12-2004

Antimicrobial Activity and Effect of Selected Essential Oil Components on Cell Membrane Lipids

Valerie W. Ling
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Valerie W. Ling entitled "Antimicrobial Activity and Effect of Selected Essential Oil Components on Cell Membrane Lipids." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

F. Ann Draughon, Major Professor

We have read this dissertation and recommend its acceptance:

P. Michael Davidson, Jochen Weiss, David C. White

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a dissertation written by Valerie W. Ling entitled "Antimicrobial Activity and Effect of Selected Essential Oil Components on Cell Membrane Lipids." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

F. Ann Draughon
Major Professor

We have read this dissertation
and recommend its acceptance:

P. Michael Davidson

Jochen Weiss

David C. White

Accepted for the Council:

Anne Mayhew
Vice Chancellor and
Dean of Graduate Studies

(Original signatures are on file with official student records.)

ANTIMICROBIAL ACTIVITY AND EFFECT OF SELECTED ESSENTIAL OIL COMPONENTS ON CELL MEMBRANE LIPIDS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Valerie W. Ling

December 2004

DEDICATION

This dissertation is dedicated to my parents, Frederick and Linda Ling, and my sister, Wendy Ling Chin for their support,

and my Heavenly Father for the dedication of His life to me.

ACKNOWLEDGEMENTS

I wish to thank Dr. F. Ann Draughon for her professional and personal support and guidance. She was the first person to inspire my interest in food safety and taught me invaluable information about food microbiology. She is the best mentor and professor that any graduate student can have. I appreciated all her encouragement and patience with me.

I extend thanks to my committee members, Dr. P. Michael Davidson, Dr. David C. White, and Dr. Jochen Weiss, for their time, technical expertise, and editing skills. I am especially grateful for use of Dr. White's Center for Biomarker Analysis lab and assistance from his staff. Training from James Cantu, Amanda Smithgall, and Aaron Peacock from Dr. White's lab were crucial to the lipids portion of my dissertation work.

I thank Carolina Naar, who helped me enormously with media preparation and Dr. Philipus Panglioli. Their assistance during long days of experiments was invaluable. I appreciated help from Willie Taylor with equipment and teaching me in lab techniques. I valued the support of everyone in Dr. Draughon's group: Kim Lamar, Min Huang, Andres Rodriguez, Omaima Ahmed, David Rasmussen, Jake Stevens, Andy Doane, Felix Jackson, and Dr. Harry Richards. I thank Jason Noh and Katie Davenport, who helped me in the lab.

I'm grateful for my friends in Knoxville: Dr. Amy White, Shelley Woods, Jennifer Viar, Connelly Hull, and Dee Lewis. The kindness, cheer, and prayers

that they invested in me have brought refreshment and hope in a long and difficult journey. They had strength and courage when I lacked them.

I thank my long-time friends Amy Hwang and Jennifer Tseung for their comfort and understanding. I appreciate Mei Ran, for her lightheartedness and delicious meals, and friends that I met through Navigators, like Paul and Jane Slay. I thank my new friends Mark Pate, Karen Ellison, and Dolly Fox who gave me an eternal perspective on trust, joy, and freedom. From my friends I learned how to receive when people give gladly and how to give goodness to others.

I thank and honor my mom and dad, Frederick and Linda Ling, who have worked and sacrificed to ensure that I was well-fed and would receive an education at Cornell University and the University of Tennessee. I respect and appreciate them for their care and love. I appreciate my sister, Wendy Ling Chin, Doctor of Pharmacy candidate, for her thoughtfulness and support.

Most of all, I thank the Father, Son, and Holy Spirit. His goodness became tangible to me through these aforementioned people. I thank Him for renewing my strength and determination, despite setbacks (ex. power outages, A/C malfunctioning, and my lack of wisdom sometimes). The Father has blessed me with intelligence and other talents and gifts. I hope to continue to develop them and use them responsibly so that I may display His excellence and kindness as I care for others and my world, which is my goal and joy. There is delight in learning and discovering the complexity He created in food and microbiology.

“Trust in the Lord and do good; dwell in the land and enjoy safe pasture. Delight yourself in the Lord and he will give you the desires of your heart. Commit your way to the Lord; trust in Him and he will do this: He will make your righteousness shine like the dawn, the justice of your cause like the noonday sun. Be still before the Lord and wait patiently for him.” (Ps. 37:3-7)

ABSTRACT

The objectives of this study were to evaluate the antimicrobial activity of essential oil components (EOC) against *Salmonella* Typhimurium and *Listeria monocytogenes* and evaluate changes in bacterial membrane composition by observing changes in the fatty acids of *Salmonella* associated with exposure and adaptation to (or growth in the presence of) cinnamaldehyde (CIN) and carvacrol (CRV) EOC. Ten EOC were tested for efficacy using the broth microdilution and macrodilution methods. Beta-caryophyllene, limonene, alpha-pinene, and thymol were not lethal to *Salmonella* and *Listeria* at 2800mg/L. Dose-response models showed that the most effective EOC against *Salmonella* were CIN and CRV (50% probability of lethality with avg. of 280mg/L and 1080mg/L, respectively) regardless of method. CIN (790mg/L) and RHO (1810mg/L) were the most lethal against *Listeria.* CIN- or CRV-adapted or non-adapted *Salmonella* were inoculated into broth containing 250mg/L CIN, 871mg/L CRV, or no EOC. At 2 and 24h, the saturated fatty acid (SFA) to unsaturated fatty acid (UFA) ratio in the membrane of CIN- or CRV-adapted cells treated with CIN or CRV was numerically higher than in the non-adapted cells treated with CIN or CRV. Significant ($p<0.05$) changes were observed with CIN or CRV exposure. After 2h of exposure to CIN, the non-adapted CIN-treated cells had lower total phospholipid fatty acid (PLFA), lower C16:1w7c, and higher C18:0 and SFA than the non-adapted control. After 24h of exposure to CIN, the non-adapted CIN-treated *Salmonella* had lower Cy17:0 and Cy19:0 than the non-adapted control

and higher C16:0 and SFA. Compared to the non-adapted control at 2h, the non-adapted CRV-treated *Salmonella* had lower PLFA, and lower C16:1w7c, Cy17:0, C18:1w9c, C18:1w7c, and UFA and higher C14:0, C18:0, SFA, and SFA/UFA ratio. At 24h, the non-adapted CRV-treated cells had lower PLFA, Cy17:0, C18:1w7c, and UFA and higher C16:1w7c and SFA than the non-adapted control. Antimicrobial treatment appeared to decrease the fluidity of the *Salmonella* membrane by increasing SFA. This decreased fluidity may prevent additional CIN and CRV from permeating. Growth in the presence of the antimicrobial had a much smaller effect on the fatty acid composition. Additional measurement of the membrane transition temperature from gel-to-liquid-crystalline phase would indicate fluidity.

TABLE OF CONTENTS

SECTION **PAGE**

TABLE OF CONTENTS

SECTION **PAGE**

LIST OF TABLES

TABLE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

LIST OF ABBREVIATIONS

CRV	carvacrol
CYP	beta-caryophyllene
CIN	trans-cinnamaldehyde
EOC	essential oil components
EtOH	ethanol
EUG	eugenol
LIM	limonene
LNO	linalool
PLFA	phospholipid fatty acid
RAP	(1R)-(+)-alpha-pinene
SAP	(1S)-(-)-alpha-pinene
RHO	rhodinol
SFA	saturated fatty acid
THY	thymol
UFA	unsaturated fatty acid

PART 1. INTRODUCTION

Introduction

In 2003 there were only 15,600 cases of foodborne infections that were diagnosed in laboratories (CDC 2004). The number of cases caused by 9 identified pathogens was: 6,017, *Salmonella*; 5,215, *Campylobacter*; 3,021, *Shigella*; 480, *Cryptosporidium*; 443, *E. coli* O157; 161, *Yersinia*; 138, *Listeria*; 110, *Vibrio*; and 15, *Cyclospora*. Of the 5,455 (91%) *Salmonella* isolates serotyped, *S.* Typhimurium (759 cases) was the serotype most often isolated and identified in human salmonellosis cases (14% of foodborne salmonellosis infections). Although 15,600 foodborne bacterial infections were diagnosed in 2003 (CDC 2004), it is estimated that there are over 2 million cases of salmonellosis and 1600 cases of listeriosis that occur in the U.S. each year (FDA-CFSAN 2003a, b).

The prevalence of *L. monocytogenes* in meats was found to be highly variable with about 16% of products testing positive from 1971-1994. *L. monocytogenes* serotype 4b has most often been associated with human listeriosis outbreaks (Jay 1996). A more recent study found that 577 of 31,700 samples of ready-to-eat foods tested positive for *L. monocytogenes* (prevalence of 1.82%) (Gombas and others 2003). The latest multistate outbreak of listeriosis was in the northeastern U.S. in 2002 with 46 confirmed cases and seven deaths (CDC 2002). Listeriosis is a concern because of the high mortality rate, which can be as high as 70% when listeric meningitis occurs or more than 80% with perinatal/neonatal infections (FDA-CFSAN 2003b).

Characteristics of Gram-positive and negative membranes

L. monocytogenes is a Gram-positive bacterium. The Gram-positive cell has an open, hydrophilic structure. It contains a thick and hydrated peptidoglycan (PG) layer, which forms 90% of the cell wall. The PG layer is covalently bonded to teichoic acids. Teichoic acids are acidic anionic polysaccharides that give the cell wall a net negative charge. Teichoic acids bind cations such as Ca^{2+} and Mg^{2+}. Lipoteichoic acid is a type of teichoic acid, which has a glycerophosphate chain that is exposed on the cell surface at one end. At the other end, lipoteichoic acid is covalently linked to a glycolipid situated on the outer side of the cytoplasmic membrane. This glycerophosphate chain extends through the cell wall. Important proteins that have a role in interacting with the environment are covalently and noncovalently linked to the PG layer. Teichuronic acids, which replace teichoic acids when phosphate concentrations are low, have carboxylate groups that carry a negative charge (Atlas 1995a; Lambert 2002).

The PG layer allows the penetration of molecules with a molecular weight of 30,000 to 57,000 Da. Phenols, alcohols, aldehydes, and quaternary ammonium compounds, which are small, can permeate readily. The permeability of the cell wall may depend on the chemical structure of the PG layer, such as the extent of cross-linking and glycan chain length (Lambert 2002).

S. Typhimurium is a Gram-negative bacterium. The Gram-negative cell wall is complex and has multiple layers. The outer membrane, a thin PG layer, and the periplasmic space form the outer layer of the cell wall. The periplasmic

space is between the PG layer and the cytoplasmic membrane. The outer membrane contains lipopolysaccharide (LPS), phospholipids, and proteins, such as porins (Atlas 1995a).

The exterior layer of the outer membrane contains LPS that carries a net negative charge. The innermost part of the LPS is lipid A that attaches the LPS to the hydrophobic section of the outer membrane. Lipid A is a N-acetylglucosamine disaccharide linked to unusual fatty acids, such as caproic and lauric acids. External to the lipid A is the central core polysaccharide, which is a complex polysaccharide, and an outermost polysaccharide layer called the O-antigen that extends into the environment. The negatively charged core of LPS is stabilized by Ca^{2+} and Mg^{2+} cations that crosslink the LPS (Atlas 1995a).

On the interior of the outer membrane is a layer of phospholipids, which consist of a phosphate polar head covalently bonded to a glycerol backbone, which is ester-linked to two fatty acids. Interior to the inner layer of phospholipid is the periplasmic space (Atlas 1995a). This space contains nutrient binding proteins and lipoproteins, which have a fatty acid portion that associates with phospholipids of the outer membrane and a protein region that is bonded to the PG layer. This space contains proteins that create an active metabolic center within the cell. Interior to the PG is the cytoplasmic membrane consisting of a phospholipid bilayer with embedded proteins, such as enzymes and efflux proteins (Denyer and Maillard 2002). The cytoplasmic membrane has an important role in maintaining energy status, solute transport, and regulation of the intracellular environment. Homeostasis is mostly affected by the fluidity of the

cell membrane. Changes in the lipid composition of the cytoplasmic membrane occur in response to external stimuli to maintain a relatively constant fluidity (Sikkema and others 1995). Membrane fluidity is the "combined expression of the effects of [fatty acid] chain conformation, lateral and rotational diffusion [of the lipids within the membrane] and the resistance of the membrane to sheer forces" (Denich and others 2003).

Porins are hydrophilic channels within the outer membrane that allow the diffusion of small hydrophilic molecules with a molecular weight of up to 800 Da for *E. coli* (Atlas 1995a; Denyer and Maillard 2002). Tightly packed LPS in membrane of the Gram-negative cell is the cause of the very low permeability of the outer membrane to hydrophobic compounds. In contrast, the cytoplasmic membrane has a low permeability for polar and charged molecules, except for water. Apolar molecules, such as cyclic hydrocarbons, can pass easily. Permeability of the cytoplasmic membrane depends on the hydrophobicity of the molecules. These hydrophobic molecules partition into the cytoplasmic membrane and accumulate or are metabolized (Sikkema and others 1995).

The oil:water partition coefficient is the ratio of the concentration of a substance in oil and in water at equilibrium and at a specified temperature. The optimal oil:water partition coefficient (log P) for Gram-negative and Gram-positive bacteria is 4.0 and 6.0 for the oil phase, respectively. Aromatic solvents with a log P of 2.0 - 4.0 changed the cytoplasmic membrane organization in Gram-negative bacteria (Denyer and Maillard 2002). Carvacrol (CRV), a component found in oregano and thyme essential oils, has a log P of 3.6, and prefers to

partition to a nonpolar solvent rather than water (Ultee and others 2002). Therefore, CRV may be more effective against Gram-negative bacteria since the optimal log P of Gram-negative bacteria is closer to that of CRV.

Because Gram-positive bacteria have a more permeable cell wall they are more susceptible to antibiotics than Gram-negative bacteria, which has the thick outer LPS layer as a permeability barrier. Gram-positive organisms seem to be more sensitive to lipophilic compounds than Gram-negative organisms because of their lack of an outer membrane. Since LPS of the outer membrane is highly hydrophilic, small hydrophilic solutes may pass through the porins, but the outer membrane acts as a barrier to most hydrophobic compounds (Sikkema and others 1995; Lambert 2002).

Essential oils and components

Natural antimicrobials, such as herbs and spices, have been used for centuries to preserve and flavor food. Today, consumers desire high-quality, minimally processed foods that have fewer preservatives and an extended shelf-life (Brul and Coote 1999). Essential oils, which are obtained from plants by extraction or distillation, contain flavor, aroma, and antimicrobial components. They are used mainly for flavoring agents. Herbs, spices, and their components are Generally Recognized As Safe food ingredients under conditions of customary use (Ultee and others 1998). Studies have reported cinnamon, clove and mustard to be highly active against various microorganisms. Other inhibitory herbs and spices include allspice, bay leaf, caraway, coriander, cumin, oregano, rosemary, sage and thyme (Zaika 1988). Carvacrol, the bioactive component

found in oregano and thyme essential oils, is used to flavor baked goods, nonalcoholic beverages, and chewing gum (Ultee and others 1999). Eugenol and cinnamic aldehyde are used as flavorings in candies and baked goods (Kim and others 1995).

Antimicrobial activity of essential oils

In the past two decades, many studies have reported the inhibitory and lethal effects of essential oils tested against a wide variety of microorganisms in various media and food products. In studies that compared various essential oils, the most effective oils against bacteria were thyme, oregano, cloves, cinnamon, lemongrass and bay (Deans and Ritchie 1987; Hammer and others 1999). For example, 0.03% (v/v) thyme and 0.12% oregano essential oils inhibited the growth of *Escherichia coli*. However, comparison of the efficacy of various oils is complex because their composition varies among the different plant genera as well as within the same genus. Variation in composition of active components may result from geographic origin, climate, processing, and variety differences (Zaika 1988). Carvacrol and thymol, which are bioactive constituents in oregano essential oil, are present in different percentages in various species of *Origanum*.

Antimicrobial activity of essential oil components

The study of essential oil components (EOC) produces more useful data about their mechanism than essential oils, which vary in their composition of components. Table 1.1 describes selected essential oils and their major antimicrobial components. Evaluation of these components may present

Table 1.1. Plants and their bioactive components (Bauer and others 1997).

