ferulic acids were lethal against S. aureus. This finding suggested that, in addition to the pH effect on undissociation of the acids, there also may be increased susceptibility of the gram-positive organism due to the lack of protection from lipids in the cell wall or membrane as present in gram-negative organisms (Lamanna, 1973). Also, the structure and related polarity of the compounds may be involved (Baranowski et al., 1980), but this will be discussed in the next section.

IV. INHIBITION OF BACILLUS CEREUS

The three hydroxycinnamic acids evaluated were found to possess significant inhibitory activity against Bacillus cereus over time with no effect of pH level. The concentrations (500 and 1000 ppm) of the acids were found to differ significantly across the acids ($p<0.01$), and differences within individual acids were identified with a t-test. The most inhibitory compound was p-coumaric acid which was significantly more effective than ferulic acid ($p<0.01$) and caffeic acid ($p<0.01$). No differences between the latter two acids was found. (The equations of the regression lines for the growth curves are presented in Appendix A).

Figures 10, 11, and 12 show the effect of the hydroxycinnamic acids on B. cereus at the three pH levels evaluated, 7.0, 6.5 and

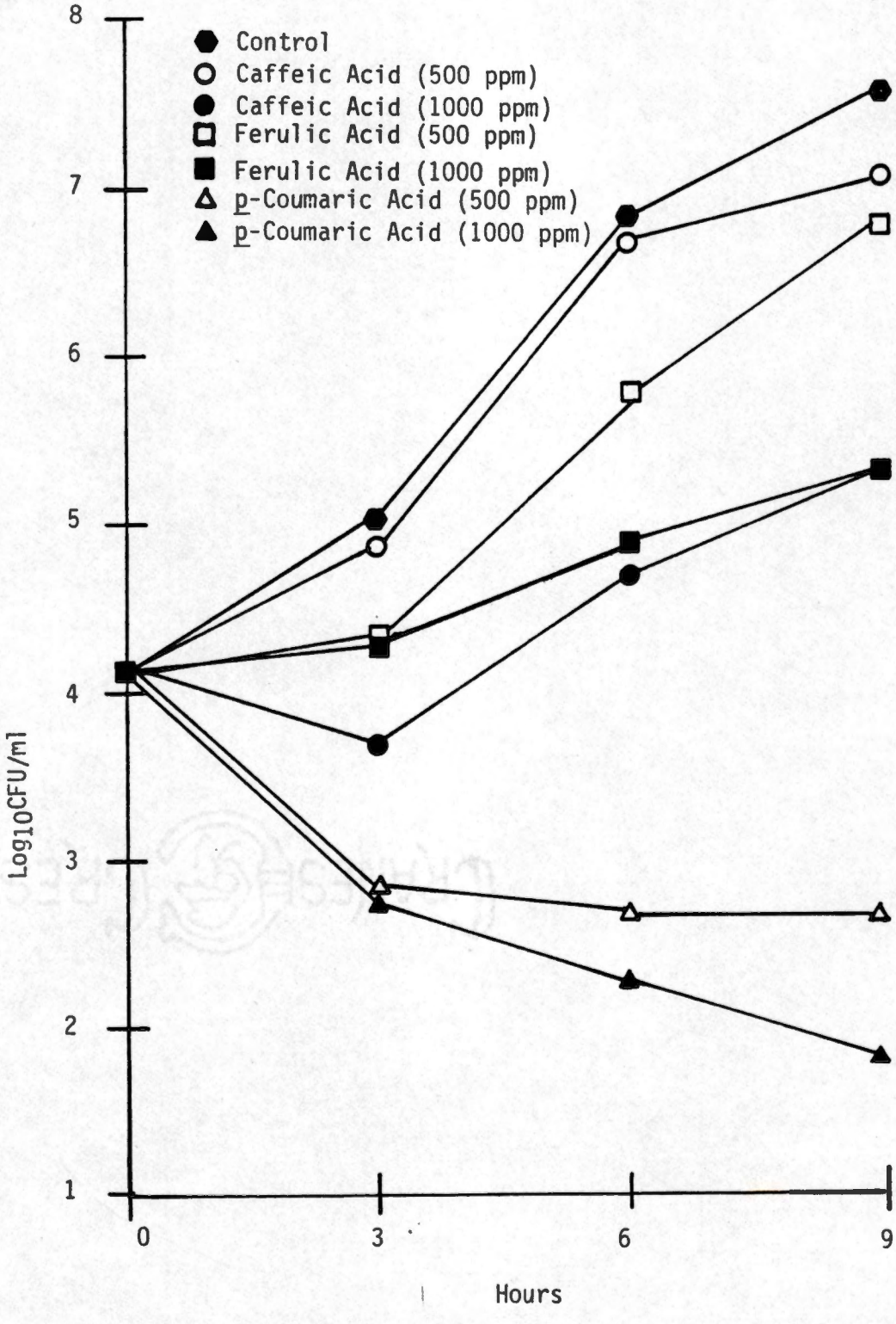


FIGURE 10. Growth of B. cereus in the presence of the hydroxycinnamic acids at pH 7.0.