Main food-related plant source	Bioactive component	Base structure
Origanum and thyme oils	Carvacrol	Phenol
Clove oil	Beta-caryophyllene	Cyclic sesquiterpene hydrocarbon
Cassia and cinnamon bark oils	Cinnamic aldehyde	Aromatic unsaturated aldehyde
Clove and cinnamon leaf oils	Eugenol	Phenol
Citrus peel and caraway oils	Limonene	Cyclic terpene hydrocarbon
Sweet basil and sage oils	Linalool	Acyclic terpene alcohol
Rose and geranium oils	Rhodinol	Acyclic terpene alcohol
Rosemary oil	Alpha-pinene	Bicyclic terpene hydrocarbon
Origanum and thyme oils	Thymol	Phenol

problems because of the low water solubility of components, such as thymol, carvacrol (CRV), eugenol, and trans-cinnamaldehyde (CIN) (Griffin and others 1999). The minimum inhibitory concentration (MIC) of CRV, CIN, and thymol against *S.* Typhimurium were 150, 396, and 150 mg/L, respectively, using the broth macrodilution method (Helander and others 1998). Eugenol was found to inhibit the growth of *E. coli* O157:H7 and *L. monocytogenes* at 1000 mg/L (Blaszyk and Holley 1998). The concentration of CIN needed to achieve a 50% decrease in 1,500-2000 CFU/well of *E. coli* O157:H7, *S*. enterica Hadar, and *L. monocytogenes* was 600, 400, and 100 mg/L, respectively (Friedman and others 2002). Although there have been several studies to investigate the efficacy of some common constituents of essential oils, there are limited studies that examine the mechanism of inhibition of essential oil constituents.

Antimicrobial activity assay

A broth dilution assay is a type of invitro method that is conducted when quantitative results are needed (Parish and Davidson 1993). A range of concentrations of antimicrobials is prepared in non-selective broth. Consecutive two-fold dilutions are typically prepared in clinical microbiology, but arithmetic intervals may be used as well. A single concentration is dispensed into a tube (broth macrodilution) or a lane in a microtiter plane (broth microdilution).

The test vessel is incubated with the test organism usually for 16-24 hours at the optimum temperature of the organism (Parish and Davidson 1993). The test vessel is observed for the lowest concentration at which there is no turbidity or no growth. This is called the minimum inhibitory concentration (MIC). To determine the minimum lethal or bactericidal concentration (MLC or MBC), 10-100 ul are plated from the last tubes or wells with growth and all those with no growth. The MLC or MBC is the lowest concentration that results in $\geq$99.9% killing of the final inoculum (NCCLS 1993).

Four factors that affect results of antimicrobial tests are the test method, medium, microorganism, and antimicrobial (Zaika 1988; Parish and Davidson 1993). Common test mediums include water, microbiological media such as broth and agar, foods, beverages, or food systems with defined composition. The pH is of particular importance since the activity of organic acids, such as benzoic acid depends on the acid being in its undissociated form, which allows the antimicrobial to penetrate the cell membrane more easily (Parish and Davidson 1993). Most EOC are slightly soluble in aqueous systems like broth, at

best (Zaika 1988). Therefore, evaluation of EOC may present problems, such as visibility of growth end-points.

The application of the spice or component may include various concentrations and botanical compositions. The form of the spice may be ground, extract, infusion, or essential oil. EOC, such as CRV, are available commercially in liquid or in crystalline form. The antimicrobial activity may be detected by applying the component to agar with a paper disk or in a cup or mixed with the test medium (Zaika 1988). The solubility of the essential oil or component and method of preparing stock solutions of the oil or component may influence antimicrobial activity. Assay methods have different sensitivity as well (Friedman and others 2002). An antimicrobial could be effective in media but exhibit lower antimicrobial activity in food, such as in high fat foods in which the antimicrobial may partition into the food lipids (Zaika 1988).

Characteristics of the organism are important factor influencing results. Strains may vary in genotype and phenotype (Parish and Davidson 1993). Microorganisms that are antibiotic resistant are a concern since antibiotic treatment for serious infections may be useless if the microbe is resistant. Microorganisms may be tested in the form of vegetative cells or spores (Zaika 1988). The inoculum can be directly applied to the agar surface or mixed with the medium. Actively growing cells in the log phase are more sensitive than stationary-phase cells or spores (Parish and Davidson 1993). A higher inoculum size (log 7 or 8) of log-phase cells are more rapidly killed than a stationary-phase inoculum diluted to low inocula (log 4) (NCCLS 1993). Although population size

and growth phase independently influence the rate of lethality, the inoculum size will have the greatest effect on results (NCCLS 1993). The National Committee for Clinical Laboratory Standards (NCCLS) suggests an inoculum of log 5.7 for testing aerobic bacteria using broth dilution assays. Studies of essential oils and components are difficult to compare since a variety of assays have been used to evaluate antimicrobials, but there is no standardized test method or format for reporting assay results of antimicrobial activity of essential oils (Friedman and others 2002).

Mechanisms of inhibition by changes in membrane function

Concentrations of essential oils that are lethal to bacteria tend to be much higher than tolerable taste thresholds. However, essential oils can be used at lower inhibitory concentrations (Brul and Coote 1999). Knowledge about the mechanism of action of antimicrobials is needed to effectively combine antimicrobial additives and food processing technology to prevent food from spoilage and growth of pathogenic microorganisms without sacrificing organoleptic qualities.

The mechanism of action of some antimicrobials, such as CRV, is the disruption of the cytoplasmic membrane, which leads to a decrease in the proton motive force of the bacterial cell (Ultee and others 2002). The energy for cell processes is generated from a proton motive force (or hydrogen ion gradient), which is established by the separation of protons across the cytoplasmic membrane. During metabolism, the cell translocates protons out of the cytoplasmic membrane that creates an excess of hydrogen ions and positive

charges outside of the membrane. The concentration and electrical differences between the inside and outside of the cell across the cytoplasmic membrane produce the proton motive force, which consists of the proton potential (or pH gradient) and electrical (or membrane) potential. Protons cannot diffuse across the cytoplasmic membrane. They can only pass into the cell again through a specific proton channel, ATPase. As protons pass through the channel, ATPase catalyzes the conversion of ADP to ATP to provide energy for the cell to do other work (Atlas 1995b).

The interaction of lipophilic compounds with the bacterial membrane affects the structure of the cytoplasmic membrane, which may increase the passive proton (ion) flux across the membrane. The leakage of protons decreases the proton gradient and therefore dissipates the proton motive force. Lipophilic compounds, such as CRV and ethanol, have been associated with ATP depletion (Sikkema and others 1995; Ultee and others 1999).

CRV partitions into the cytoplasmic membrane. Ultee and others (1999) investigated the mechanisms of action of CRV when it was added to a *Bacillus cereus* cell pellet suspended in buffer. Viable cell numbers decreased exponentially when vegetative *B. cereus* cells were incubated for 30 min with 150 - 451 mg/L CRV. This reduction was related to a change in the membrane potential. CRV (2 mg/L) significantly decreased the membrane potential and dissipated the potential at concentrations greater than 23 mg/L. CRV (38 mg/L) reduced the pH gradient and dissipated the gradient at 150 mg/L and higher. The intracellular potassium pool decreased and extracellular potassium pools

increased with 150 mg/L CRV. Within 7 min, 300 mg/L CRV depleted the intracellular ATP pool without a proportional increase in extracellular ATP. It was concluded that CRV acted at the cytoplasmic membrane to increase permeability to protons and potassium ions.

CRV and thymol totally disrupted the outer membrane of *E. coli* O157:H7 and *S.* Typhimurium as indicated by increased uptake of 1-*N*-phenylnaphthylamine (NPN), which is a probe that fluoresces in a hydrophobic environment, and the release of LPS. Uptake occurred since the membrane was no longer acting as a strong permeability barrier. CRV (300 mg/L) disrupted the cytoplasmic membrane since it decreased the intracellular ATP pool and induced a small, but not proportional increase, in the extracellular ATP pool of *E. coli* O157:H7. Changes that occurred with thymol were similar, but less prominent. This indicates that they had a measurable effect on the cytoplasmic membrane. CIN did not affect NPN uptake and did not significantly affect intracellular or extracellular ATP (Helander and others 1998).

Therefore, it appears that the mechanism of action of CIN is not associated with an increase in permeability of the cell membrane. CIN is an aromatic aldehyde. It has been suggested that the carbonyl group of aldehydes can bind to metal ions, sulfhydryl groups, amino acids, and proteins (Bowles and Miller 1993). CIN was found to be an effective inhibitor of histidine decarboxylase activity of *Enterobacter aerogenes*. Therefore it was suggested that binding of the CIN carbonyl group to the decarboxylase protein resulted in loss of decarboxylase activity (Wendakoon and Sakaguchi 1995).

Ramos-Nino and others (1996) developed multiple regression equations that described the effects of physicochemical and structural properties of benzaldehyde and benzoic and cinnamic acids on antibacterial activity. Steric parameters contributed more to benzaldehyde activity, while lipophilicity and ionization (pK_a) parameters did not. Therefore, benzaldehyde was suggested to act on the outside of the cytoplasmic membrane. In contrast, lipophilicity and pK_a of benzoic and cinnamic acid contributed most to their activity. They also found that the greater the molecular size of the aldehyde, the lower the antibacterial activity (Ramos-Nino and others 1996). This is the opposite of organic acids, which show increased antimicrobial activity with increased chain length (Jay 2000).

One study found that CIN at 0.3 ml/L was lethal to *B. cereus* cells but no significant amount of protein was lost. It was reported that treatment with CIN caused exponential phase cells to become long and filamentous and inhibited them from separating from each other (Kwon and others 2003).

Mechanisms of inhibition by changes in lipids

Lipophilic compounds, such as EOC, partition in the lipid bilayer membrane of microorganisms. The polarity of the compound determines where it accumulates in the membrane. Different compounds have preferential accumulation sites and cause different perturbations of the membrane. Accumulation of the compound correlates with its lipophilicity (Sikkema and others 1995).

Lipophilic molecules disrupt the interaction between lipids molecules and the membrane proteins (enzymes) that they border. These compounds may interact with hydrophobic regions of the enzymes. The interaction of these compounds affect fluidity and fatty acid composition of the bacterial membrane, which regulates the activity of membrane-embedded enzymes. This interaction can also change protein conformation. It has been proposed that the accumulation of lipophilic molecules in the membrane disrupts lipid-protein and lipid-lipid interaction, leading to toxicity (Sikkema and others 1995).

Bilayer membranes can exist in three phases: gel (ordered lipid chains), liquid-crystalline (disordered lipid chains) and hexagonal II (Ultee and others 2000b). Bacterial responses to the accumulation of hydrophobic compounds are an attempt to: 1) maintain a sufficient ratio of liquid-crystalline lipid in the membrane to restore membrane fluidity, 2) keep a balance between phospholipids that facilitate or do not promote a bilayer, and 3) extrude the hydrophobic compound through drug efflux systems. The fluidity of the membrane affects the growth of the cell and the proper functioning and structural integrity of the membrane (Denich and others 2003). For example, the percentage of unsaturated fatty acids in *E. coli* phospholipids is usually about 50%. This percentage can decrease to 30% without ceasing bacterial growth. If the percentage of saturated fatty acids decreases to less than 15% the membrane permeability increases (Seltmann and Holst 2002).

Cells adapt to hydrophobic compounds by changing the fatty acid composition (type of branching, chain length, and degree of unsaturation) and by

changing the phospholipid head-group composition (Ultee and others 2000a). The changes in bacterial lipids in the membrane are a defense mechanism used to maintain optimal fluidity in the liquid-crystalline phase, which is required for important cell processes. Lipids directly affect passive permeability and indirectly affect cell processes because of their interactions with membrane proteins (Russell and others 1995).

A modified fatty acid composition affects the lipid membrane melting point, which influences membrane fluidity (Li and others 2002). For example, the melting point of unsaturated fatty acids and branched-chain fatty acids is lower than that of saturated fatty acids. Therefore an increase in these fatty acids causes a decrease in the phase transition temperature and an increase in membrane fluidity (Casadei and others 2002). Fatty acids with high melting points, such as saturated, long, and straight-chain fatty acids, decreases membrane fluidity (Juneja and Davidson 1993).

Changes in the fatty acid composition are influenced by various environmental factors such as growth temperature and pressure (Casadei and others 2002; Li and others 2002). At decreasing temperatures the lipids in the membrane become ordered and the regions consisting only of lipids partly separate from the regions rich with proteins (Seltmann and Holst 2002). Changes in fatty acid composition of bacterial membranes have also been studied in the presence of various antimicrobials, such as nisin (Mazzotta and Montville 1997; Li and others 2002), sodium chloride, tertiary butylhydroxyquinone, methyl paraben, and propyl paraben (Juneja and Davidson

1993), acidulants (Juneja and others 1998), and phenolic compounds (acid phenols and tannins) (Rozes and Peres 1998). Phenolic compounds were shown to increase membrane fluidity, which influences membrane permeability (Diefenbach and Keweloh 1994).

Post and Davidson (1986) reported a relationship (R=0.89) between the ratio of saturated to unsaturated fatty acids (SFA /UFA) in the total lipid fraction of the membrane of Gram-positive bacteria (*S. aureus*, *B. cereus*, and *C. perfringens*) and susceptibility to butylated hydroxyanisole (BHA). SFA/UFA ratio in Gram-negative bacteria (*P. fluorescens*, *P. fragi*, *E. coli*, and *S. "anatum"*) was not related to their susceptibility. The highest SFA/UFA ratio was found in the two most resistant strains, which were *S. aureus* 12600 and LP (LD_{90} =293 and 304 mg/L BHA, respectively). *C. perfringens* was the most susceptible (LD_{90} =110 mg/L BHA) and had the lowest SFA/UFA ratio. It was suggested that the higher SFA/UFA ratio increased the ordering of fatty acid alkyl chains, which enhanced resistance. There was no relationship found between susceptibility and individual fatty acids or SFA/UFA ratios of the polar lipid fraction.

S. aureus LP was more resistant to methyl:propyl (2:1) paraben (MIC = 900 mg/L), which is a phenolic compound, and had a higher percentage of total lipid than sensitive *S. aureus* strains (Bargiota and others 1987). This resistant strain also had lower cyclopropyl 17:0 and cyclopropyl 19:0 than sensitive strains. It was suggested that parabens partition into the membrane lipids. This absorption into the lipids may protect the cells from additional penetration. A

decrease in cyclopropyl fatty acids may lower the permeability or interaction of parabens by affecting membrane fluidity.

Keweloh and others (1991) also found that phenolic compounds increase the permeability of the cell membrane. This study concluded that sublethal concentrations of phenol in the growth medium for 3 hours impaired cell growth and caused an increase in the saturated to unsaturated fatty acid ratio (increase in 16:0 and decrease in 16:1 and 18:1) in the exponential growth phase, which decreased the fluidity of the *E. coli* K-12 membrane. The authors suggested that this decrease in fluidity was a defense mechanism to decrease the damage by phenol. Additional phenol molecules would not reach the lipid bilayer to disrupt it as easily because of tightly packed acyl chains in the more saturated membrane. The increase in saturation would also reduce leakage of cellular metabolites.

When *Pseudomonas putida* adapted to phenol, the fatty acids converted from *cis*- to *trans*- form. Since this conversion increased membrane ordering, the membrane fluidity decreased (Heipieper et al., 1992; Sikkema et al., 1995). *Cis-trans* conversion also occurred in bacteria deprived of nutrients. The *trans/cis* ratio may be applied as an indication of starvation or stress (Guckert et al., 1986; Sikkema et al., 1995).

EOC, such as CRV, disrupt the membrane (Brul and Coote 1999). In liposomes prepared from *E. coli* phospholipids, cyclic hydrocarbons, such as alpha-pinene (9300 mg/L), accumulated in the bilayer interior, which caused swelling of the bilayer and therefore increased bilayer surface area (Sikkema and others 1994). The accumulation disrupted interactions between the acyl chains,

leading to increased membrane fluidity. It also caused decreased activity of cytochrome c oxidase and increased permeability to protons, which decreased the proton motive force. Cytochrome c oxidase is a membrane enzyme. The partitioning of the hydrocarbon into the cytoplasmic membrane may have caused the enzyme activity to decrease. This partitioning may have changed the interactions between proteins and lipids, thickness of the membrane, membrane fluidity, and/or phospholipid headgroup hydration (Sikkema and others 1994).

It has been suggested that CRV occupies more area than the typical space between the fatty acid chains of two adjoining phospholipid molecules. This interferes with the van der Waal interactions between the chains and therefore increases fluidity (Ultee and others 2000b; Ultee and others 2002). If the membranes become too fluid, additional CRV can continue to permeate the membrane, accumulate, and cause more damage (Ultee and others 1998).

Ultee and others (2002) reported that CRV expanded model liposomal membranes using fluorescent probes. They suggested that this swelling leads to a destabilized membrane and therefore a leakage of ions. The phenolic hydroxyl group of CRV is required for its antimicrobial activity since structurally similar compounds were not as inhibitory. Since Griffin and others (1999) categorized CRV in a group of oxygenated terpenoids of high antimicrobial activity associated with higher hydrogen binding capacity, this hypothesis is reasonable. Also, during exposure to CRV an influx of H^+ and efflux of K^+ ions in *B. cereus* was reported. The leakage of ions led to a drop in the pH gradient, destruction of the membrane potential and depletion of intracellular ATP (Ultee and others 1999).

Adaptation

If a factor or condition negatively influences bacterial growth or survival, it is considered a stress (Yousef and Courtney 2003). Mild or sublethal stresses are those that slow or stop bacterial growth without affecting viability. Stresses that cease growth and result in some loss of viability are defined as moderate. Extreme or severe stresses cause lethality to a majority of the bacterial population. Stresses affecting bacterial growth or survival may be physical, chemical, or biological.

When bacterial cells detect stresses that affect membrane fluidity, cell protein structure, ribosomes, or nucleic acids, they respond both genetically and phenotypically. This response involves transcription that results in regulatory proteins being synthesized. Production of regulatory proteins may lead to synthesis of other proteins that deal with the stress. Stress adaptation includes "1) production of proteins that repair damage, maintain the cell, or eliminate the stress agent, 2) transient increase in resistance or tolerance to deleterious factors, 3) cell transformation to a dormant state, 4) evasion of host organism defenses, and 5) adaptive mutations" (Yousef and Courtney 2003).

According to Yousef and Courtney (2003), stress adaptation is an "increase of an organism's resistance to deleterious factors following exposure to mild stress." In the food processing environment and in foods, pathogens are frequently exposed to sublethal stresses. When a microorganism is exposed to these stresses it may respond to protect itself, or adapt, to increase its tolerance to the same or different type of stress. Other terms for adaptation include

adaptive response, induced tolerance, habituation, acclimization, and stress hardening.

The ability of an organism to survive a stress is defined as tolerance, which is similar to resistance. When a microorganism is adapted to a stress, it may also resist a similar or different stress that was previously lethal or injurious to the cell (Yousef and Courtney 2003). For example, heating raw milk at temperatures below pasteurization may cause bacteria to adapt to mild heat. When the milk is used to make cheese, the bacteria are highly resistant to further heat processing. Therefore, adaptations to sublethal stresses are important to public health.

Mechanism of stress response

Stress responses depend on the stress and bacterial species. Microorganisms respond to stress immediately due to a sudden experience of stress or adapt to the stress over the long-term. To respond, a microorganism must sense a stress through a sensor such as a lipid, protein, or nucleic acid. A microorganism detects a stress when the stress affects the folding of mRNA, a change in a protein's half-life or structure, alterations in cellular metabolites, and changes in the membrane structure or fluidity. The sensor causes a change in transcription or translation of stress-related proteins, which alter cell physiology to increase tolerance (Yousef and Courtney 2003).

The response can be general, which protects against various stresses, or specific. Numerous changes in cell physiology, such as changes in cell envelope composition and morphology, are caused by the general stress response. This

results in decreased growth rate and entry into the stationary phase. General stress response is controlled by sigma factors in *E. coli* and other Gram-negative bacteria (Yousef and Courtney 2003).

Microorganisms have been shown to respond to heat, cold, acid, osmotic stress, and oxidative stress (Yousef and Courtney 2003). One of the changes in response to cold is a major alteration of the fatty acid composition of lipids in the bacterial membrane to increase membrane fluidity. To increase fluidity in response to cold temperatures, bacteria increase unsaturation or decrease the chain length. Only minor changes occur in the head-group composition (Russell and others 1995).

Monitoring stress response in research can be useful. The effect of mild stresses may reveal if the organism becomes resistant or sensitized to further stress. Adaptation is best observed at sublethal levels and during the exponential phase of growth, although adaptation has been induced in the stationary phase (Yousef and Courtney 2003). Research on the mechanisms and effects on the membrane lipids by food antimicrobials will add to the knowledge needed to keep foods safer yet minimize the use of additives and processing (Russell and others 1995).

CRV is lipophilic and was shown to accumulate and expand membranes treated with CRV. This swelling may cause a destabilized membrane in *B. cereus*. Swelling increased fluidity as indicated by a decrease in the temperature at which the membrane transitions from gel to liquid-crystalline phase (T_m). This led to increased passive permeability and therefore a leakage of ions. In

contrast, growth of *B. cereus* cells in the presence of 60 mg/L CRV reduced the membrane fluidity since the T_m was higher than non-adapted control cells (Ultee and others 2000a).

In the same study, *iso*-$C_{13:0}$ and *iso*-$C_{15:0}$ fatty acids increased in the membrane lipid composition of *B. cereus* cells incubated with 60 mg/L CRV while cis-$C_{16:1}$ fatty acid decreased. These changes correlated with a decrease in membrane fluidity of cells adapted to CRV. However, the decrease in long chain fatty acids ($C_{18:0}$) did not correlate with the decrease in fluidity. Therefore, more information is needed to understand these changes. Cells adapted to CRV had additional phospholipids compared to non-adapted cells. There were no noticeable differences in proportions of phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), and phosphatidylglycerol (PG) (Ultee and others 2000b).

Further information is needed about the targets of essential oil components. For example, since CRV accumulates in the membrane specific information about where it inserts itself in the membrane may elucidate how CRV may affect lipid-lipid and protein-lipid interactions. Responses to antimicrobials, such as a change in fatty acid composition, should be determined as to whether they enhance cell survival (adaptive response) or present evidence of toxicity.

Herbal or botanical antimicrobials offer an exciting new array of chemicals, which may help to make foods safer by inhibiting growth of pathogenic bacteria. Some herbs and essential oils have been used for thousands of years for both flavoring and medicinal purposes. It is only now that we are beginning to

understand the physiological activity of essential oils at the cellular level. Information is needed on the mechanism and mode of action of herbal antimicrobials to safely and economically use these powerful chemicals to not only flavor foods but also to ensure safety and preservation.

Research objectives

The objectives of this research were to:

1) Evaluate the antimicrobial activity of ten essential oil components against *L. monocytogenes* and S. Typhimurium

2) Compare results between the macro- to microdilution methods within an essential oil component by organism

3) Compare the antimicrobial concentrations needed to be effective against the two organisms

4) Determine the effect of exposure to cinnamaldehyde and carvacrol on the fatty acid composition of the bacterial membrane of *Salmonella*

5) Determine whether changes in the fatty acid composition of the bacterial membrane of *Salmonella* occur due to growth in the presence of cinnamaldehyde and carvacrol as a possible adaptive response

6) Determine whether reversal of these changes in fatty acid composition occurs when the stressors, cinnamaldehyde and carvacrol, are removed.

REFERENCES

Atlas R. 1995a. Organization and structure of microorganisms. In: R. Callanan. Principles of microbiology. St. Louis: Mosby-Year Book, Inc. 68-117.
Atlas R. 1995b. Cellular metabolism: The generation of ATP. In: R. Callanan. Principles of microbiology. St. Louis: Mosby-Year Book, Inc. 118-169.
Bargiota E, Rico-Munoz E, Davidson PM. 1987. Lethal effect of methyl and propyl parabens as related to *Staphylococcus aureus* lipid composition. International Journal of Food Microbiology 4:257-266.
Bauer K, Garbe D, Surburg H. 1997. Common fragance and flavor materials. New York: Wiley-VCH.
Blaszyk M, Holley RA. 1998. Interaction of monolaurin, eugenol and sodium citrate on growth of common meat spoilage and pathogenic organisms. International Journal of Food Microbiology 39:175-183.
Bowles BL, Miller AJ. 1993. Antibotulinal properties of selected aromatic and aliphatic aldehydes. Journal of Food Protection 56:788-794.
Brul S, Coote P. 1999. Preservative agents in foods: Mode of action and microbial resistance mechanisms. International Journal of Food Microbiology 50:1-17.
Casadei MA, Manas P, Niven G, Needs E, Mackey BM. 2002. Role of membrane fluidity in pressure resistance of *Escherichia coli* NCTC 8164. Applied and Environmental Microbiology 68:5965-5972.
CDC. 2002. Public health dispatch: outbreak of listeriosis. MMWR 51:950-951.
CDC. 2004. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food. MMWR 53:338-343.
Deans SG, Ritchie G. 1987. Antibacterial properties of plant essential oils. International Journal of Food Microbiology 5:165-180.
Denich TJ, Beaudette LA, Lee H, Trevors JT. 2003. Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. Journal of Microbiological Methods 52:149-182.
Denyer SP, Maillard J-Y. 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. Journal of Applied Microbiology Symposium Supplement 92:35S-45S.
Diefenbach R, Keweloh H. 1994. Synthesis of trans unsaturated fatty acids in *Pseudomonas putida P8* by direct isomerization of the double bond of lipids. Archives of Microbiology 162:120-125.
FDA-CFSAN 2003a. *Salmonella spp.* Bad bug book. Available from http://www.cfsan.fda.gov/~mow/chap1.html. Posted January 10, 2003.
FDA-CFSAN 2003b. *Listeria monocytogenes*. Bad bug book. Available from http://www.cfsan.fda.gov/~mow/chap6.html. Posted January 10, 2003.
Friedman M, Henika PR, Mandrell RE. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes,* and *Salmonella enterica*. Journal of Food Protection 65:1545-1560.
Gombas DE, Chen Y, Clavero R, Scott V. 2003. Survey of *Listeria monocytogenes* in ready-to-eat foods. Journal of food protection 66:559-569.

Griffin S, Wyllie S, Markham J, Leach D. 1999. The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. Flavour and Fragrance Journal 14:322-332.

Hammer K, Carson C, Riley T. 1999. Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology 86:985-990.

Helander I, Alakomi H, Latva KK, Mattila ST, Pol I, Smid E. 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. Journal of Agricultural and Food Chemistry 46:3590-3595.

Jay J. 1996. Prevalence of *Listeria* spp. in meat and poultry products. Food Control 7:209-214.

Jay J. 2000. Food preservation with chemicals. In: J. Jay. Modern food microbiology. Gaithersburg: Aspen Publishers, Inc. 253-275.

Juneja VK, Davidson PM. 1993. Influence of altered fatty acid composition on resistance of *Listeria monocytogenes* to antimicrobials. Journal of Food Protection 56:302-305.

Juneja VK, Foglia TA, Marmer BS. 1998. Heat resistance and fatty acid composition of *Listeria monocytogenes*: Effect of pH, acidulant, and growth temperature. Journal of Food Protection 61:683-687.

Kim JM, Marshall MR, Cornell JA, Preston JF, Wei CI. 1995. Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella typhimurium* in culture medium and on fish cubes. Journal of Food Science 60:1364-1368.

Kwon JA, Yu CB, Park HD. 2003. Bacteriocidal effects and inhibition of cell separation of cinnamic aldehyde on *Bacillus cereus*. Letters in Applied Microbiology:61-65.

Lambert P. 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. Journal of Applied Microbiology Symposium Supplement 92:46S-54S.

Li J, Chikindas ML, Ludescher RD, Montville TJ. 2002. Temperature and surfactant induced membrane modifications that alter *Listeria monocytogenes* nisin sensitivity by different mechanisms. Applied and Environmental Microbiology 68:5904-5910.

Mazzotta AS, Montville TJ. 1997. Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10 degree C and 30 degree C. Journal of Applied Microbiology 82:32-38.

NCCLS. 1993. Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically - approved standard. In: NCCLS Antimicrobial Susceptibility Testing. 13. Villanova,PA.: National Committee for Clinical Laboratory Standards.

Parish ME, Davidson PM. 1993. Methods for evaluation. In: P.M. Davidson and A. L. Branen. Antimicrobials in foods. Marcel Dekker.

Post LS, Davidson PM. 1986. Lethal effect of butylated hydroxyanisole as related to bacterial fatty acid composition. Applied and Environmental Microbiology 52:214-216.

Ramos-Nino ME, Clifford MN, Adams MR. 1996. Quantitative structure activity relationship for the effect of benzoic acids, cinnamic acids and benzaldehydes on *Listeria monocytogenes*. Journal of Applied Bacteriology 81:608-612.

Rozes N, Peres C. 1998. Effects of phenolic compounds on the growth and the fatty acid composition of *Lactobacillus plantarum*. Applied Microbiology and Biotechnology 49:108-111.

Russell NJ, Evans RI, ter Steeg PF, Hellemons J, Verheul A, Abee T. 1995. Membranes as a target for stress adaptation. International Journal of Food Microbiology 28:255-261.

Seltmann G, Holst O. 2002. The bacterial cell wall. Berlin: Springer-Verlag.

Sikkema J, de Bont JAM, Poolman B. 1994. Interactions of cyclic hydrocarbons with biological membranes. Journal of Biological Chemistry 269:8022-8028.

Sikkema J, de Bont JAM, Poolman B. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews 59:201-222.

Ultee A, Gorris LG, Smid EJ. 1998. Bactericidal action of carvacrol towards the food-borne pathogen *Bacillus cereus*. Journal of Applied Bacteriology 85:211-218.

Ultee A, Kets EPW, Smid EJ. 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 65:4606-4610.

Ultee A, Bennik MHJ, Moezelaar R. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 68:1561-1568.

Ultee A, Slump RA, Steging G, Smid EJ. 2000a. Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice. Journal of Food Protection 63:620-624.

Ultee A, Kets EPW, Alberda M, Hoekstra FA, Smid EJ. 2000b. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. Archives of Microbiology 174:233-238.

Wendakoon CN, Sakaguchi M. 1995. Inhibition of amino acid decarboxylase activity of *Enterobacter aerogenes* by active components in spices. Journal of Food Protection 58:280-283.

Yousef AE, Courtney PD. 2003. Basics of stress adaptation and implications in new-generation foods. In: A. E. Yousef and V. K. Juneja. Microbial stress adaptation and food safety. Boca Raton: CRC Press. 31-54.

Zaika LL. 1988. Spices and herbs: their antimicrobial activity and its determination. Journal of Food Safety 1998:97-118.

PART 2. ANTIMICROBIAL ACTIVITY OF SELECTED CHEMICAL COMPONENTS FROM ESSENTIAL OILS AGAINST *SALMONELLA* TYPHIMURIUM AND *LISTERIA MONOCYTOGENES*

Abstract

The antimicrobial activity of essential oils is due to the presence of various chemical components in the oil. The objective of this study was to evaluate the antimicrobial activity of active chemical components of essential oils against *Salmonella* Typhimurium and *Listeria monocytogenes* using broth microdilution and macrodilution assays. Carvacrol (CRV), beta-caryophyllene, trans-cinnamaldehyde (CIN), eugenol (EUG), limonene, linalool (LNO), alpha-pinene, rhodinol (RHO), and thymol were tested for antimicrobial efficacy using a 21-h or 24-h inoculum diluted to log 5.7 CFU/ml in phosphate-buffered brain-heart infusion broth (pH 7.2) with 1% Tween 20 in microtiter plates (microdilution) and glass tubes (macrodilution). Tween was used to solubilize the antimicrobial in the aqueous phase. SAS/PROBIT dose-response models were used to analyze the data. Beta-caryophyllene, limonene, alpha-pinene, and thymol were not lethal to *S.* Typhimurium and *L. monocytogenes* at 2800 mg/L and 3200 mg/L, respectively. LNO was more highly antimicrobial using the macrodilution method. The most effective essential oil components against *S.* Typhimurium were CIN and CRV (50% probability of lethality using an avg. of 280 mg/L and 1080 mg/L, respectively) with both methods. CIN (avg. 790 mg/L) and RHO (avg. 1810 mg/L) were the most lethal essential oil components against *L. monocytogenes*. *L. monocytogenes* required higher doses of antimicrobial components to achieve lethality regardless of method.