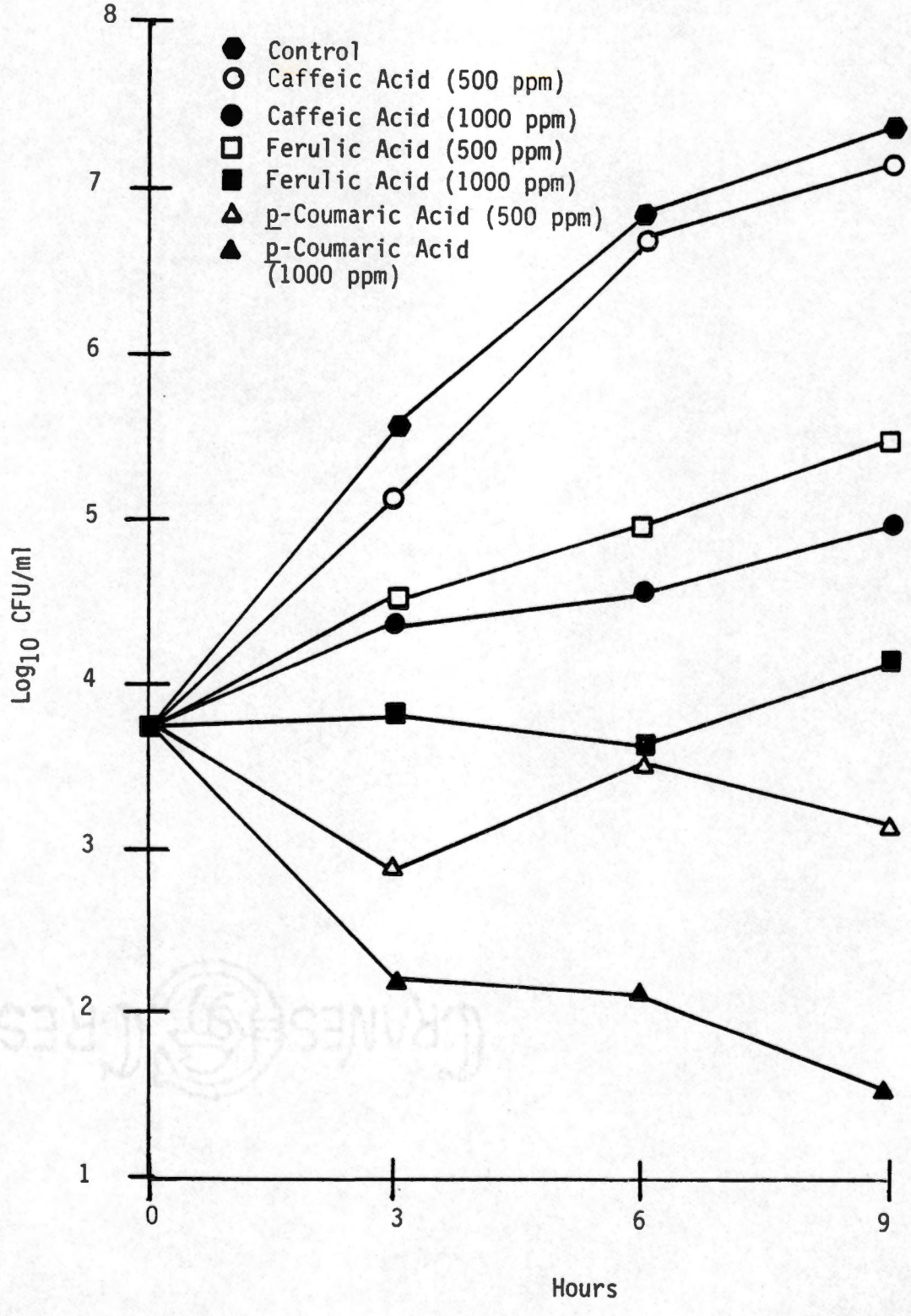


FIGURE 11. Growth of B. cereus in the presence of the hydroxycinnamic acids at pH 6.5.

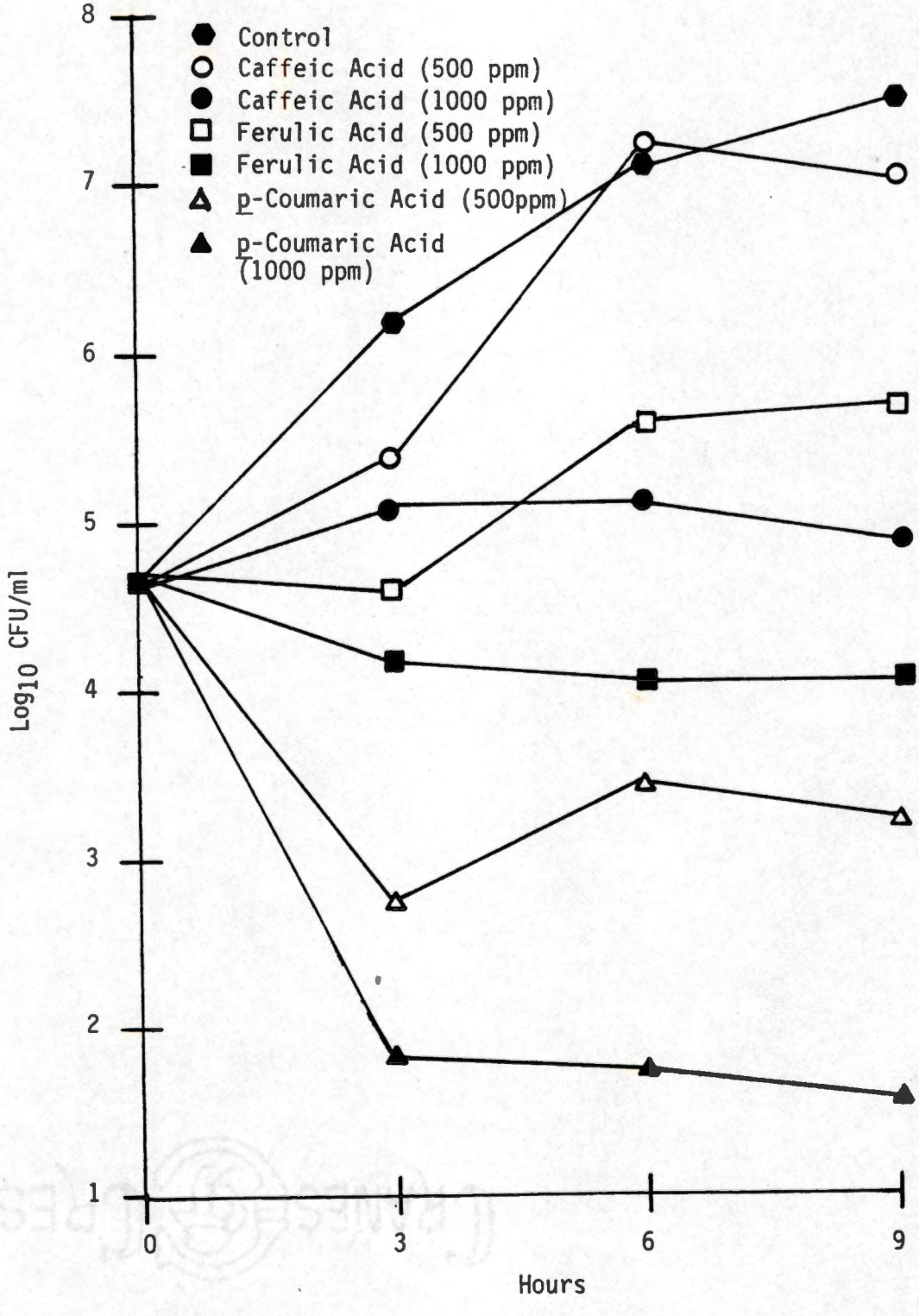


FIGURE 12. Growth of B. cereus in the presence of the hydroxycinnamic acids at pH 6.0.

6.0, respectively. (These pH levels were selected because preliminary studies showed poor growth of B. cereus in TSB at pH levels below 6.0). The concentrations of the acids are separated in the growth curves because they differed from one another over time and pH levels.

With 1000 ppm p-coumaric acid, a sudden drop in viable cells occurred between 0 and 3 hours, and a slight decrease continued throughout the test period at pH 7 (Figure 10). Ferulic and caffeic acids (1000 ppm) were approximately equivalent in their inhibitory effect on B. cereus growth. Similar trends were observed with 500 ppm of the acids, with the exception of caffeic acid which appeared to have little or no activity. Growth rates of B. cereus in the presence of the test compounds at pH 6.5 and pH 6.0 closely resembled those at pH 7.0 (Figures 11 and 12). The activities of 1000 ppm caffeic and ferulic acids increased to where, at pH 6.0, they were virtually bacteriostatic. The higher concentrations of all the acids provided greater activity at all three pH levels.