Introduction

Consumers desire high-quality, minimally processed foods that have fewer preservatives and an extended shelf-life (Brul and Coote 1999). Essential oils, which are obtained from plants by distillation, contain flavor, aroma, and antimicrobial components. They are used mainly for flavors and fragrances. Carvacrol (CRV), the bioactive component found in oregano and thyme essential oils, is a flavoring agent in baked goods, nonalcoholic beverages, and chewing gum (Ultee and others 1999). Eugenol and cinnamaldehyde (CIN) are used in candies and baked goods (Kim and others 1995b). Essential oils of clove, cinnamon, and allspice contain eugenol. CIN is a constituent of cinnamon and cassia oils (Davidson and Naidu 2000).

In the past two decades, many studies have reported the inhibitory and lethal effects of essential oils tested against a wide variety of microorganisms in various culture media and food products. Studies have reported oregano, thyme, clove, and cinnamon essential oils to be highly active against various microorganisms (Lis-Balchin and Deans 1997; Hammer and others 1999 ; Friedman and others 2002; Valero and Salmerón 2003). However, comparing the efficacy of various oils is complex because the composition of bioactive components within a genus, or even species, can vary due to a number of factors such as climate and geographic origin (Zaika 1988).

Evaluation of these EOC presents challenges because of the low water solubility of components, such as thymol, carvacrol, eugenol, and

cinnamaldehyde (Griffin and others 1999). Kim and others (1995a) determined that the minimum bactericidal concentration (MBC) of carvacrol against *Listeria monocytogenes* and *Salmonella* Typhimurium was 500 mg/L and 250 mg/L, respectively, using macrodilution broth method. The minimum inhibitory concentration (MIC) of carvacrol, trans-cinnamaldehyde, and thymol against *S.* Typhimurium were 150, 396, and 150 mg/L, respectively, using the broth macrodilution method (Helander and others 1998). Eugenol was found to inhibit the growth of *E. coli* O157:H7 and *L. monocytogenes* at 1000 mg/L (Blaszyk and Holley 1998). The concentrations of CIN need to achieve a 50% decrease in CFU in *E. coli* O157:H7, *S*. enterica Hadar, and *L. monocytogenes* were 600, 400, and 100 mg/L, respectively (Friedman and others 2002). Although several studies have investigated the efficacy of some common constituents, little information exists on the evaluation of less well-known components and their mechanisms of action.

Although several studies have investigated the efficacy of EOC, only a few have examined their mechanisms of inhibition. CRV was shown to cause potassium ions to leak, reduce intracellular pH, destroy the membrane potential, and deplete the intracellular ATP of *Bacillus cereus* cells, which would result in lethality (Ultee and others 1999). Ultee and others (1999) investigated the effect of carvacrol and cymene of the membrane of *B. cereus*. Cymene is structurally the same as CRV except that it lacks the hydroxyl group. Both expanded the membrane and decreased the membrane potential. While 150 mg/L CRV completely destroyed the pH gradient, 268 mg/L of cymene did not. In addition,

carvacrol decreased the membrane potential more than cymene. Therefore, it was concluded that the hydroxyl group in CRV was important for its antimicrobial activity. One study found that CIN caused exponential phase cells to become long and filamentous and inhibited them from separating from each other (Kwon and others 2003). CRV disrupted the outer membrane of *E. coli* O157:H7 and *S.* Typhimurium since it decreased intracellular ATP and increased extracellular ATP in *E. coli* O157:H7. CIN did not disrupt the outer membrane or influence intra- or extracellular ATP pools (Helander and others 1998).

Common methods used to test antibacterial activity are filter paper disk diffusion, double plate, gradient plate, agar cup, and broth macro- and microdilution (Zaika 1988). Since some components are hydrophobic, disk and agar methods are less suitable and broth dilution assays are preferred (Davidson and Naidu 2000). Difficulties encountered when working with essential oils and their components include poor solubility, turbidity, and volatility. The measurement accuracy of EOC has not been previously compared between the two dilution methods. The objectives of this study were to evaluate the effectiveness of the antimicrobial activity of selected EOC against *L. monocytogenes* and *S.* Typhimurium using dose-response curves and to compare the minimum lethal concentrations obtained from broth macro- and microdilution assays.

Materials and methods

Essential oil components

Materials and methods for microdilution or macrodilution assays were based on the National Committee for Clinical Laboratory Standards Broth Dilution Procedures with modifications as described below (NCCLS 1993). The efficacy of carvacrol, beta-caryophyllene, trans-cinnamaldehyde, eugenol, (S)-(-)-limonene, linalool, (1R)-(+)-alpha-pinene, (1S)-(-)-alpha-pinene, rhodinol (mixture of citronellol and geraniol), and thymol were tested. Chemicals were purchased from Aldrich (Milwaukee, WI), except trans-cinnamaldehyde (Fisher Scientific - Acros, Fairlawn, NJ) and eugenol (Fluka, Buchs, Switzerland). Table of essential oil component abbreviations and solubilities are in Table 2.1.

Stock solutions of EOC were prepared in ethanol, absolute (Aldrich, St. Louis, MO). Sterile water dissolved into ethanol served as the control stock solution. Aliquots ($\leq$160 µl) of freshly prepared EOC or control stock solutions were added into 8 ml Brain Heart Infusion Broth, Modified (BHI, BBL, Sparks MD) with 2% Tween 20 (Fisher Scientific, Fairlawn, NJ) with glass beads such that the final concentration of ethanol did not exceed 1%. Tween and glass beads were used to aid with dispersion of EOC in BHI. All BHI was prepared with phosphate buffer (0.1M, pH 7.2 $\pm$ 0.1) instead of water to prevent pH changes. The pH of BHI was adjusted to 7.35 ($\pm$ 0.1) prior to autoclaving with 2N NaOH, after which the final pH was pH 7.2 ($\pm$ 0.1). Glass tubes containing the EOC and BHI were shaken vigorously forty times to disperse the oil-based components. Dispersions

Table 2.1. Abbreviations, purities, and solubilities of essential oil components (Merck & Co, Inc. 2001).

Essential oil component	Abbreviation	Purity (%)	Solubility
Carvacrol	CRV	98	Practically insoluble in H_2O, freely soluble in alcohol
Beta-caryophyllene	CYP	>98	Not available
Trans-cinnamaldehyde	CIN	99	Dissolves in 700 parts H_2O, 7 volumes of 60% alcohol
Eugenol	EUG	>99	1 ml dissolves in 2 ml 70% alcohol
Limonene	LIM	96	Practically insoluble in H_2O, miscible with alcohol
Linalool	LNO	97	Soluble in 4 volumes 60% a alcohol
(1R)-(+)-alpha-pinene	RAP	98	Practically insoluble in H_2O
(1S)-(-)-alpha-pinene	SAP	>99	Practically insoluble in H_2O
Rhodinol	RHO	>83	Very slightly soluble in H_2O, miscible with alcohol
Thymol	THY	98	1 g dissolves in 1L H_2O or 1 ml alcohol
Essential oil components	EOC		---

containing some components, such as CRV or RHO, became cloudy-white or cloudy-yellow, respectively.

Preparation of EOC concentrations for assays

For the microdilution broth assay, 50 µl of a specific EOC in BHI were dispensed into a microtiter plate (MIC 2000 Thermo Labsystems VWR Scientific Products, West Chester, PA) with each lane containing a different concentration, covered with tape and a lid, and frozen at -14.3°C in freezer bags to decrease volatility of the 10 antimicrobials during preparation and storage. Plates were stored for less than one week. To prepare for the macrodilution assay, 1 ml of each antimicrobial concentration in BHI was dispensed into 4 glass tubes and stored at 4°C. Stocks and dispersions for the macrodilution assay were prepared two days before an experiment. Both microtiter plates and tubes were warmed to room temperature half an hour before inoculation. The inoculum volume diluted the antimicrobial concentrations by one-half.

A wide range of concentrations was initially tested using all selected EOC. Less effective EOC were eliminated from the study. Then the more effective EOC were tested in a narrower range in smaller increments. The lowest concentration tested allowed growth. No growth was detected at the highest concentration. The final Tween concentration was 1% and ethanol concentration was less than 1%. The concentrations tested for each EOC are shown in Tables 2.2 and 2.3. Some of the EOC were tested in smaller increments because they were found to be more lethal in preliminary experiments. The effective EOC

Table 2.2. Concentrations of essential oil components tested against *L. monocytogenes* using microdilution and macrodilution broth methods.

	Concentration range in mg/L (increments tested)	
Component	Microtiter plates	Glass tubes
CRV	2200-3200 (200mg/L)	2400-3200 (200mg/L)
CYP	400-2000 (400mg/L)	2400-3200 (400mg/L)
CIN	400-1200 (200mg/L)	200-1200 (200mg/L)
EUG	1800-2800 (200mg/L)	2400-3000 (200mg/L)
LIM	400-2000 (400mg/L)	2400-3200 (400mg/L)
LNO	400-2800 (400mg/L)	2400-3200 (200mg/L)
RAP	400-2000 (400mg/L)	2400-3200 (400mg/L)
SAP	400-2000 (400mg/L)	2400-3200 (400mg/L)
RHO	1400-2200 (200mg/L)	1400-2200 (200mg/L)
THY	400-2000 (400mg/L)	2400-3200 (400mg/L)

Table 2.3. Concentrations of essential oil components tested against *S.* Typhimurium using microdilution and macrodilution broth methods

	Concentration in mg/L (increments tested)	
Component	Microtiter plates	Glass tubes
CRV	800-2000 (200mg/L)	800-1600 (200mg/L)
CYP	400-2000 (400mg/L)	2000, 2800
CIN	200-800 (200mg/L)	200, 250, 300, 400, 600
EUG	1000-1600 (200mg/L)	1200-1800 (200mg/L)
LIM	400-2000 (400mg/L)	2000, 2800
LNO	1200-2000 (200mg/L)	1000-1800 (200mg/L)
RAP	400-2000 (400mg/L)	2000, 2800
SAP	400-2000 (400mg/L)	2000, 2800
RHO	800-1800 (200mg/L)	1200-3000 (200mg/L)
THY	400-2000 (400mg/L)	2000, 2800

were more of interest. Therefore, smaller increments were tested to determine a concentration at which there may be a threshold to its antimicrobial activity.

Bacterial culture preparation

Listeria monocytogenes serotype 4b ATCC 19115 and *Salmonella* Typhimurium DT104 ATCC BAA-186 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Experimental cultures were maintained on Tryptic Soy Agar slants. A loopful of culture was to inoculate 9 ml of BHI with *L. monocytogenes* or *S.* Typhimurium. The BHI tube was incubated for 24h at 35℃. Then a loopful was transferred into 9 ml BHI. The BHI tube inoculated with *L. monocytogenes* or *S.* Typhimurium was incubated at 35℃ for 21h or 24h, respectively, to prepare the inoculum for experiments. The 21h or 24h culture was serially diluted to 10^{-3} in 9 ml and 99 ml BHI to prepare the inoculum. A one ml aliquot from the 10^{-3} diluted culture was serially diluted in 0.1% peptone water and spread plated onto Standard Methods Agar (SMA) to determine the inoculum size. The inoculum size in the10^{-3} diluted culture was approximately log 6 CFU/ml.

Microdilution method. The 10^{-3} diluted inoculum (50 µl) was added to the 50 µl of EOC in the microtiter plate to achieve a final concentration of bacteria of approximately log 5.7 CFU/ml or log 4.7 CFU/well. Final volume in the well was 100 µl. Microtiter plates were incubated at 35°C for 24 h on a microtiter plate shaker.

Macrodilution method. One ml of the 10^{-3} diluted inoculum was added to the tube containing 1 ml of EOC to achieve a final inoculum of approximately log

5.7 CFU/ml. Final volume in the tube was 2 ml. Tubes were vortexed and incubated in a shaking water bath at 35°C for 24h.

The MIC was determined as the lowest concentration of antimicrobial agent that completely inhibited growth of the organism in the wells as detected by the unaided eye. The MIC was difficult to determine in macrodilution and especially microdilution methods because some EOC would appear turbid due to the low solubility of EOC rather than due to bacterial growth. CRV and RHO were two such antimicrobials. Therefore, it was necessary to subculture from the wells to clearly determine if there was growth and to test antimicrobials using macrodilution broth instead.

Although macrodilution method improved visibility of results, tubes containing RHO and CRV appeared turbid with pellet, but when subcultured, contained no detectable colonies. Within the tubes small droplets of carvacrol were visible, especially at higher concentrations. The pellets may have been due to the phosphate precipitation. Therefore, it was easier to evaluate effectiveness by detecting growth on agar plates and finding the minimum lethal concentration (MLC). MLC was determined as the lowest concentration that permitted no growth after subculture.

To determine the MLC 10 µl was obtained from the microtiter wells or glass tubes and plated onto SMA. Plates were incubated 35°C for 24h and counted. MLC was determined according to the NCCLS Guidelines for the Rejection Value for Each Initial Inoculum Concentration on the Basis of a Single 0.01-ml Sample. These guidelines suggest that when the number of colonies

from a test vessel is equal to or less than the rejection value (which is $\geq$11 CFU for a log 5.7 CFU/ml inoculum) the concentration of the antimicrobial in the test vessel is considered lethal (99.9% or greater reduction in the final inoculum (NCCLS 1993).

Statistical analysis

Three replications were performed for each antimicrobial and method. The data were analyzed using SAS procedure PROBIT analysis (Statistical Analysis System Institute, Cary, NC) to develop dose-response models. Since the response of interest is lethality or nonlethality, the dependent variable is a binary discrete variable. PROBIT is a type of logistic regression that fits regression equations to categorical responses. Bacterial response, such as probability of lethality, was plotted as a function of the dose (López-Malo and others 2002).

The concentration of antimicrobial was transformed to $\log_{10}$ to obtain a more linear regression line. First, PROBIT analysis was conducted to evaluate if there was a difference among EOC stock solutions of a certain antimicrobial that were prepared at different times within a certain method and organism. The data were summarized across the EOC stock solutions, for each antimicrobial, within a method and by organism. PROBIT analysis used the summarized data to determine if there were dose effects and to generate dose-response curves to compare the effectiveness across antimicrobials. Next, the data were analyzed to compare methods within each organism. Lastly, the doses of each chemical

were compared to determine if an organism required higher doses than the other organism for the macrodilution method.

Results

PROBIT dose-response curves

The dose-response curves for *L. monocytogenes* and S. Typhimurium exposed to EOC have a sigmoid shape since there is no response from the population until the dose reaches a threshold response. When the higher saturation dose has been applied no increase in response occurs. In this study the initial region at lower doses is one of insignificant lethality. Then there is a linear region of exponential inactivation (after a logarithmic transformation), and then a final region where higher concentrations do not cause additional lethality (López-Malo and others 2002).

The PROBIT procedure estimates the probability of successful response (lethality) for a dose (concentration of antimicrobial), which is the independent variable. Zero to 100% probability is the range of response. This probability is based on regression parameters of the number of successful responses and the total number of events and the threshold response rate. Dose-response curves are useful when deciding what probability of lethality is desired in cost-benefit analysis and risk assessment. Dose-response curves for *L. monocytogenes* exposed to EOC are presented in Figures 2.1 and 2.2. The solid line represents the regression line, which is used to obtain the prediction for the mean probability

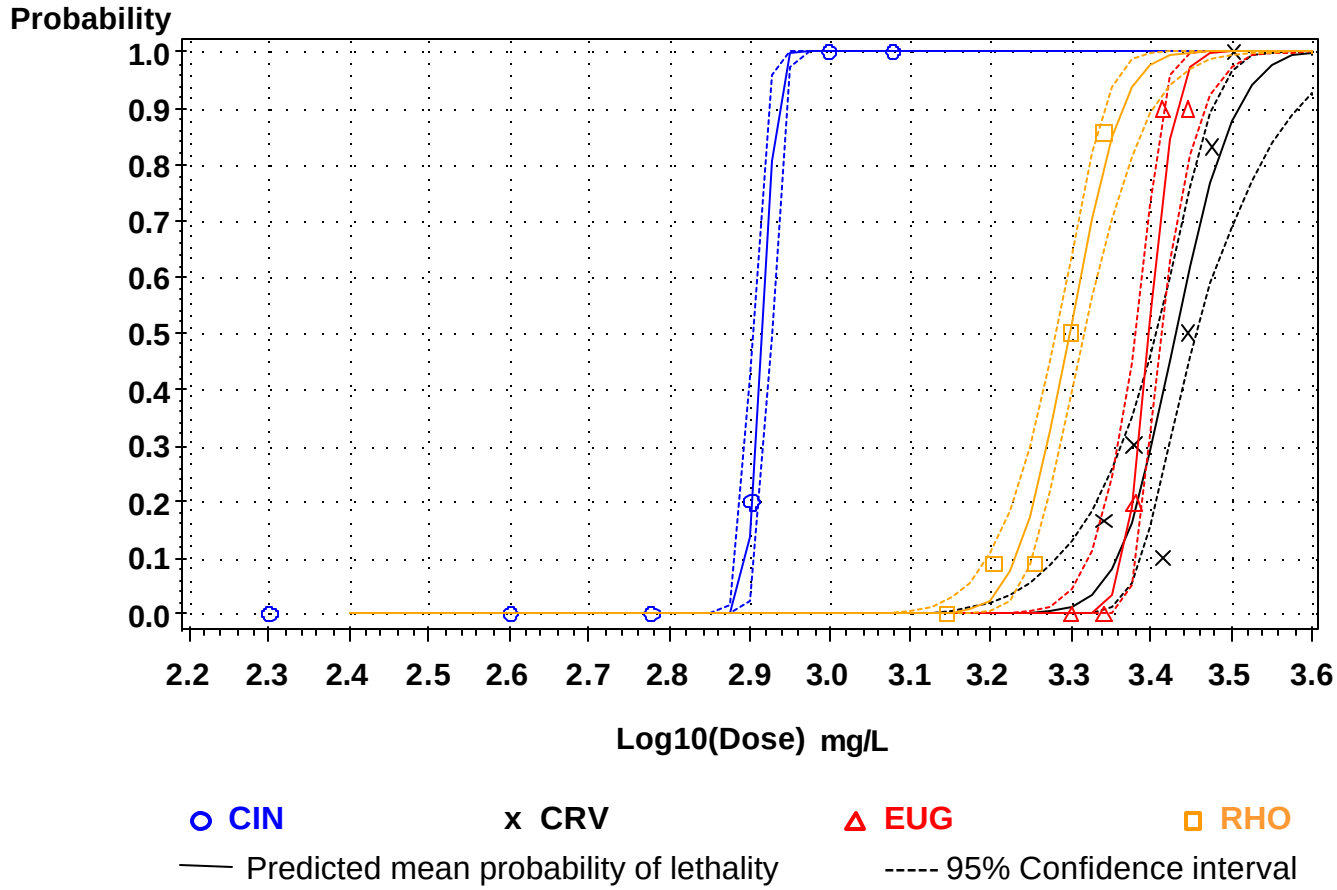


Figure 2.1. Probability of lethality of *Listeria monocytogenes* treated by essential oil components using microdilution method (microtiter plates).

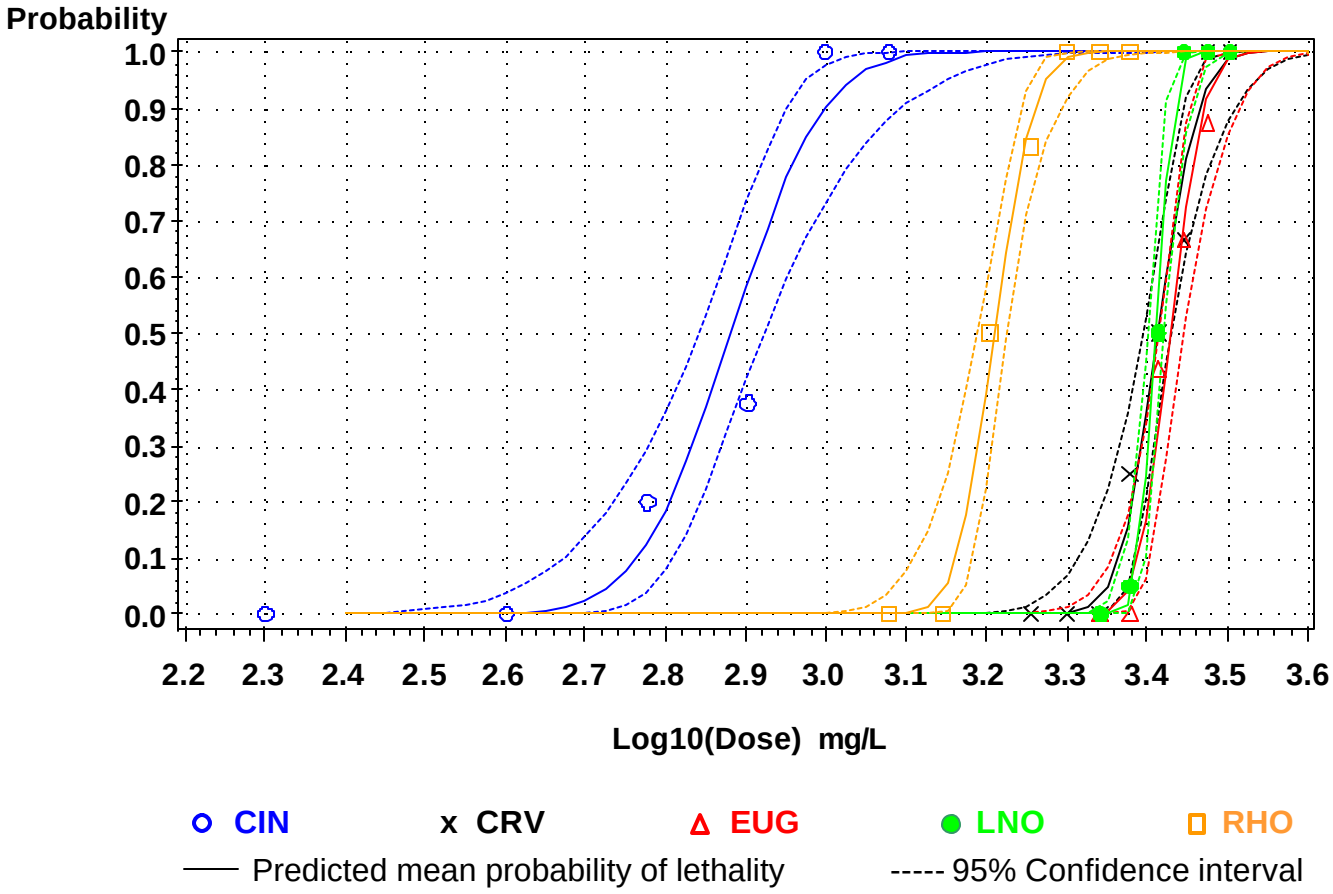


Figure 2.2. Probability of lethality of *Listeria monocytogenes* treated by essential oil components using macrodilution method (glass tubes).

of lethality at a given dose. The dotted lines on each side of the mean predicted probability represent the 95% confidence interval. The confidence interval is a range of values that is expected to contain the mean predicted probability of lethality in 95% of experiments.

L. monocytogenes

Microdilution method

The essential oil components (EOC) CYP, LIM, RAP, SAP, and THY were not lethal to *L. monocytogenes* at 2000 mg/L and LNO was not lethal at 2800 mg/L against *L. monocytogenes* using the microtiter plate assay (Table 2.4). CIN, CRV, EUG, and RHO exhibited lethality to *L. monocytogenes* at concentrations greater than 760 mg/L. CIN was the most effective EOC, followed by RHO (Table 2.4 and Figure 2.1). The difference between the doses of CIN and RHO at 50% probability of lethality was approximately 1200 mg/L (Table 2.4). At 99% probability of lethality, this difference was greater than 1700 mg/L. The MIC values of EUG and CRV were similar but less than that of CIN and RHO.

Macrodilution method

CYP, LIM, RAP, SAP, and THY were not lethal at 3200 mg/L (Table 2.4). Colonies grew abundantly on SMA plates after these samples were subplated. Implementation of the macrodilution method determined that CIN was the most effective EOC against *L. monocytogenes* followed by RHO (Table 2.4 and Figure 2.2). The difference between their doses at 50% probability was >800 mg/L (Table 2.4). At 99% probability of lethality, this difference decreased to

Table 2.4. Antimicrobial doses corresponding to 50% and 99% probability of lethality against *L. monocytogenes.*

	Dose (mg/L) corresponding to 50% probability of lethality		Dose (mg/L) corresponding to 99% probability of lethality	
Component*	Microtiter plate	Tubes	Microtiter plate	Tubes
CIN	818	760	875	1236
CRV	2704	2600	3698	3214
EUG	2500	2698	2877	3199
LNO	---	2582	---	2838
RHO	1986	1626	2600	1995

* Microtiter plate: CYP, LIM, RAP, SAP, and THY at 2000 mg/L and LNO at 2800 mg/L were not lethal.
Tubes: CYP, LIM, RAP, SAP, and THY at 3200 mg/L were not lethal.

>700mg/L. CRV, EUG, and LNO and were similar in antimicrobial activity, but not as effective as CIN and RHO.

Comparison of micro- and macrodilution methods for an antimicrobial

In both the micro- and macrodilution methods, CIN was the most effective against *L. monocytogenes* followed by RHO. CIN and CRV were as active in the microtiter plates as in the tubes (Table 2.4). Statistically, it appeared that EUG was more effective in the microtiter plates ($p<0.01$) than in the tubes and that RHO was more effective in the glass tubes ($p<0.01$) than in the microtiter plates. However, these differences were probably due to pipetting error. Since ethanol has no surface tension, the volume transferred from the EOC stock solutions in ethanol to the BHI broth was likely inconsistent. The differences between the micro- and macrodilution methods for RHO and EUG were less than two-fold. A two-fold difference in MIC values is considered to be within experimental error (Inouye and others 2001).

***S.* Typhimurium**

Microdilution method

CYP, LIM, RAP, SAP, and THY were not lethal at 2000 mg/L against *S.* Typhimurium in the microtiter plates (Table 2.5). CIN was the most effective EOC, followed by CRV, EUG, RHO, and LNO at 50% probability pf lethality (Figure 2.3 and Table 2.5). The difference between CIN and CRV doses was >700 mg/L at 50% probability of lethality and increased to >1000 mg/L at 99%

Table 2.5. Antimicrobial doses corresponding to 50% and 99% probability of lethality against *S.* Typhimurium.

	Dose (mg/L) corresponding to 50% probability of lethality		Dose (mg/L) corresponding to 99% probability of lethality	
Component*	Microtiter plate	Tubes	Microtiter plate	Tubes
CIN	284	280	312	419
CRV	1033	1130	1380	1556
EUG	1227	1294	1282	2094
LNO	2056	1567	2576	1845
RHO	1535	---	2495	---

* Microtiter plate: CYP, LIM, RAP, SAP, and THY at 2000 mg/L were not lethal.

Tubes: CYP, LIM, RAP, SAP, and THY at 2800 mg/L and RHO at 3000 mg/L were not lethal.

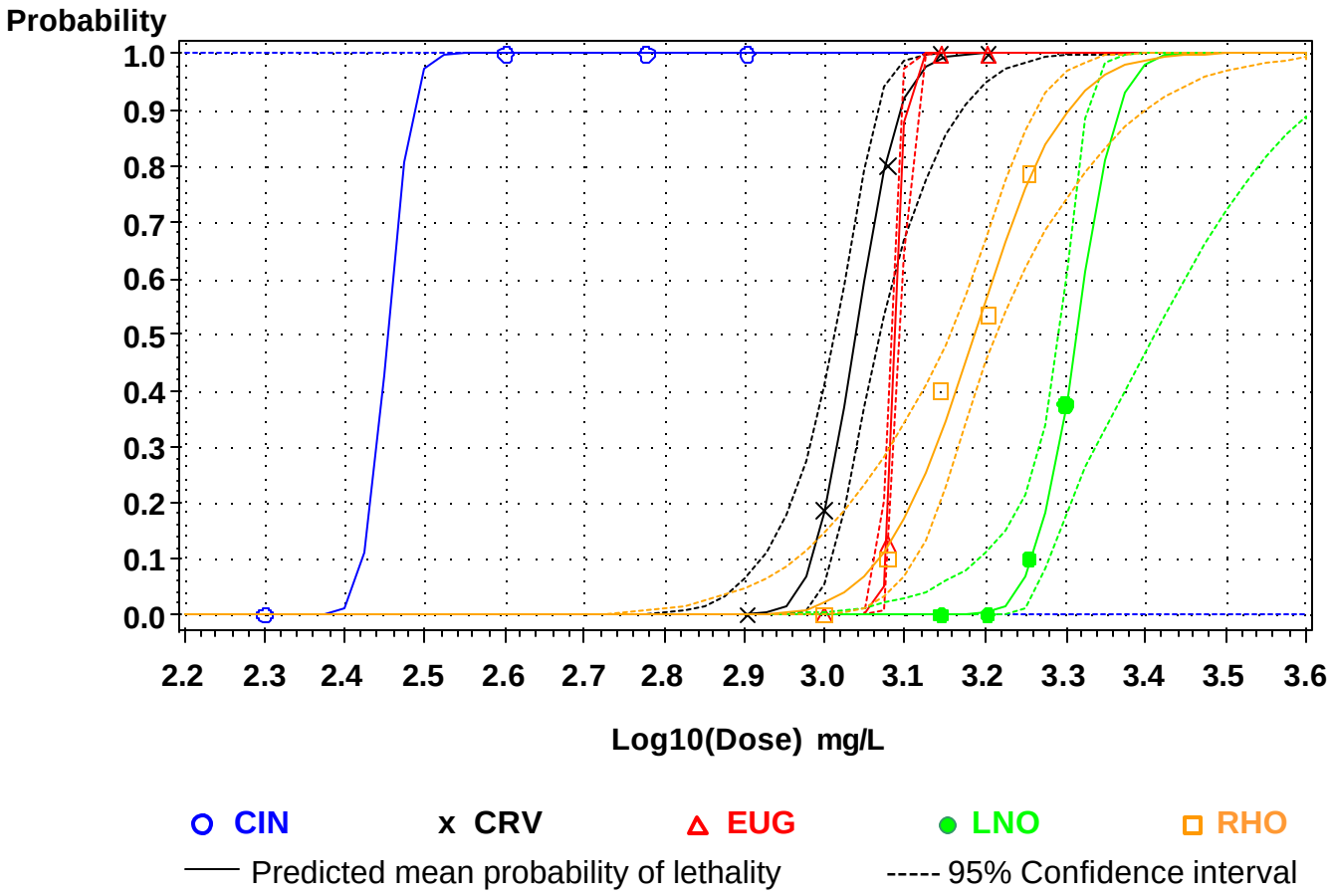


Figure 2.3. Probability of lethality of *Salmonella* Typhimurium treated by essential oil components using microdilution method (microtiter plates).

probability (Table 2.5). CIN lacked a 95% confidence interval because there were fewer distinct doses tested in the sublethal range. The 95% confidence interval for LNO was wider since the concentration that may achieve a 100% lethal dose was not prepared for the assay.

Macrodilution method

CYP, LIM, RAP, SAP, and THY were not lethal at 2800 mg/L in the glass tube assay (Table 2.5). RHO was not lethal at 3000 mg/L. CIN was the most effective EOC against *S.* Typhimurium followed by CRV, LNO, and EUG at 50% probability of lethality (Figure 2.4 and Table 2.5). The difference between CIN and CRV doses was >800 mg/L at 50% probability of lethality and increased to >1100 mg/L at 99% probability (Table 2.5).