When evaluated over time for all pH levels, differences were found between concentrations for individual hydroxycinnamic acids. These differences are depicted in Figure 13 for each of the acids. A t-test showed that 1000 ppm caffeic acid differed significantly from the control ($p<0.05$) but not from 500 ppm. There was inhibition with both concentrations of ferulic acid, and 1000 ppm caused significantly increased inhibition over 500 ppm. This suggested that inhibition was proportional to the concentration of caffeic

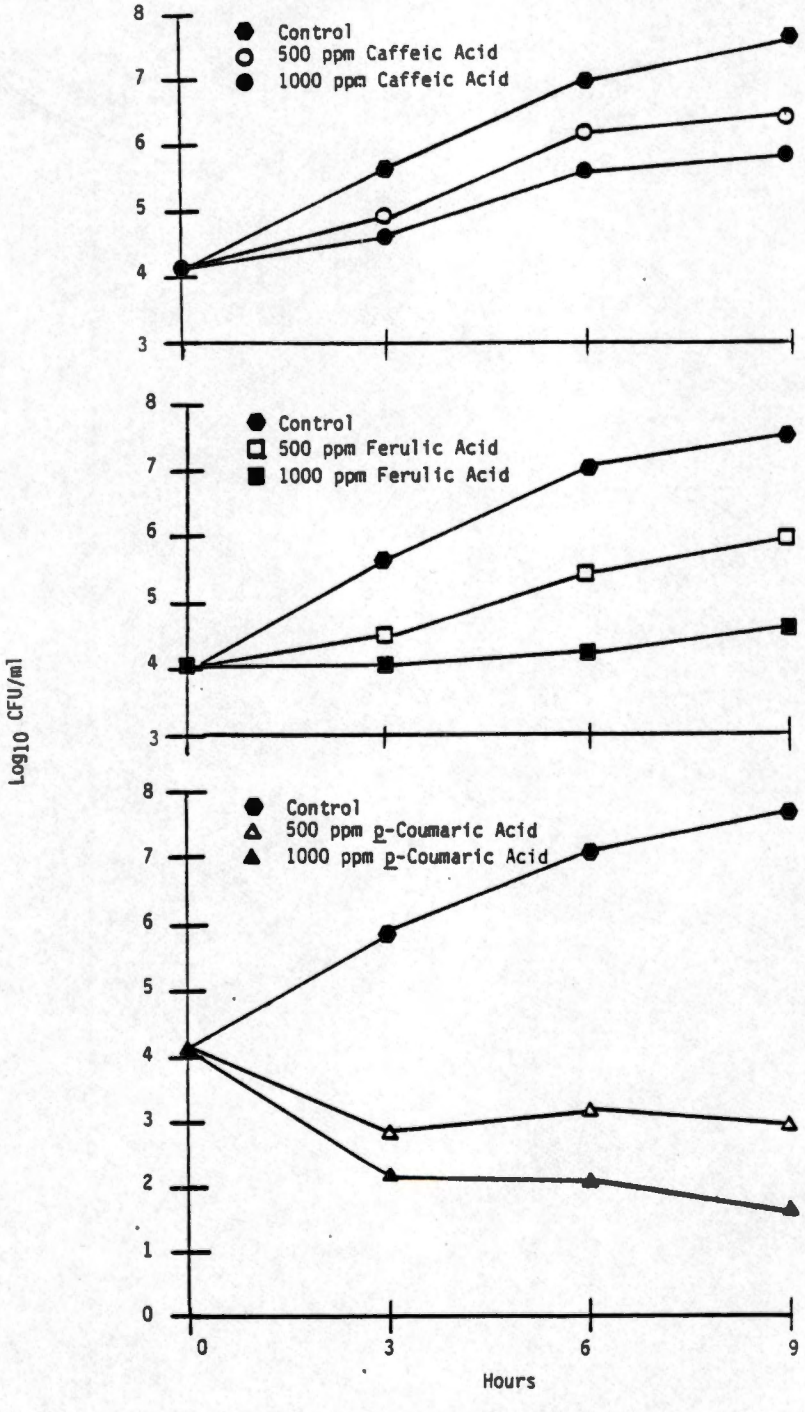


FIGURE 13. Differences in the effect of 500 and 1000 ppm of the hydroxycinnamic acid on the growth of B. cereus.

and ferulic acids, and greater inhibition may be obtainable with concentrations higher than those used in this study. This was not true with concentrations of p-coumaric acid. Both concentrations were inhibitory, however, no significant difference was observed with 1000 ppm over 500 ppm. This indicated that by whatever mechanism inhibition occurred, once the cell was inhibited or damaged, an increased concentration of p-coumaric acid provided no significant increase in activity.

B. cereus was more sensitive to the presence of the hydroxycinnamic acids than the other organisms tested as determined by the greater differences in log counts between the control and acids. Also, B. cereus was the only organism whose inhibition by the hydroxycinnamic acids was not affected by pH. This suggested that the maximum effect of the acids was obtained at pH 7.0. The gram-positive cell wall, containing few lipids (Lamanna, 1973), was less protective to the bacterium as stated in the discussion on S. aureus inhibition. This supports the findings of Grodzinska-Zachwieja and Kahl (1966) who found caffeic acid to be inhibitory to gram-positive organisms tested, including B. cereus and S. aureus. These researchers did not evaluate ferulic or p-coumaric acids. Results from this undertaking were in contrast to these of Leifertova and others (1975) who found only negligible inhibition of various bacteria with hydroxycinnamic acids.

The activity of the compounds against B. cereus, or any of the other organisms tested, may have been related to the polarity

of the compound. Baranowski and coworkers (1980) found inhibition of Saccharomyces cerevisiae was inversely related to the compounds' polarity. They found ferulic acid, the most non-polar compound, had the greatest activity. In contrast to Baranowski and co-workers, p-coumaric acid, with polarity in between caffeic and ferulic acids was the most inhibitory in this study. This suggests that increased solubility in both aqueous and lipid phases may be beneficial to inhibition or prevention of growth (Davidson and Branen, 1980).

V. INJURY TO STAPHYLOCOCCUS AUREUS

A determination of the extent of cell injury was made by exposing the cells to the hydroxycinnamic acids and subsequently plating on a non-selective (TSA) and selective (Staph 110) media. The results of the study are presented in Tables 7, 8, and 9 with percent injury calculated at each time interval. No significant differences were detected between the mean log counts on the two media for any of the pH's levels used. Further analysis with a t-test showed that there were no significant differences in mean log counts over time for each acid and concentration used, except p-coumaric at 500 ppm, pH 7.0. This indicated that, in general, there was little or no injury to the cells which were recovered on the non-selective medium, TSA.

The recovery of S. aureus exposed to p-coumaric acid at pH 7.0 in TSA and Staph 110 is presented in Figure 14. The most injury (96.28%) occurred with 500 ppm at 3 hours which suggested that the

TABLE 7. Log Count Means and Percent Injury of S. aureus at pH 7.0 Recovered in TSA and Staph 110 Medium

Hydroxycinnamic Acid (ppm)	Time (Hrs)	Log Count Mean[1]		Percent Injury[2]
		TSA	Staph 110	
Control (0 ppm)	0	4.88	4.80	16.82
	3	5.14	5.14	0.00
	6	6.41	5.94	66.12
	9	7.07	7.04	6.67
Caffeic (500)	0	4.88	4.80	16.82
	3	5.26	5.20	12.90
	6	6.25	6.04	38.34
	9	6.98	7.25	0.00
Caffeic (1000)	0	4.88	4.80	16.82
	3	5.09	4.82	46.30
	6	5.40	5.33	14.88
	9	5.14	5.96	0.00
p-Coumaric (500)	0	4.88	4.80	16.82
	3	4.63	3.20	96.28
	6	4.18	2.86	95.21
	9	3.80	3.10	80.05
p-Coumaric (1000)	0	4.88	4.80	18.82
	3	4.65	3.79	86.20
	6	3.97	2.82	92.92
	9	3.34	2.40	88.52
Ferulic (500)	0	4.88	4.80	16.82
	3	5.28	5.25	6.67
	6	5.72	6.25	0.00
	9	7.35	6.85	68.37

TABLE 7. (Continued)

Hydroxycinnamic Acid (ppm)	Time (Hrs)	Log Count Mean[1]		Percent Injury[2]
		TSA	Staph 110	
Ferulic (1000)	0	4.88	4.80	16.82
	3	4.82	4.27	71.81
	6	4.88	5.05	0.00
	9	5.64	5.33	51.02