Comparison of micro- and macrodilution methods for an antimicrobial

The inhibitory activity of CIN, CRV, and EUG was not different between the macro- and microdilution methods when tested against *S.* Typhimurium. LNO was statistically more effective against S. Typhimurium using the macrodilution method ($p<0.0001$) and required 490 mg/L less to achieve a 50% probability of lethality (Table 2.5). Since this difference was less than two-fold the difference may have been due to pipetting error. Although the comparison was not statistically analyzed, RHO seemed to be more effective in the microtiter plates, since there was abundant growth of *S.* Typhimurium when samples were subplated from glass tubes containing 3000 mg/L RHO. There was a strong stock solution effect among different stocks of RHO when tested against *L.*

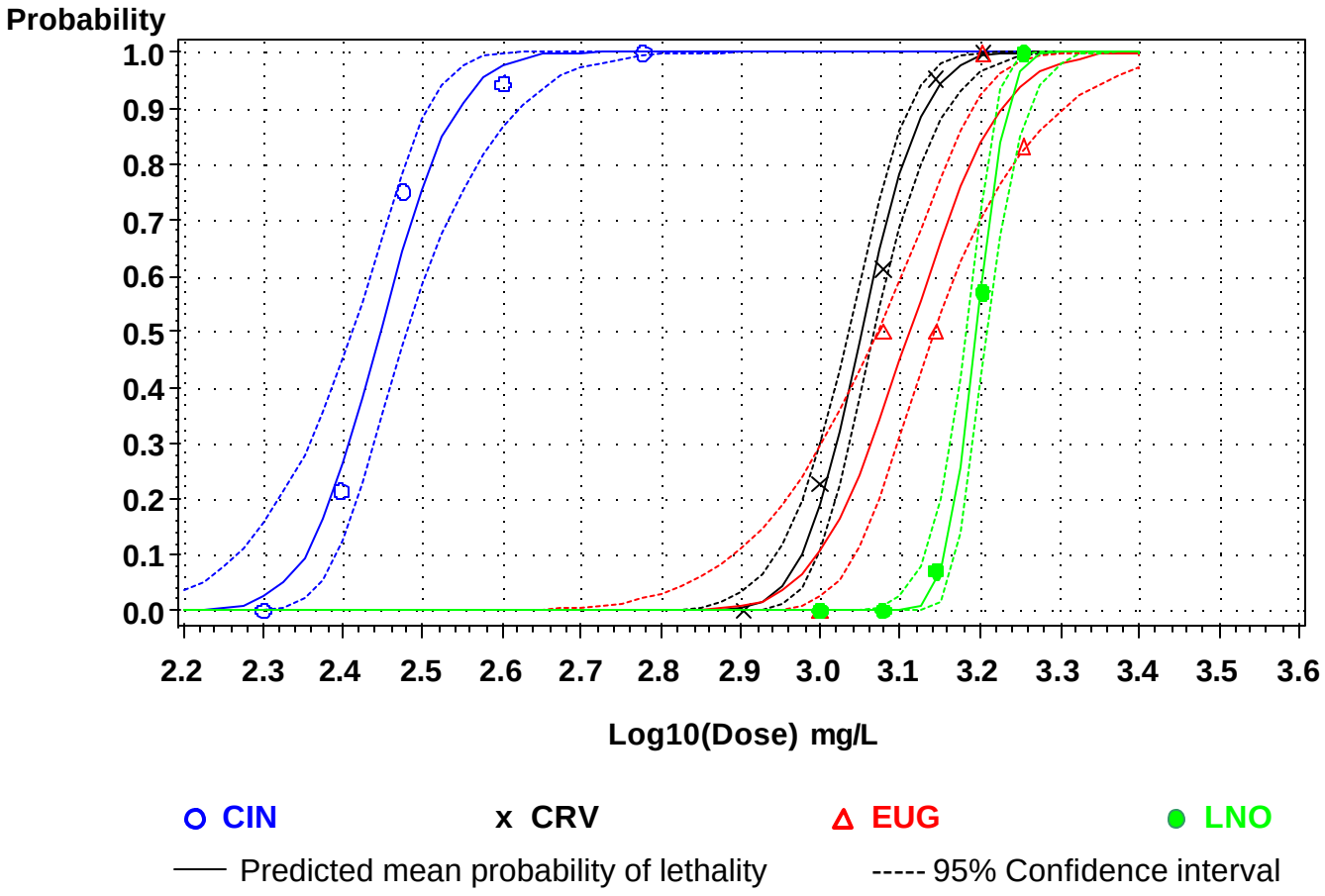


Figure 2.4. Probability of lethality of *Salmonella* Typhimurium treated by essential oil components using macrodilution method (glass tubes).

monocytogenes in microtiter plates. The variation may be due to inconsistency when pipetting stock solutions because of the low surface tension of ethanol. In addition, it was difficult to determine if the variation in RHO antimicrobial activity was due to loss of RHO during storage or incubation of plates because of the volatility of RHO.

Comparison of effectiveness against *L. monocytogenes* and *S. Typhimurium*

All figures show that CIN was much more bactericidal than the other antimicrobials. RHO and CRV were the next most effective antimicrobials against *L. monocytogenes* and *S.* Typhimurium, respectively. *L. monocytogenes* required higher doses of CIN, CRV, EUG, LNO and RHO regardless of which method was used. At 50% probability of lethality, >500 mg/L more of CIN was needed to be effective against *L. monocytogenes* than *S.* Typhimurium. At 99%, the concentration increased to >600 mg/L. For CRV to be lethal with 50% probability, >1600 mg/L CRV was needed against *L. monocytogenes*. At 99%, the concentration increased to >1900 mg/L.

Discussion

Statistical differences compared to scientific differences

A two-fold difference in MIC values is considered to be within experimental error (Inouye and others 2001). Although the results of our study showed statistical differences between the micro- and macrodilution methods for EUG,

LNO, and RHO, they may not be scientifically significant since the differences were much lower than a two-fold difference and may be due to experimental error instead. The doses of CIN, CRV, and EUG needed to be lethal against *L. monocytogenes* were more than two-fold higher than those against *S.* Typhimurium and therefore scientifically significant.

Comparisons with other studies

When these results were compared with those of other studies, similarities and differences were found. Studies of essential oils and components were difficult to compare since there are various assays used to evaluate antimicrobials, but there is no standardized test method or format for reporting assay results of bactericidal activity (Friedman and others 2002). Sensitivities of bacterial strains, method of growth, and phase of growth when the antimicrobial was applied, and enumeration of survivors may affect results. Solubility of the oil or component and method of preparing stocks may influence activity (Friedman and others 2002). Variations in experimental procedures, composition of medium, and preparation of slightly soluble phenolics lead to wide differences in results (Wen and others 2003).

In a microtiter plate assay of 96 essential oils and 23 oil components, CIN, EUG, THY, CRV, citral, geraniol, perillaldehyde, carvone S, estragole, and salicylaldehyde were the most effective components tested against *L. monocytogenes* F2379 (Friedman and others 2002). Similarly, our study found that CIN, EUG, and CRV were most lethal to *L. monocytogenes*. However, doses of CIN, EUG, and CRV at 50% probability of lethality were about three to

four times higher than the BA50 values of Friedman and others (2002) (Table 2.6). The BA50 values were determined from the linear regression model as the dose that resulted in a 50% decrease in the number of CFU compared to that of the negative control rather than through a dose-response PROBIT analysis. BA50 value of THY was 770 mg/L, but our study did not find THY to be as effective as the other antimicrobials. LIM and LNO were not evaluated above 2080 mg/L and 2890 mg/L respectively. The lower activity of LIM and LNO agrees with these authors' findings that LIM and LNO were not effective at 6700 mg/L. Friedman and others (2002) did not evaluate RHO.

They tested these components against *S. enterica* serovar Hadar (isolated from ground turkey). They found that THY, CIN, CRV, EUG, salicylaldehyde, geraniol, isoeugenol, terpineol, perillaldehyde, and estragole were the most active. Doses of CIN, EUG, and CRV at 50% probability of lethality of our study were about two times higher than their BA50 values (Table 2.6). However, the LNO dose at 50% lethality was about two-fold lower than the BA50 value. THY was found to be effective at a BA50 value 340 mg/L but our study did not find it to be effective. Friedman and others (2002) reported that LIM was not effective up to 6700 mg/L and our study did not test it above 3300 mg/L. Similarly, they found that CRV and LNO doses needed to inactivate *S. enterica* were lower than that of *L. monocytogenes,* which agrees with our findings of the relative susceptibility of *Salmonella* compared to *Listeria*.

Table 2.6. Comparison of BA50 values adapted from Friedman and others (2002) and concentrations to reach 50% probability of lethality from this present study using microdilution assay.

	BA50 values (mg/L) from Friedman and others (2002)			Concentration (mg/L) to reach 50% probability of lethality	
	L. monocytogenes		*S. enterica*	*L. monocytogenes*	*S.* Typhimurium
	F2379	RM2388	serovar Hadar MH136	serotype 4b	DT104
CIN	190 ± 10	80 ± 10	330 ± 140	818	284
CRV	830 ± 100	860 ± 100	540 ± 0.01	2704	1033
EUG	610, 500	810 ± 10	870 ± 350	2500	1227
LNO	6700, 8500	6700, 6800	3700 ± 400	-	2056
THY	770 ± 100	770 ± 200	340 ± 20	-	-

These differences in the lethality values between the two studies may be due to their preparation in phosphate-buffered saline (PBS; pH=7.0) (Friedman and others 2002). For example, to prepare the THY treatment, they vortexed the THY solids in a microcentrifuge tube with PBS, warmed it for 4s, and vortexed it. The preparation led to a more mixed suspension and therefore a higher antibacterial activity than in this study. This may explain why our study found that THY was less active than other antimicrobials, although it appeared to dissolve in ethanol.

The variation in efficacy may be due to differences in inoculum size, incubation time, and the- formation of mixed micelles from Tween and <1% ethanol from the stock solution. The most influential factor in these assays is the inoculum size (NCCLS 1993). Larger bacterial populations may be more likely than smaller populations to have some more resistant cells, which can survive and grow. Therefore, smaller populations will be more quickly and fully inhibited (Amsterdam 1996). The susceptibility of antimicrobials increases with a smaller inoculum size (Collins and others 1995). For example, the antimicrobial activity of essential oils of oregano, thyme, and clove were found to increase with decreasing inoculum level of *Staphylococcus aureus* and *Salmonella* Hadar (Remmal and others 1993). The mean suggested inoculum is log 6 CFU/ml for microdilution (NCCLS 1993). Friedman and others (2002) added 50-µl of 1,500 to 2,000 CFU/well in PBS to 100-µl of antimicrobial in microtiter plates. Since the inoculum size in our study (log 5.7CFU/ml or 50,000 CFU/well) was considerably

larger as recommended by NCCLS, this may explain the higher concentrations required for lethality.

Another important difference between the studies was the incubation time. Friedman and others (2002) incubated the microtiter plates for one hour, a significantly shorter time compared to 24 h. They had found in previous experiments with *E. coli* and *S. enterica* that the % kill by cinnamon oil, oregano oil, and eugenol approached a maximum at 60 min and reached a plateau. The strong influence of incubation time was demonstrated when ampicillin was tested against *S. aureus* and *E. coli* strains. The MIC values for 24-h incubation were at least two-fold more than those of 12-h incubation (Amsterdam 1996). The method of determining survivors was different as well as the statistical model used for the dose-response curve. It can be argued that lethality at 60 min should also be demonstrated at 24h if the dose is actually lethal, rather than inhibitory.

Furthermore, Friedman and others (2002) did not use Tween 20 to increase solubility of the hydrophobic antimicrobials, but used vigorous shaking in PBS. In our study the concentration of Tween was 1%. Tween 20 has been reported to decrease the antimicrobial activity of oregano, thyme, and clove essential oils and EUG. Dispersions of oregano, thyme, and clove essential oils in 0.2% agar solution produced lower MIC values than those with 2% ethanol and 2.5% Tween. MIC values were lower with Tween 20 than with Tween 80 (Remmal and others 1993). In another study, Tween 20 was used at >0.25% to increase eugenol solubility, but it decreased inhibitory activity (Blaszyk and

Holley 1998). Blaszyk and Holley (1998) hypothesized that while Tween increases the solubility of thymol in water, it decreases its solubility in the cytoplasmic membrane since the membrane is hydrophobic. This may help explain the much higher values of our study.

Friedman and others (2002) also tested various strains of *S.* enterica serovars Meleagridis, Hadar, and Newport and *L. monocytogenes* F2379 (isolated from cheese associated with outbreak) and RM2388 (isolated from a retail mint herb, serotype 1). They calculated the BA50 values for cinnamon bark oil, oregano origanum oil, and eugenol. The differences in BA50 values were less than two-fold among strains within a species. These authors noted that the *L. monocytogenes* F2379, which was isolated from cheese associated with an outbreak, seemed more resistant than the *L. monocytogenes* RM2388. They believed that the F2379 strain had virulence factors that RM2388 lacked. It was hypothesized that F2379 developed resistance while in the cheese, but RM2388 did not develop resistance in the mint. The authors emphasized the need to ascertain if resistance is associated with the source of the isolate or a response of the strain to adapt to the food processing environment. Since the strains in our study are known to be associated with outbreaks, these strains may be more resistant than the previous study because of their source or virulence factors.
In a study by Kim and others (1995a), the MBC value for CRV was three to five times lower than the concentrations to achieve 50% probability of lethality against *S.* Typhimurium and *L. monocytogenes* in our study (Table 2.7). The MBC values for EUG and LNO were about three times lower against *S.* Typhimurium

Table 2.7. Comparison of minimum bactericidal concentrations (MBC) adapted from Kim and others (1995) and concentrations to reach 50% and 99% probability of lethality from this present study using macrodilution assay.

			Concentration (mg/L) to reach:			
	MBC (mg/L) from Kim et al (1995)		50% probability of lethality		99% probability of lethality	
	L. monocytogenes Scott A	*S.* Typhimurium ATCC 6539	*L. monocytogenes* serotype 4b	*S.* Typhimurium DT104	*L. monocytogenes* serotype 4b	*S.* Typhimurium DT104
CRV	500	250	2600	1130	3214	1556
EUG	>1000	500	2698	1294	3199	2094
LNO	>1000	1000	2582	1567	2838	1845

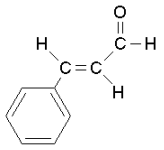
(Kim and others 1995a). Similarly, they found that higher concentrations of antimicrobials were required to inactivate *L. monocytogenes.* It was unclear from the article at which sampling time the MBC was determined. Although the concentration of 1% Tween and inoculum size of log 6 were similar to our study, the assay was conducted with a much larger volume of 20 ml of tryptic soy broth (TSB) in flasks.

Structure and molecular characteristics associated with activity

In our study, the aldehyde component (CIN) was the most effective, followed by the phenolics (CRV and EUG) and acyclic terpene alcohols (LNO and RHO) (Figure 2.5). This agreed with the findings of Friedman and others (2002), who found that the aldehydes and phenolics were very active. Phenolics are believed to be the main antimicrobial components in many essential oils (López-Malo and others 2002). CRV and THY are both phenolic and differ only in the position of the hydroxyl group. The hydroxyl group is the necessary characteristic required for the action of CRV against *B. cereus* to decrease the membrane potential and pH gradient (Ultee and others 2002).

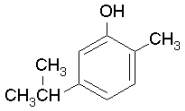
The carbonyl group of aldehydes has the capacity to undergo oxidation and reduction reactions and nucleophilic additions which allows it to bind metal ions, sulfhydryl and amino groups of membrane proteins (Bowles and Miller 1993). Cinnamaldehyde was found to be an effective inhibitor of histidine decarboxylase activity of *Enterobacter aerogenes* and its carbonyl group was suggested as the functional group (Wendakoon and Sakaguchi 1995).

Aromatic unsaturated aldehyde

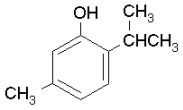


Cinnamaldehyde

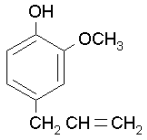
Phenolics



Carvacrol

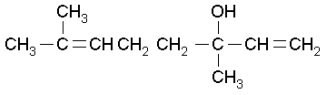


Thymol

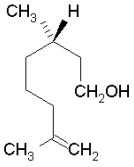


Eugenol

Acyclic terpene alcohols

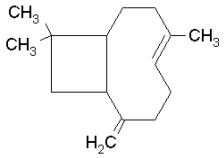


Linalool

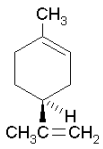


Rhodinol

Terpene hydrocarbons

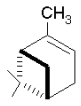


ß-caryophyllene



(S)-(?)-Limonene

(1R)-(+)-a-pinene



(1S)-(-)-a-pinene

Figure 2.5. Structures of selected essential oil components.

In a study of the effect of structure and molecular characteristics on antimicrobial activity, hierarchical cluster analysis arranged 60 terpenoids into five groups based on their activity patterns against *Pseudomonas aeruginosa, E. coli* O157:H7*, Staphylococcus aureus,* and *Candida albicans* (Griffin and others 1999). THY and CRV were in group I, EUG and LNO in group II, and LIM, RAP, and SAP in group IV. Group I, which inhibited all four microorganisms, was more soluble in water (although low in solubility in general) and had a higher capacity to form hydrogen bonds than group IV, which was not inhibitory. Overall group I and II were similar in structure and molecular characteristics, but group I was more active. Compared to group II, group I was more capable of forming a hydrogen bond, had a smaller surface area, and a lower hydrophilic/lipophilic balance. Group IV included hydrocarbons like CYP, RAP, SAP, and LIM which are inactive because of their lower water solubility and lower H-bonding capacity (Griffin and others 1999).

Hydrophobic molecules cannot easily diffuse through the LPS of Gram-negative bacteria. In contrast, small hydrophilic compounds can pass through porins. Group I is more likely to permeate because of its smaller size and capacity to form H-bonds to interact with the water layer on the exterior of bacteria (Griffin and others 1999). Griffin and others (1999) conclude that "specificity and activity was not always defined by the functional group present but were associated with H-bonding parameters." However, they cautioned that solubility and H-bonding did not explain all the patterns in antimicrobial activity and there were other molecular properties to be measured.

Sensitivity of Gram-negative and Gram-positive organisms

Most studies have found that Gram-positive organisms are more susceptible than Gram-negative to essential oils and their components (as reviewed by Burt 2004). This might be expected since the dense LPS of Gram-negative organisms is hydrophilic and acts as an impermeable barrier to hydrophobic molecules, which can diffuse slowly (Vaara 1992). However, other studies that that *S.* Typhimurium was more sensitive than *L. monocytogenes* (Kim and others 1995a). Our study also found S. Typhimurium was more susceptible than *L. monocytogenes*. Perhaps, it is more difficult for hydrophobic EOC to penetrate the thick peptidoglycan layer of *L. monocytogenes* in order to act on the cytoplasmic membrane. It is hypothesized that after they diffuse past the LPS in Gram-negative bacteria, they can act on the proteins in the periplasmic space and then pass through only a thin peptidoglycan layer to act on the cytoplasmic membrane. More research is needed on the main target on which these essential oils act.

The partitioning of EOC in the membrane may also help explain the different susceptibility of Gram-negative and positive bacteria to EOC. The optimal oil:water partition coefficient (log P) for Gram-negative and Gram-positive bacteria is 4.0 and 6.0 for the oil phase, respectively. Aromatic solvents of 2.0 - 4.0 log P changed the cytoplasmic membrane organization in Gram-negative bacteria (Denyer and Maillard 2002). CRV has a log P of 3.6, preferring to partition in the nonpolar solvent more than water (Ultee and others 2002).

Therefore, CRV may be more effective against Gram-negative bacteria since the optimal log P of Gram-negative bacteria is closer to the log P of CRV.

Mechanisms of action

It has been suggested that carvacrol occupies more area than the typical space between the fatty acid chains of two adjoining phospholipid molecules. This interferes with the van der Waals interactions between the chains and therefore affects fluidity (Ultee and others 2000; Ultee and others 2002). Liquid-crystalline is the optimal state of the membrane lipids in which fatty acyl chains are loosely packed and interactions between the chains are not at a maximum because the chains or its $-CH_2-$ groups is rotated (Seltmann and Holst 2002). If the membranes become too fluid, additional carvacrol can continue to permeate the membrane, accumulate, and cause more damage (Ultee and others 1998).

It was found that carvacrol expanded the single-membrane liposomes prepared from phosphatidylethanoloamine shown by an increase in fluorescence of a probe (Ultee and others 2002). It is believed that this swelling leads to a destabilized membrane and therefore a leakage of ions. During exposure to carvacrol, an influx of protons from outside the cytoplasmic membrane and efflux of potassium ions in *Bacillus cereus* was reported (Ultee and others 1999). This leakage was also evident in the decrease of the pH gradient across the membrane (Ultee and others 2002). The leakage of ions leads to a drop in the membrane potential and depletion of the intracellular ATP pool (Ultee and others 2002). It was found that 300 mg/L CRV reduced viable counts of *Bacillus cereus*

cells in 30 min and depleted intracellular ATP within 7 min without a proportional increase in extracellular ATP (Ultee and others 1999).

Similarly, Helander and others (1998) reported that CRV at 300 mg/L (MIC = 451 mg/L) increased the uptake of hydrophobic fluorescent probe NPN in *E. coli* O157:H7 and S. Typhimurium. Uptake of NPN occurred because the outer membrane was no longer acting as a strong permeability barrier. CRV also decreased intracellular ATP in *E. coli* O157:H7, but with a smaller increase in extracellular ATP. These effects indicated that CRV disrupted the outer membrane. In contrast, it was suggested that CIN did not disrupt the outer membrane because at 264 mg/L (MIC = 396 mg/L) it did not increase the uptake of NPN or influence intra- or extracellular ATP pools (Helander and others 1998). CRV was reported to impair the cell membrane and increase permeability because of the increased fluorescence of nuclear stain EB after *Staphyloccus aureus* and *Pseudomonas aeruginosa* were exposed to 0.1% CRV (Lambert and others 2001).

Technical limitations of broth dilution assays

Compared to the macrodilution assay, the microtiter plate assay was less laborious to prepare the concentrations and inoculate (which NCCLS suggests to do within 15 min). Results were more difficult to reproduce with the microtiter plates, especially due to the low solubility of CRV and RHO, which contributed to turbidity. Oil droplets settled within CRV dispersions in tubes and wells, which may not have contacted the cells uniformly during incubation. If the plates were not uniformly manufactured, interaction between the antimicrobials and lipophilic

components in the wells may have been inconsistent. It is more difficult to assess the MLC with the microdilution method, but less broth is required (NCCLS 1993). The macrodilution method is more appropriate when there are fewer assays to conduct, since preparing many sets of tubes can be tedious and more operators are needed to inoculate during a short time (Collins and others 1995).

Limitations of minimum lethal or bactericidal concentration

There are microbiologic and technical problems with the MLC determination (Amsterdam 1996). The definition of MLC is the concentration resulting in a 99.9% decrease in the number of initial bacteria in the inoculum (NCCLS 1993). Amsterdam (1996) asserted that it has not been demonstrated that 99% is more clinically relevant than 98%. NCCLS (1993) noted that this distinction might not have biological relevance. Bacteria also tend adhere and proliferate above the meniscus of the medium to possibly avoid contact with the antimicrobial (Amsterdam 1996). This happens more often with plastic test tubes than with acid-treated borosilicate glassware or with plastic microtiter plates (NCCLS 1993). In addition, the adhesion of bacteria to the interior of the test vessel depends on the type of material (NCCLS 1993). NCCLS cautioned, "the determination of the MLC is subject to methodological variables such that the clinical relevance of MLC values is nearly impossible to assess.

A microbiologic complication is the "persistence" phenomenon, which describes a small number (<0.1% of the inoculum) of cells, or "persisters," that survive the bactericidal activity of the antimicrobial because they are metabolically inactive or replicating slowly (NCCLS 1993). If the persisters are

retested, they are as susceptible as the original strain and no greater percentage of cells that persist. The persistence phenomenon is a type of tolerance. Tolerance is defined as the ability of the microorganism to escape lethal effect of the antimicrobial, but the MIC remains the same since it can grow (NCCLS 1993; Amsterdam 1996). Other types of tolerance are genotypic and phenotypic, which can occur more often and are more relevant (Amsterdam 1996).

Applications of components to food

One of the limitations of this study is that the broth dilution assay is an artificial way of bringing the antimicrobial and the pathogen in contact, but outside of food. Once an antimicrobial is found to be effective with in vitro methods, its action in a food system can be tested. Because of the interactions of the antimicrobial with food components, the inhibitory effect is lower in foods than in microbiological media. Foods high in fat would require higher concentrations to inhibit growth (Zaika 1988).

Since essential oils are components are pungent, they may cause undesirable flavors or aromas (López-Malo and others 2002). Although 300-400 mg/L was lethal to *S.* Typhimurium in-vitro in our study, higher levels would be needed if the control microorganisms was intended. The concentrations needed for lethal action by essential oils exceed tolerable taste thresholds. Therefore, to achieve desired effects against microorganisms essential oils and their components may be added to foods at inhibitory levels and be combined with other antimicrobials or processing conditions (Brul and Coote 1999). Since these

inhibitory levels are greater than those used for flavoring, manufacturers would be required to submit a GRAS notice to FDA (Draughon 2004).

Although NCCLS suggested an inoculum of log 5.7 for clinical assays, this may or may not be applicable in food. The selection of the inoculum size depends on the food intended for application of the antimicrobial. Various foods differ in their initial microflora as well as possible contaminating microorganisms. The selection of the microorganism depends on the target food and whether the natural microflora and/or contaminating microorganisms are of interest. The intended use of the antimicrobial should be defined, such as to increase shelf life. At normal usage levels most food antimicrobials do not inactivate pathogens (Parish and Davidson 1993). Therefore, it would be beneficial to combine other intervention strategies if antimicrobials are used. Food processing conditions such as with those with heat will affect the activity of EOC due to their volatility. At 60°C pure CIN was converted to benzaldehyde, but CIN in cinnamon oil was stable (Friedman and others 2000). There is a shortage of knowledge about these effects (Friedman and others 2002). More research is needed on essential oils and components in foods as well as in combinations with other intervention strategies for synergistic action while maintaining acceptable quality and safety.

Significant findings

Beta-caryophyllene, limonene, alpha-pinene, and thymol were not lethal to *S.* Typhimurium and *L. monocytogenes* at 2800 mg/L and 3200 mg/L, respectively. The most effective essential oil components against *S.* Typhimurium were CIN and CRV (50% probability of lethality using an avg. of 280 mg/L and 1080 mg/L, respectively) with both methods. CIN (avg. 790 mg/L) and RHO (avg. 1810 mg/L) were the most lethal against *L. monocytogenes*. *L. monocytogenes* required higher doses of antimicrobial components to achieve lethality regardless of which method was used to determine MLC. Since selected components of essential oils were inhibitory to *S.* Typhimurium and *L. monocytogenes*, combinations of these components may be useful as antimicrobials and should be evaluated.

REFERENCES

Amsterdam D. 1996. Susceptibility testing of antimicrobials in liquid media. In: V. Lorian. Antibiotics in laboratory medicine. 4th edition. Baltimore: Williams & Wilkins. 52-111.

Blaszyk M, Holley RA. 1998. Interaction of monolaurin, eugenol and sodium citrate on growth of common meat spoilage and pathogenic organisms. International Journal of Food Microbiology 39:175-183.

Bowles BL, Miller AJ. 1993. Antibotulinal properties of selected aromatic and aliphatic aldehydes. Journal of Food Protection 56:788-794.

Brul S, Coote P. 1999. Preservative agents in foods: Mode of action and microbial resistance mechanisms. International Journal of Food Microbiology 50:1-17.

Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods - a review. International Journal of Food Microbiology: *In press*.

Collins CH, Lyne PM, Grange JM. 1995. Antimicrobial susceptibility tests. In: C. H. Collins. Collins and Lyne's microbiological methods. 7th edition. Oxford, Great Britain: Butterworth-Heinemann Ltd. 178-205.

Davidson PM, Naidu AS. 2000. Phyto-phenols. In: A. S. Naidu. Natural food antimicrobial systems. Boca Raton, Fla: CRC Press.

Denyer SP, Maillard J-Y. 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. Journal of Applied Microbiology Symposium Supplement 92:35S-45S.

Draughon FA. 2004. Botanicals as biopreservatives in foods. Journal of Food Science 58:20-28.

Friedman M, Kozukue N, Harden L. 2000. Cinnamaldehyde content in foods determined by gas-chromatography-mass spectrometry. Journal of Agricultural and Food Chemistry 48:5702-5709.

Friedman M, Henika PR, Mandrell RE. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes,* and *Salmonella enterica*. Journal of Food Protection 65:1545-1560.

Griffin S, Wyllie S, Markham J, Leach D. 1999. The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. Flavour and Fragrance Journal 14:322-332.

Hammer K, Carson C, Riley T. 1999. Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology 86:985-990.

Helander I, Alakomi H, Latva KK, Mattila ST, Pol I, Smid E. 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. Journal of Agricultural and Food Chemistry 46:3590-3595.

Inouye S, Tsuruoka T, Uchida K, Yamaguchi H. 2001. Effect of sealing and Tween 80 on the antifungal susceptibility testing of essential oils. Microbiology and Immunology 45:201-208.

Kim J, Marshall MR, Wei CI. 1995a. Antibacterial activity of some essential oil components against five foodborne pathogens. Journal of Agricultural and Food Chemistry 43:2839-2845.

Kim JM, Marshall MR, Cornell JA, Preston JF, Wei CI. 1995b. Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella typhimurium* in culture medium and on fish cubes. Journal of Food Science 60:1364-1368.

Kwon JA, Yu CB, Park HD. 2003. Bacteriocidal effects and inhibition of cell separation of cinnamic aldehyde on *Bacillus cereus*. Letters in Applied Microbiology:61-65.

Lambert R, Skandamis P, Coote P, Nychas G. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of Applied Microbiology 91:453-462.

Lis-Balchin M, Deans SG. 1997. Bioactivity of selected plant essential oils against *Listeria monocytogenes*. Journal of Applied Microbiology 82:759-762.

López-Malo A, Alzamorab SM, Palou E. 2002. *Aspergillus flavus* dose–response curves to selected natural and synthetic antimicrobials. International Journal of Food Microbiology 73:213-218.

NCCLS. 1993. Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically - approved standard. In: NCCLS Antimicrobial Susceptibility Testing. 13. Villanova,PA.: National Committee for Clinical Laboratory Standards.

Parish ME, Davidson PM. 1993. Methods for evaluation. In: P.M. Davidson and A. L. Branen. Antimicrobials in foods. Marcel Dekker.

Remmal A, Bouchikhi T, Rhayour K, Ettayebi M. 1993. Improved method for the determination of antimicrobial activity of essential oils in agar medium. J Essent Oil Res 5:179-184.

Seltmann G, Holst O. 2002. The outer membrane of the Gram-negative bacteria. In: O. Holst. The bacterial cell wall. Berlin: Springer-Verlag. 9-29.

Ultee A, Gorris LG, Smid EJ. 1998. Bactericidal action of carvacrol towards the food-borne pathogen *Bacillus cereus*. Journal of Applied Bacteriology 85:211-218.

Ultee A, Kets EPW, Smid EJ. 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 65:4606-4610.

Ultee A, Bennik MHJ, Moezelaar R. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. Applied and Environmental Microbiology 68:1561-1568.

Ultee A, Kets EPW, Alberda M, Hoekstra FA, Smid EJ. 2000. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. Archives of Microbiology 174:233-238.

Vaara M. 1992. Agents that increase the permeability of the outer membrane. Microbiological Reviews 56:395-411.

Valero M, Salmeró n MC. 2003. Antibacterial activity of 11 essential oils against *Bacillus cereus* in tyndallized carrot broth. Journal of Food Microbiology 85:73-81.
Wen A, Delaquis P, Stanich K, Toivonen P. 2003. Antilisterial activity of selected phenolic acids. Food Microbiology 20:305-311.
Wendakoon CN, Sakaguchi M. 1995. Inhibition of amino acid decarboxylase activity of *Enterobacter aerogenes* by active components in spices. Journal of Food Protection 58:280-283.
Zaika LL. 1988. Spices and herbs: their antimicrobial activity and its determination. Journal of Food Safety 1998:97-118.