[1]Mean of 2 replications

[2]Percent Injury = $\frac{\text{TSA cell count - Staph 110 cell count}}{\text{TSA cell count}} \times 100.$

TABLE 8. Log Count Means and Percent Injury of S. aureus at pH 6.0 Recovered in TSA and Staph 110 Medium

Hydroxycinnamic Acid (ppm)	Time (Hrs)	Log Count Mean[1]		Percent Injury[2]
		TSA	Staph 110	
Control (0)	0	5.08	5.08	0.00
	3	5.85	6.00	0.00
	6	7.05	6.92	25.87
	9	7.71	7.25	65.33
Caffeic (500)	0	5.08	5.08	0.00
	3	5.87	5.97	0.00
	6	5.79	6.68	0.00
	9	7.36	6.54	84.86
Caffeic (1000)	0	5.08	5.08	0.00
	3	5.63	5.64	0.00
	6	5.40	5.63	0.00
	9	7.06	6.40	78.12
p-Coumaric (500)	0	5.08	5.08	0.00
	3	5.10	4.21	87.12
	6	5.30	4.00	94.99
	9	5.69	4.15	97.12
p-Coumaric (1000)	0	5.08	5.08	0.00
	3	4.84	4.54	49.88
	6	5.08	3.90	93.39
	9	4.73	3.15	97.36
Ferulic (500)	0	5.08	5.08	0.00
	3	5.64	5.64	0.00
	6	5.99	6.50	0.00
	9	6.06	3.82	99.42

TABLE 8. (Continued)

Hydroxycinnamic Acid (ppm)	Time (Hrs)	Log Count Mean[1]		Percent Injury[2]
		TSA	Staph 110	
Ferulic (1000)	0	5.08	5.08	0.00
	3	5.81	5.06	82.22
	6	5.40	5.27	25.87
	9	5.00	5.56	0.00

[1]Mean of 2 replications.

[2]Percent Injury = $\frac{\text{TSA cell count - Staph 110 cell count}}{\text{TSA cell count}} \times 100.$

TABLE 9. Log Count Means and Percent Injury of S. aureus at pH 5.0 Recovered in TSA and Staph 110 Medium

Hydroxycinnamic Acid (ppm)	Time (Hrs)	Log Count Mean[1]		Percent Injury[2]
		TSA	Staph 110	
Control (0)	0	4.84	4.93	0.00
	12	6.26	6.15	22.38
	24	6.93	7.07	0.00
	36	7.34	7.18	30.81
	48	7.34	7.11	41.16
Caffeic (500)	0	4.84	4.93	0.00
	12	5.37	5.12	43.76
	24	5.83	5.10	80.50
	36	5.61	5.48	25.87
	48	5.49	4.93	72.46
Caffeic (1000)	0	4.84	4.93	0.00
	12	4.72	5.12	0.00
	24	5.34	5.10	42.45
	36	4.95	5.00	0.00
	48	4.96	5.84	0.00
p-Coumaric (500)	0	4.84	4.93	0.00
	12	4.41	4.31	20.57
	24	3.99	3.92	14.89
	36	4.40	3.69	80.50
	48	4.63	4.32	51.02
p-Coumaric (1000)	0	4.84	4.93	0.00
	12	3.62	1.76	98.62
	24	2.89	1.55	95.43
	36	1.67	3.04	0.00
	48	2.24	3.06	0.00
Ferulic (500)	0	4.84	4.93	0.00
	12	4.87	4.61	45.04
	24	4.72	4.71	2.28

TABLE 9. (Continued)

Hydroxycinnamic Acid (ppm)	Time (Hrs)	Log Count Mean[1]		Percent Injury[2]
		TSA	Staph 110	
Ferulic (500)	36	4.67	4.44	41.12
	48	4.60	3.28	95.21
Ferulic (1000)	0	4.84	4.93	0.00
	12	3.04	4.00	0.00
	24	2.54	3.04	0.00
	36	3.15	1.66	96.76
	48	3.61	2.65	89.04

[1]Mean of 2 replications.

[2]Percent Injury = $\frac{\text{TSA cell count - Staph 110 cell count}}{\text{TSA cell count}} \times 100.$

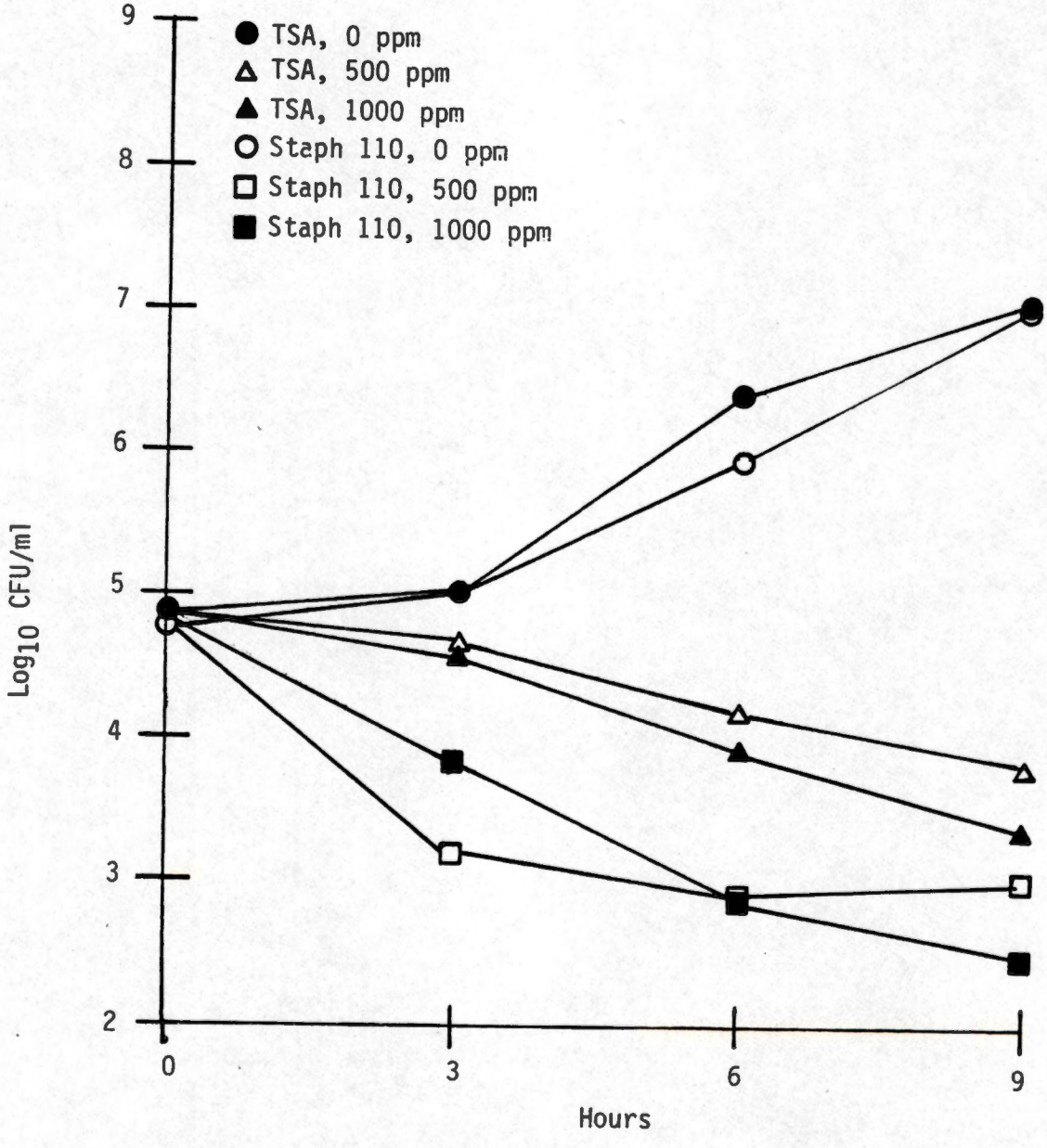


FIGURE 14. Recovery of S. aureus exposed to p-coumaric acid at pH 7.0 in TSA and Staph 110.

cells were most sensitive to the high sodium chloride content of Staph 110 medium during the lag phase of growth. A similar trend also was observed with 1000 ppm p-coumaric acid at pH 5.0 (98.62% at 12 hours, Figure 15). Also, there was a trend for the injury to be minimum at the last enumeration interval with either concentration of p-coumaric acid at both pH levels (Figures 14 and 15). These findings indicate that the organisms may be able to repair injury (Gray, et al., 1974) after prolonged exposure to the compound. However, the injury of S. aureus with the caffeic and ferulic acids did not follow any specific trend.

The results from this study of injury are contrary to other reports on recovery of S. aureus in selective media (Gray, et al., 1974; Ray, 1979; Smith and Palumbo, 1982). Generally, there were larger differences (>1-2 log cycles) found between log counts of injured cells in a selective medium and a non-selective medium (Gray, et al., 1974). Ray (1979) explains that this difference is due to the sensitivity of an injured cell to selective compounds (sodium chloride) as a result of damage to permeability barriers in the cell wall and cell membrane after sublethal physical or chemical treatments. Results from the present study found no significant injury over time with S. aureus exposed to the hydroxycinnamic acids, except with 500 ppm p-coumaric acid at pH 7.0. Because caffeic acid was the least effective inhibitor of S. aureus, only small levels of injury were expected. The insignificant injury observed demonstrated that the cells could repair injury even in the presence of a selective

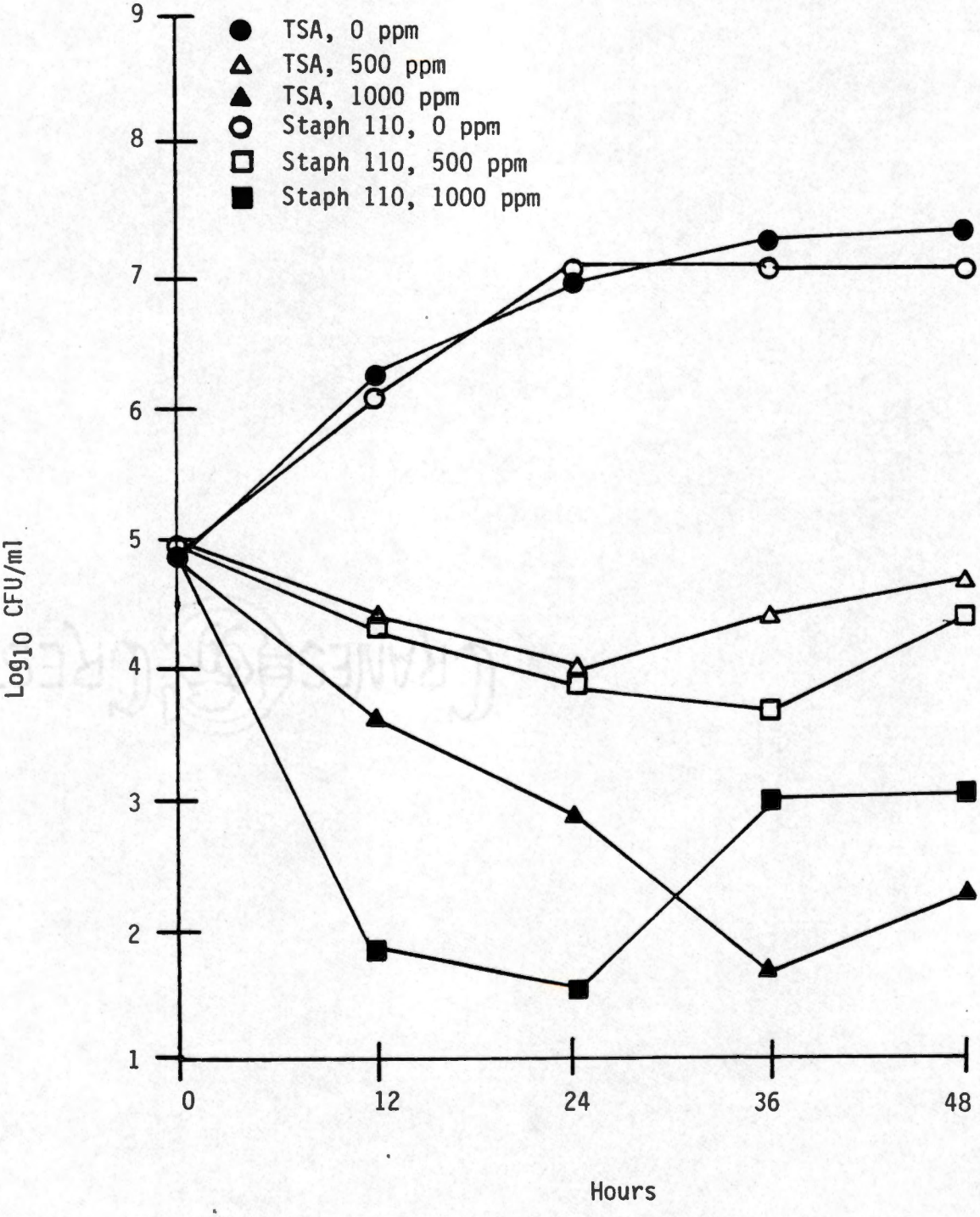


FIGURE 15. Recovery of S. aureus exposed to p-coumaric acid at pH 5.0 in TSA and Staph 110.

agent (sodium chloride). However, ferulic and p-coumaric acids were very effective in preventing the growth of S. aureus, and a lethal effect was observed in most instances. This lethal effect, and the low levels of injury observed, suggested that there may have been lethal injury (non-reversible) (Ray, 1979). This means that most of the survivor cells were able to multiply in the Staph 110 medium at levels comparable to those in TSA, and the cells that were lethally injured (killed) would not be expected to be enumerated on either medium.

Another explanation of the small amount of injury measured after high inhibition levels (>99.99%) were observed is that the cells were subject to metabolic injury (Ray, 1979) or inhibition of essential enzyme systems (Davidson and Branen, 1981) when in the presence of the hydroxycinnamic acids. When removed from the environment containing the compounds, the cells may have been able to over come the inhibitory effect of the acids and grow on either medium (reversible inhibition). Those that were inactivated or killed by the inhibition of essential enzyme systems would not be expected to be enumerated, even on TSA.

VI. LEAKAGE OF ^{14}C-LABELED COMPOUNDS FROM ESCHERICHIA COLI

In the presence of 1000 ppm p-coumaric acid, E. coli showed slight leakage of incorporated ^{14}C-labeled compounds (Figure 16). While much of the leakage came at the first sampling period,

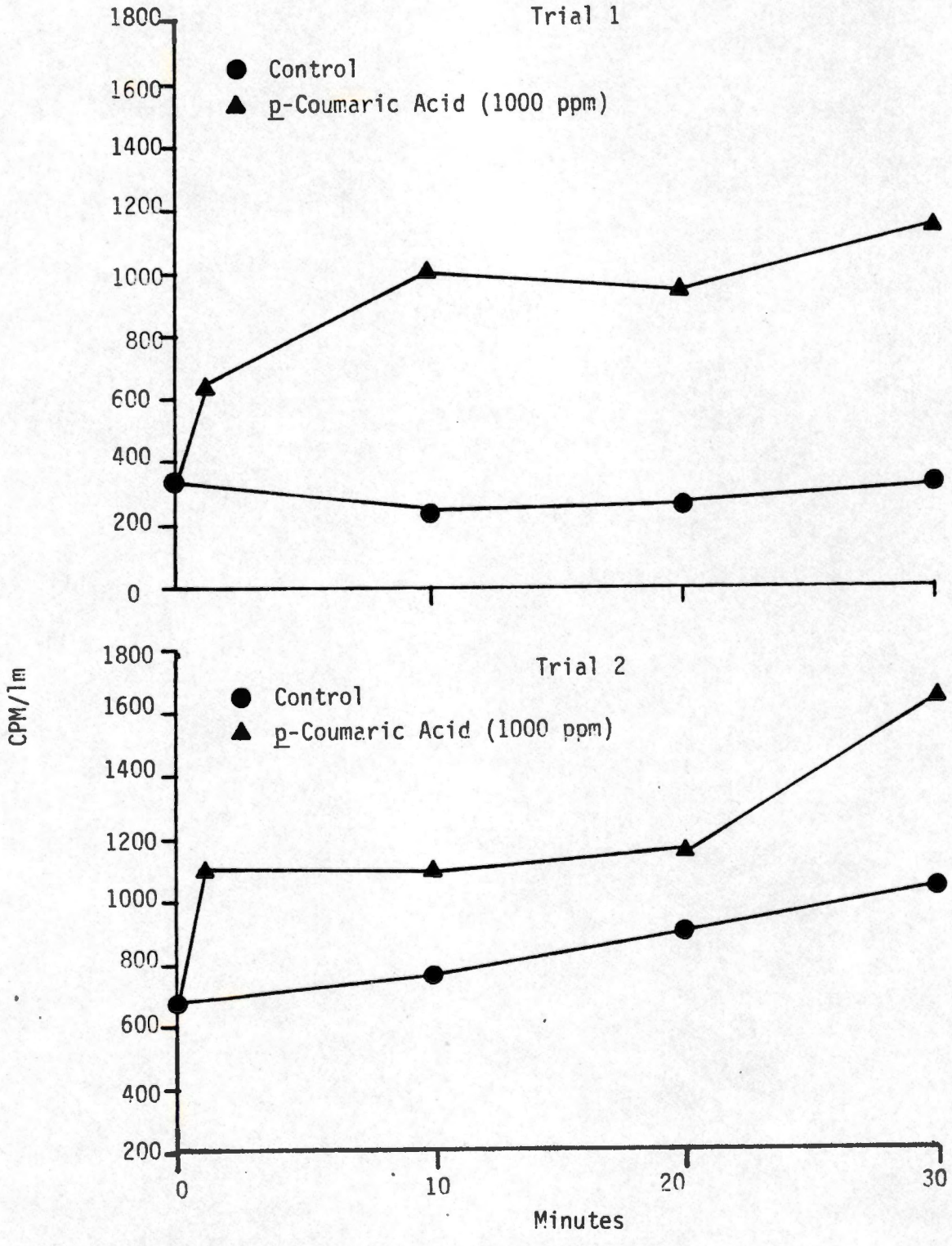


FIGURE 16. Leakage of ^{14}C-labeled compounds from E. coli after exposure to 1000 ppm p-coumaric acid in 0.05 M citrate buffer, pH 6.0. The cpm/ml for whole cells was 46,177 in Trial 1 and 42,743 in Trial 2.

(assumed to be 1 min due to the come up time of the centrifuge) losses of ^{14}C-labeled compounds also occurred at later sampling times. The maximum loss of incorporated ^{14}C, expressed as a percentage of the counts per min (cpm) per ml of the whole cells, was 2.51% at 30 min in Trial 1 and 3.92% in Trial 2 (Figure 16). In the second trial, there was also a slight loss of the ^{14}C-labeled compounds from the control (ethanol) cells over time. The exact reason for this was unknown.

The loss of incorporated ^{14}C was in agreement with previous work which reported leakage of intracellular constituents in the presence of other phenolics (Davidson and Branen, 1980; Judis, 1963). However, the maximum loss of ^{14}C-labeled compounds with p-coumaric acid (3.92%) observed in the present study was much lower than the levels found with P. fragi and P. fluorescens, 11.5% and 24.8% respectively, after exposure to butylated hydroxyanisole reported by Davidson and Branen (1980). This small amount of leakage may be related to the slightly depressed growth rate of E. coli at pH 6.0 (Figure 5, p. 33). Since p-coumaric acid slowed the growth rate of E. coli inhibition of the cells could have been partially a result of leakage.

Because B. cereus and S. aureus were more sensitive to p-coumaric acid, it would have been desirable to measure the loss of ^{14}C-labeled compounds from those cells. However, these organisms required B vitamins and other nitrogen-containing compounds for growth (Guirard and Snell, 1981; Buchanan and Gibbons, 1974) which made their cultivation in a simple defined medium impossible.

CHAPTER V

SUMMARY

The three most common hydroxycinnamic acids, caffeic, p-coumaric, and ferulic, were investigated for their antibacterial activity against two gram-negative organisms, P. fluorescens and E. coli, and two gram-positive food poisoning organisms, B. cereus and S. aureus. The bacteria were grown at optimum conditions in TSB at three pH levels, depending upon the organism. Growth was monitored by plate counts in TSA.

The hydroxycinnamic acids did not affect the growth of P. fluorescens significantly. The growth of the organisms in the presence of the last compounds closely followed that of the control. Growth of E. coli was inhibited by the hydroxycinnamic acids when grown at pH 6.0 and 5.0, but not at 7.0, and inhibition was greater at pH 5.0. Results suggested that inhibition was inversely related to the pH. Staphylococcus aureus growth was inhibited by the hydroxycinnamic acids at pH 7.0, 6.0, and 5.0 with the level of inhibition increasing at pH 5.0. The most effective acid was p-coumaric acid which exhibited lethal activity at pH 5.0. Bacillus cereus was sensitive to all of the compounds at pH 7.0, 6.5, and 6.0, and a lethal effect was observed with p-coumaric acid. Higher concentrations of ferulic acid had increased activity while 1000 ppm p-coumaric acid had no significant increase in activity over 500 ppm.

Injury of S. aureus with the test compounds was determined in TSA and Staph 110 medium. No significant injury over time was found with any of the compounds, except 500 ppm p-coumaric acid at pH 7.0. Results suggested that the cells were able to repair damage after exposure to the acids, or once removed from the environment containing the compounds, the cells were able to grow on a selective medium at levels comparable to those on a non-selective medium.

Escherichia coli was grown in a glucose salts medium containing a ^{14}C-labeled amino acid mixture and leakage of incorporated ^{14}C from the cells after exposure to 1000 ppm p-coumaric acid for 30 min was monitored with liquid scintillation counting. A maximum of 3.92% of the ^{14}C-labeled compounds, expressed as a percentage of the whole cells, was lost by E. coli. These results suggested then inhibition of E. coli, and others, by p-coumaric acid may be at least partially due to leakage of intracellular constituents.

It was concluded from the findings of this investigation that the hydroxycinnamic acids, especially p-coumaric acid, may have potential for use as antimicrobial agents. Because the concentrations used in this study (0.05 and 0.1%) were comparable levels of antimicrobial compounds used in foods, and considering their potential antioxidant activity, these naturally occurring compounds may have applications in reduced lipid-containing food products. Because the compounds were tested under optimum conditions in a nutrient medium, a study of the effect of food components and environmental

conditions upon the antimicrobial activity of the compounds would be beneficial.

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APPENDICES

APPENDIX A

Equations for log count (y) of bacterial growth in the presence of the hydroxycinnamic acids as a function of time (x)[1]:

B. cereus, combined pH 7.0, 6.5, and 6.0, 500 ppm:

Control	$y = 0.38x + 3.94^{a}$
Caffeic	$y = 0.26x + 5.02^{b}$
p-Coumaric	$y = -0.10x + 3.35^{c}$
Ferulic	$y = 0.21x + 3.62^{b}$

B. cereus, combined pH 7.0, 6.5, and 6.0, 1000 ppm:

Control	$y = 0.38x + 3.94^{a}$
Caffeic	$y = 0.19x + 3.32^{b}$
p-Coumaric	$y = -0.26x + 4.11^{c}$
Ferulic	$y = 0.04x + 4.25^{b}$

E. coli, pH 6.0, 500 and 1000 ppm:

Control	$y = 0.42x + 5.61^{a}$
Caffeic	$y = 0.34x + 5.50^{ab}$
p-Coumaric	$y = 0.21x + 5.82^{bc}$
Ferulic	$y = 0.27x + 5.53^{bc}$

E. coli, pH 5.0, 500 and 1000 ppm:

Control	$y = 0.64x + 6.45^{a}$
Caffeic	$y = 0.17x + 6.65^{b}$
p-Coumaric	$y = -0.003x + 6.21^{b}$
Ferulic	$y = 0.02x + 6.31^{b}$

S. aureus, combined pH 6.0 and 7.0, 500 and 1000 ppm:

Control	$y = 0.32x + 4.95^{a}$

Caffeic	$y = 0.27x + 4.86^{a}$
p-Coumaric	$y = 0.07x + 4.94^{b}$
Ferulic	$y = 0.18x + 4.72^{c}$

S. aureus, pH 5.0, 500 ppm:

Control	$y = 0.05x + 3.56^{a}$
Caffeic	$y = 0.004x + 5.04^{b}$
p-Coumaric	$y = - 0.03x + 5.15^{b}$
Ferulic	$y = - 0.02x + 4.87^{b}$

S. aureus, pH 5.0, 1000 ppm:

Control	$y = 0.05x + 3.67^{a}$
Caffeic	$y = - 0.005x + 3.80^{b}$
p-Coumaric	$y = - 0.68x + 3.75^{c}$
Ferulic	$y = - 0.04 x + 3.86^{bc}$

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[1]Equations followed by the same letter in a series are not significantly different at the 95% level.

APPENDIX B

TABLE 10. Log Count Means and Percent Inhibition of B. cereus at pH 7.0 in the Presence of the Hydroxycinnamic Acids

Hydroxycinnamic Acid (ppm)	Log Count Mean[1] (Percent Inhibition) Hours 0	3	6	9
Caffeic (500)	4.15	4.85 (32.39)	6.68 (29.20)	7.07 (69.10)
(1000)	4.15	3.69 (95.32)	4.69 (99.28)	5.34 (99.42)
p-Coumaric (500)	4.15	2.83 (99.35)	2.67 (>99.99)	2.64 (>99.99)
(1000)	4.15	2.76 (99.45)	2.30 (>99.99)	1.78 (>99.99)
Ferulic (500)	4.15	4.30 (80.94)	5.76 (91.49)	6.76 (84.86)
(1000)	4.15	4.29 (81.38)	4.89 (98.85)	5.34 (99.42)
Control (0)	4.15	5.02	6.83	7.58

[1]Mean of 2 replications.

TABLE 11. Log Count Means and Percent Inhibition of B. cereus at pH 6.5 in the Presence of the Hydroxycinnamic Acids

Hydroxycinnamic Acid (ppm)	Log Count Mean[1] (Percent Inhibition) Hours 0	3	6	9
Caffeic (500)	3.74	5.12 (64.52)	6.71 (35.43)	7.21 (36.90)
(1000)	3.74	4.35 (93.97)	4.51 (99.59)	4.95 (99.65)
p-Coumaric (500)	3.74	2.88 (99.80)	3.52 (99.96)	3.15 (>99.99)
(1000)	3.74	2.15 (99.96)	2.12 (>99.99)	1.53 (>99.99)
Ferulic (500)	3.74	4.52 (91.09)	4.95 (98.88)	5.50 (98.77)
(1000)	3.74	3.84 (98.14)	3.65 (99.94)	4.15 (99.94)
Control (0)	3.74	5.57	6.90	7.41

[1]Mean of 2 replications.

TABLE 12. Log Count Means and Percent Inhibition of B. cereus at pH 6.0 in the Presence of the Hydroxycinnamic Acids

Hydroxycinnamic Acid (ppm)	Log Count Mean[1] (Percent Inhibition) Hours			
	0	3	6	9
Caffeic (500	4.61	5.08 (85.46)	7.24 (0.00)	7.03 (66.12)
(1000)	4.61	5.39 (92.92)	5.13 (99.02)	4.78 (99.89)
p-Coumaric (500)	4.61	2.75 (99.97)	3.43 (99.98)	3.21 (>99.99)
(1000)	4.61	1.84 (>99.99)	1.74 (> 99.99)	1.56 (>99.99)
Ferulic (500)	4.61	4.65 (97.37)	5.56 (97.37)	5.69 (98.45)
(1000)	4.61	4.17 (99.13)	4.10 (99.91	4.09 (99.61)
Control (0)	4.61	6.23	7.14	7.50

[1]Mean of 2 replications.

TABLE 13. Log Count Means and Percent Inhibition of E. coli at pH 7.0 in the Presence of the Hydroxycinnamic Acids

Hydroxycinnamic Acid (ppm)	Log Count Mean[1] (Percent Inhibition) Hours			
	0	3	6	9
Caffeic (500)	5.62	7.03 (0.00)	7.98 (12.90)	8.74 (18.72)
(1000)	5.62	7.08 (0.00)	7.46 (73.69)	8.21 (76.01)
p-Coumaric (500)	5.62	6.80 (8.80)	7.96 (16.82)	8.12 (80.50)
(1000)	5.62	6.65 (35.43)	7.17 (86.51)	8.24 (74.30)
Ferulic (500)	5.62	6.87 (0.00)	7.87 (32.39)	8.70 (25.87)
(1000)	5.62	6.78 (12.90)	7.83 (38.34)	8.78 (10.87)
Control (0)	5.62	6.84	8.04	8.83

[1]Mean of 2 replications.

TABLE 14. Log Count Means and Percent Inhibition of E. coli at pH 6.0 in the Presence of the Hydroxycinnamic Acids

Hydroxycinnamic Acid (ppm)	Log Count Mean[1] (Percent Inhibition) Hours			
	0	3	6	9
Caffeic (500)	5.21	6.54 (18.72)	7.68 (24.14)	8.64 (56.35)
(1000)	5.21	6.12 (69.10)	7.15 (77.61)	7.90 (92.06)
p-Coumaric (500)	5.21	5.66 (85.21)	7.04 (82.62)	7.86 (92.76)
(1000)	5.21	5.51 (92.41)	5.94 (98.62)	6.19 (99.84)
Ferulic (500)	5.21	6.47 (30.82)	7.46 (54.29)	7.94 (91.29)
(1000)	5.21	6.09 (71.15)	6.39 (96.12)	7.44 (97.24)
Control (0)	5.21	6.63	7.80	9.00

[1]Mean of 2 replications.

TABLE 15. Log Count Means and Percent Inhibition of E. coli at pH 5.0 in the Presence of the Hydroxycinnamic Acids

Hydroxycinnamic Acid (ppm)	Log Count Mean[1] (Percent Inhibition) Hours				
	0	12	24	36	48
Caffeic (500)	6.16	6.68 (19.50)	6.58 (97.31)	7.36 (89.28)	7.58 (98.85)
(1000)	6.16	6.37 (96.02)	6.37 (98.34)	6.30 (99.07)	6.32 (99.94)
p-Coumaric (500)	6.16	6.12 (94.63)	6.45 (98.00)	6.79 (97.12)	6.91 (99.75)
(1000)	6.16	6.08 (95.10)	5.65 (99.68)	6.03 (99.50)	4.74 (>99.99)
Ferulic (500)	6.16	7.11 (59.26)	7.11 (90.88)	7.22 (92.24)	7.15 (99.57)
(1000)	6.16	6.42 (93.97)	6.42 (98.14)	6.60 (98.14)	6.65 (99.87)
Control (0)	6.16	7.39	8.15	8.33	9.52

[1]Mean of 2 replications.

TABLE 16. Log Count Means and Percent Inhibition of P. fluorescens in the Presence of the Hydroxycinnamic Acids at pH 7.0

Hydroxycinnamic Acid (ppm)	Log Count Mean[1] (Percent Inhibition) Hours 0	3	6	9
Caffeic (500)	5.06	5.24 (0.00)	5.89 (0.00)	7.03 (54.29)
(1000)	5.06	5.04 (30.82)	5.46 (22.37)	6.21 (93.08)
p-Coumaric (500)	5.06	4.73 (66.12)	5.44 (25.87)	6.60 (83.02)
(1000)	5.06	4.74 (65.33)	5.00 (73.08)	5.46 (98.77)
Ferulic (500)	5.06	4.91 (48.17)	5.82 (0.00)	6.94 (62.85)
(1000)	5.06	5.05 (29.20)	5.21 (56.35)	6.89 (66.89)
Control (0)	5.06	5.20	5.57	7.37

[1]Mean of 2 replications.

TABLE 17. Log Count Means and Percent Inhibition of P. fluorescens at 6.5 in the Presence of the Hydroxycinnamic Acids

Hydroxycinnamic Acid (ppm)	Log Count Mean[1] (Percent Inhibition) Hours 0	3	6	9
Caffeic (500)	5.01	5.10 (0.00)	5.14 (77.09)	6.45 (64.52)
(1000)	5.01	5.59 (0.00)	4.97 (84.51)	6.15 (82.22)
p-Coumaric (500)	5.01	5.18 (0.00)	4.94 (85.54)	5.95 (88.78)
(1000)	5.01	4.93 (0.00)	4.85 (88.25)	5.59 (95.10)
Ferulic (500)	5.01	5.07 (0.00)	5.24 (71.16)	6.62 (47.52)
(1000)	5.01	4.60 (47.52)	5.13 (77.61)	6.04 (86.20)
Control (0)	5.01	4.88	5.78	6.90

[1]Mean of 2 replications.

TABLE 18. Log Count Means and Percent Inhibition of P. fluorescens at pH 6.0 in the Presence of Hydroxycinnamic Acids

Hydroxycinnamic Acid (ppm)	Log Count Mean[1] (Percent Inhibition) Hours			
	0	3	6	9
Caffeic (500)	5.05	5.07 (0.00)	5.03 (0.00)	5.99 (0.00)
(1000)	5.05	4.94 (25.87)	4.77 (39.74)	5.90 (0.00)
p-Coumaric (500)	5.05	4.91 (30.82)	4.94 (10.87)	5.23 (78.12)
(1000)	5.05	4.81 (45.04)	4.59 (60.19)	5.37 (69.80)
Ferulic (500)	5.05	5.07 (0.00)	4.87 (24.14)	4.94 (88.71)
(1000)	5.05	4.90 (32.39)	4.89 (20.57)	4.87 (90.45)
Control (0)	5.05	5.07	4.99	5.89

TABLE 19. Log Count Means and Percent Inhibition of S. aureus at pH 7.0 in the Presence of the Hydroxycinnamic Acids

Hydroxycinnamic Acid (ppm)	Log Count Mean[1] (Percent Inhibition) Hours			
	0	3	6	9
Caffeic (500)	5.03	5.69 (33.93)	6.50 (58.31)	7.80 (0.00)
(1000)	5.03	5.01 (86.20)	6.45 (96.28)	6.61 (98.95)
p-Coumaric (500)	5.03	5.30 (73.08)	5.32 (97.24)	5.80 (98.95)
(1000)	5.03	5.26 (75.45)	5.91 (89.28)	5.87 (98.77)
Ferulic (500)	5.03	5.64 (41.16)	6.28 (74.88)	6.69 (91.87)
(1000)	5.03	5.51 (56.35)	6.10 (83.40)	6.69 (92.76)
Control (0)	5.03	5.87	6.88	7.78

[1]Mean of 2 replications.

TABLE 20. Log Count Means and Percent Inhibition of S. aureus at pH 6.0 in the Presence of the Hydroxycinnamic Acids

Hydroxycinnamic Acid (ppm)	Log Count Mean[1] (Percent Inhibition) Hours			
	0	3	6	9
Caffeic (500)	4.84	6.01 (0.00)	7.06 (8.80)	7.70 (20.56)
(1000)	4.84	5.76 (33.93)	6.68 (61.98)	7.52 (47.52)
p-Coumaric (500)	4.84	5.14 (84.15)	5.22 (98.68)	5.87 (98.82)
(1000)	4.84	4.96 (89.53)	4.99 (99.22)	4.70 (99.92)
Ferulic (500)	4.84	5.68 (45.04)	6.38 (80.94)	7.15 (77.61)
(1000)	4.84	4.97 (89.28)	5.49 (97.54)	5.78 (99.04)
Control (0)	4.84	5.94	7.10	7.80

[1]Mean of 2 replications.

TABLE 21. Log Count Means and Percent Inhibition of S. aureus at pH 5.0 in the Presence of the Hydroxycinnamic Acids

Hydroxycinnamic Acid (ppm)	Log Count Mean[1] (Percent Inhibition) Hours				
	0	12	24	36	48
Caffeic (500)	5.46	6.28 (94.38)	6.76 (90.23)	6.41 (98.00)	6.19 (98.45)
(1000)	5.46	5.41 (99.24)	4.71 (99.91)	4.89 (99.94)	5.44 (99.72)
p-Coumaric (500)	5.46	5.10 (99.63)	5.30 (99.66)	4.98 (99.92)	4.62 (99.96)
(1000)	5.46	4.18 (99.96)	4.70 (99.91)	2.53 (>99.99)	2.21 (>99.99)
Ferulic (500)	5.46	5.60 (98.82)	6.02 (98.27)	5.92 (99.35)	6.80 (93.69)
(1000)	5.46	4.49 (99.91)	4.18 (99.97)	3.32 (>99.99)	3.35 (>99.99)
Control (0)	5.46	7.53	7.77	8.11	8.00

[1]Mean of 2 replications

VITA

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