PART 3. CHANGES IN FATTY ACID COMPOSITION ASSOCIATED WITH EXPOSURE AND ADAPTATION TO TRANS-CINNAMALDEHYDE

Abstract

Trans-cinnamaldehyde (CIN) is a major component found in the essential oil fraction of cinnamon cassia oils. It has been reported to be inhibitory to various pathogens. The mechanism of this inhibition is not fully understood, but is assumed to occur at the membrane level. To increase understanding of the inhibitory effect of CIN, the objective of this study was to evaluate changes in bacterial membrane composition by observing changes in total phospholipid fatty acid (PLFA) and fatty acid composition associated with exposure and adaptation to CIN. *Salmonella* Typhimurium DT104 cells were grown for 24h at 35°C with or without 250mg/L CIN in 9-ml brain heart infusion broth. Log 5.7 CFU/ml of *S.*Typhimurium was inoculated into 200ml broth containing 250mg/L CIN. At 2h and 24h, samples were centrifuged, washed, lyophilized, and extracted for lipids. Fatty acid methyl esters prepared from phospholipids were quantified by GC-MS. After 2h of exposure to CIN, the non-adapted treatment group had lower ($p<0.05$) total phospholipid fatty acid (PLFA), lower C16:1w7c and unsaturated fatty acid (UFA) and higher ($p<0.05$) C18:0, saturated fatty acid (SFA), and Cy17:0 to C16:1w7c ratio than the non-adapted control. After 24h of exposure to CIN, the non-adapted treatment group had lower ($p<0.05$) Cy17:0, Cy19:0, and UFA than the non-adapted control and higher ($p<0.05$) C16:0 and SFA. At 2h, SFA/UFA ratio in the membranes of adapted cells exposed to CIN was numerically higher than in the non-adapted cells treated with CIN. At 24h the SFA /UFA ratios of the adapted and non-adapted *Salmonella* were similar. Since the fatty acid

composition and total PLFA of the non-adapted control and adapted controls were similar at 2 and 24h, the adaptive response had been reversed since the stressor, CIN, was not used to treat the adapted controls. It appears that CIN treatment at sub-lethal concentrations decreased the fluidity of the *Salmonella* membrane by increasing SFA. This decreased fluidity may prevent additional CIN from permeating. The effect of bacterial growth in the presence of CIN on the fatty acid composition was much smaller. Measurement of the membrane transition temperature from gel-to-liquid-crystalline phase would indicate fluidity. Investigation about the target site that CIN acts upon and the interactions of CIN with membrane lipids and proteins would contribute to the understanding of its mechanism of action and the bacterial response.

Introduction

Consumers desire high-quality, minimally processed foods that have fewer preservatives and an extended shelf-life (Brul and Coote 1999). Studies have frequently reported cinnamon essential oil to be highly active against various microorganisms (Lis-Balchin and Deans 1997; Friedman and others 2002; Valero and Salmerón 2003). Trans-cinnamaldehyde (CIN), which is a component found in cinnamon oil, is used in candies and baked goods (Kim and others 1995).

Cinnamon has been used in human foods for thousands of years and has been used as a deodorizer and sanitizer. The minimum inhibitory concentration (MIC) of CIN against *S.* Typhimurium and *E. coli* O157:H7 was 396 mg/L

(Helander and others 1998). The concentrations of CIN needed to achieve a 50% decrease in CFU in *E. coli* O157:H7, *S*. enterica Hadar, and *L. monocytogenes* were 600, 400, and 100 mg/L, respectively (Friedman and others 2002).

Concentrations that are lethal to bacteria are much higher than tolerable taste thresholds. Therefore, it is preferable to use them at inhibitory concentrations rather than at lethal concentrations (Brul and Coote 1999). Knowledge about the mechanisms of action of antimicrobials is needed to develop effective and economical combinations of antimicrobial additives and formulations to prevent food from spoilage and pathogenic microorganisms without sacrificing organoleptic qualities.

Compounds from essential oils are believed to disrupt the membrane (Brul and Coote 1999), however, there are diverse chemical families representing essential oil components. Lipophilic compounds, such as essential oil components, have been shown to accumulate and act on the cell membrane (Sikkema and others 1994, 1995; Ultee and others 1999). As they interact with the phospholipid bilayer of the cell membrane, they may increase membrane permeability and cause cellular constituents to leak (Sikkema and others 1994; Ultee and others 1998).

Although a few studies have investigated the efficacy of CIN, only a couple have examined its mechanism of inhibition. One study found that CIN at 0.3 ml/L was lethal to *Bacillus cereus* cells but no significant amount of protein was lost. It was reported that treatment with CIN caused exponential phase cells

to become long and filamentous and inhibited them from separating from each other (Kwon and others 2003). It was suggested that CIN did not disrupt the outer membrane of *E. coli* O157:H7 and *S.* Typhimurium because at 264 mg/L it did not increase the uptake of a hydrophobic fluorescent probe or influence intra- or extracellular ATP pools (Helander and others 1998).

When a microorganism is exposed to sublethal doses of a chemical that negatively influences bacterial growth or survival, it may respond to protect itself, or adapt, to increase its tolerance to the same or different type of stress. A lipid can act as a sensor of stress and cause a change in transcription or translation of stress-related proteins, which alter cell physiology to increase tolerance to stress. A change in fluidity or membrane structure can be detected and the cell may respond with alterations in bacterial membrane composition (Yousef and Courtney 2003).

Changes in bacterial lipids in the membrane are a defense mechanism used to maintain optimal fluidity in the liquid-crystalline phase for important cell processes. Membrane fluidity is the "combined expression of the effects of [fatty acid] chain conformation, lateral and rotational diffusion [of the lipids within the membrane] and the resistance of the membrane to sheer forces" (Denich and others 2003). The fluidity of the membrane affects the growth of the cell and the proper functioning and structural integrity of the membrane (Denich and others 2003). For example, the percentage of unsaturated fatty acids in *E. coli* phospholipids is usually about 50%. This percentage can decrease to 30% without ceasing bacterial growth. If the percentage of saturated fatty acids

decreases to less than 15% the membrane permeability increases (Seltmann and Holst 2002). Lipids directly affect passive permeability and indirectly influence cell processes because of their interactions with membrane proteins (Russell and others 1995).

To maintain optimal fluidity the cell may change the fatty acid and phospholipid head-group composition which affect the lipid membrane melting point (Keweloh and Heipieper 1996). As melting point decreases, fluidity increases (Casadei and others 2002). For example, fatty acids with high melting points, such as saturated, long, and straight-chain fatty acids, decrease membrane fluidity (Juneja and Davidson 1993). A change in the fatty acid composition to decrease the fluidity has been shown to be an adaptive response to the compensate for the fluidizing effect of an essential oil component (Ultee and others 2000). No studies about the adaptive response to CIN have been published. The objectives of this study were to determine:

1) the effect of exposure to CIN on the fatty acid composition of the bacterial membrane of *Salmonella*,

2) whether changes in the fatty acid composition of the bacterial membrane of *Salmonella* occur due to growth in the presence of CIN as a possible adaptive response to CIN, and

3) whether reversal of these changes in fatty acid composition occur when the stressor, CIN, was removed.

Materials and methods

Bacterial culture preparation

Salmonella Typhimurium DT104 ATCC BAA-186 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Experimental cultures were maintained on Tryptic Soy Agar slants. A loopful of culture was used to inoculate 9 ml of BHI with *S.* Typhimurium. The BHI tube was incubated for 24 h at 35℃. Then a loopful was transferred into 9 ml BHI and incubated for 21 h at 35℃.

Essential oil component

Stocks solutions of CIN (Fisher Scientific, Fairlawn, NJ) were prepared in ethanol, absolute (Aldrich, St. Louis, MO). Sterile water dissolved into ethanol served as the control stock solution. Aliquots of CIN stock solution were added into 8 ml brain heart infusion broth, modified (BHI, BBL, Sparks MD) with 2% Tween 20 (Fisher Scientific, Fairlawn, NJ) with glass beads. The final concentration of Tween was 1% and ethanol was 0.5% during adaptation and treatment. Tween and glass beads were used to aid with dispersion of CIN in BHI. All BHI was prepared with phosphate buffer (0.1M, pH 7.2 $\pm$ 0.1) instead of water to prevent pH changes, which could affect the fatty acid composition of bacterial membranes. The pH of BHI was adjusted to 7.35 ($\pm$ 0.1) prior to autoclaving, after which the final pH was pH 7.2 ($\pm$ 0.1). Glass tubes containing the stock and BHI were shaken vigorously forty times to disperse the oil-based components.

Adaptation

In our study adaptation was defined as growth in sublethal concentrations of CIN for 24h. One ml of the 21-h culture was serially diluted 10^{-3} with three tubes of 9 ml BHI. This dilution was repeated with another set of BHI tubes. The diluted cultures from the two tubes of the 10^{-3} dilution were combined in a large tube. Five ml of the diluted culture were used to inoculate a 5-ml BHI tube containing CIN and a 5-ml BHI tube containing 0.5% ethanol control. Both tubes had 1% Tween.

The stock and 5-ml CIN dispersions in BHI were prepared the day before, refrigerated, and allowed to warm to room temperature for 30 min before the 5-ml diluted culture was added. This inoculation halved the concentrations and the final inoculum was approximately log 5.7 CFU/ml. The final concentration of Tween was 1% and ethanol was 0.5%. The two inoculated BHI tubes containing CIN or ethanol control were vortexed and the cells were allowed to adapt to CIN or ethanol (non-adapted) by incubating in a shaking waterbath at 125 rpm for 24 h at 35°C.

Extrapolating from dose-response curves, the concentration that would achieve a 25% probability of lethality was used to adapt cells. This low probability of lethality was chosen to induce changes in the membrane lipid composition yet also allow enough growth for harvesting cells for lipid analysis. This 25% probability of lethality corresponded to 251 mg/L CIN. After a 24-hour growth in or adaptation to 251 mg/L CIN, adapted cells were inoculated into 200 ml BHI in bottles to treat adapted cells with 251 mg/L CIN for 2 and 24 hours.

After inoculation, the desired inoculum in these experiments was about log 5.7 CFU/ml to adhere to the same inoculum size as in the adaptation procedure.

The population of cells resulting after 24-h adaptation was tested since these cells would be used to inoculate the bottles. The population of cells adapted to CIN was log 8.5 CFU/ml (average of three repetitions). The control population exposed to 0.5% ethanol was log 8.8 CFU/ml (average).

Treatment of adapted cells

A total of twelve screw-capped glass bottles containing 190 ml BHI were prepared for two and twenty-four hour exposures to CIN. Ten ml CIN dispersion were added to six bottles for a final concentration of 251 mg/L. *Salmonella* adapted to CIN would be added to two bottles. *Salmonella* "adapted" only to ethanol would be added to two bottles. Two bottles would not be inoculated and were designated as sterile CIN controls since the BHI broth media contained fatty acids. Ten ml of ethanol solution were added to six bottles (with no CIN). *Salmonella* adapted to CIN would be added to two bottles. *Salmonella* exposed only to ethanol (non-adapted) would be added to two bottles. Two bottles were not to be inoculated and were designated as sterile ethanol controls since the BHI broth media contained fatty acids from the manufacturer formulation. The final concentrations would contain about 1% Tween and 0.5% ethanol, similar to those used to adapt cells. These twelve bottles containing CIN or ethanol were shaken and stored at 4°C until two hours before use, at which time they were allowed to warm to 25°C. Before adding adapted *Salmonella*, bottles were shaken. The treatments for 2 and 24h incubation were: Sterile controls: sterile +

CIN, sterile + ethanol, treatments: non-adapted *Salmonella* + CIN, adapted *Salmonella* + CIN, inoculated controls: non-adapted + ethanol, adapted + ethanol.

Tubes containing adapted cells were vortexed prior to inoculating *Salmonella* into the designated bottles. Based on the preliminary data of adapted cell counts, 212 µl of cells adapted to CIN and 166 µl of cells "adapted" to ethanol were added to designated bottles for a final inoculum size of about log 5.7 CFU/ml. The bottles were shaken and incubated in a shaking waterbath at 125 rpm for either two or twenty-four hours. After incubation the samples were centrifuged on a large volume centrifuge (Beckman J2-HS, Schaumberg, IL) at 12,000 rpm for 30 minutes at 4°C. The pellet was transferred from the centrifuge bottles to centrifuge tubes and washed three times with 0.1 M phosphate buffer (pH 7.2) at 10,000 rpm for 10 minutes at 4°C using a table-top centrifuge (Baxter Biofuge 17 R, VWR Scientific Products, West Chester, PA). The pellet was suspended in less than 1.5 ml phosphate buffer in small ampoules, wrapped in parafilm and foil and frozen at -30°C. The pellet was lyophilized and ampoules were wrapped until extraction.

Lipid extraction

Lipid extraction and analysis procedures were developed by Dr. David C. White's lab, the University of TN Center for Biomarker Analysis. Lipids from 8-18 mg lyophilized cells were extracted using a modified Bligh/Dyer method (Guckert and others 1985; Guckert and others 1986). The extraction solvent contained 4 ml phosphate buffer, 10 ml methanol, and 5 ml chloroform. After three hours the

phases were split with 5 ml water and 5 ml chloroform and allowed to sit overnight for layer separation. The lower chloroform layer was removed and dried under nitrogen. The total lipid extract was suspended in chloroform and separated into the lipid classes (neutral lipids, glycolipids, and polar lipids) with silicic acid (500 mg) columns using three solvents of increasing polarity (5 ml chloroform, 5 ml acetone, and 10 ml methanol) to elute the lipid classes from the silicic acid stationary phase (Guckert and others 1985; Guckert and others 1986).

Analysis of fatty acid methyl esters

To prepare fatty acid methyl esters (FAME) in a mild alkaline methanolic transesterification reaction, 0.5 ml chloroform, 0.5 ml methanol, and 1 ml of methanolic KOH were added to the dried polar lipids and heated at 60℃ for 30 minutes. To extract the FAME, 2 ml hexanes, 200 µl glacial acetic acid, and 2 ml nanopure water was added. After centrifuging, the hexane layer was removed. This was repeated twice. The FAME were dried under nitrogen and transferred three times with 100 µl hexanes to autosampling vials.

The samples were diluted with hexanes containing a 21:0 internal standard (50pmol/µl). The samples and a vial of GC-MIX (mixture of 20 FAME standards) were analyzed on a HP6890 gas chromatograph (GC) coupled to a HP5973 mass spectrophotometer (MS). The GC was equipped with a RJX1 fused silica capillary column (60m, 250µm internal diameter x 25 µm film thickness, Restek, Bellefonte, PA). The column was programmed from an initial temperature of 60℃ for 2 min then raised at a rate of 10℃/min to 150℃. The temperature was increased at 30℃/min to a final temperature of 312℃. The

injector port temperature was 230°C in split-less mode. Helium was used as the carrier gas at a flow rate of 1.41 ml/min. Fatty acids were identified by comparison of retention times to the FAME standards and a mass spectral data library. Fatty acids were quantified based on comparison to the 21:0 internal standard and area under each sample peak and standard peak.

Statistical analysis

Box and whisker plots were generated with Statistica (StatsSoft, Inc., Tulsa, OK) to compare total phospholipid fatty acid (PLFA) and fatty acid composition. The 2 and 24h data were divided and a randomized block design with blocking on each of three replications was used to test the effect of treatments or controls on total PLFA and fatty acids for 2h or 24h. Data were analyzed using SAS (Statistical Analysis System, Cary, NC) to compare differences in total PLFA and fatty acids between treatment and control groups within 2h or 24h with alpha value at 0.05. The generalized linear model (GLM) was used to test for differences in total PLFA and fatty acids between 2 and 24h within each treatment or control group with a Bonferonni correction factor. This correction minimized Type I error, which increases with the number of statistical tests. The Bonferonni p value was calculated as the alpha value at 0.10 divided by 6 (the number of follow-up tests conducted to test the interaction between the 6 treatments at 2h or 24h), which equals 0.017.

Results

Total phospholipid fatty acid (PLFA)

Total phospholipid fatty acid (PLFA) is the sum of all fatty acids that were hydrolyzed from phospholipids in the membrane. The mean total PLFA is shown in Table 3.1. Depicted in Figures 3.1 and 3.2 are box and whisker plots representing the mean, standard error, and standard deviation of the total PLFA of the sterile groups of *Salmonella* under each of the experimental treatments. The boundaries of the box around the mean consist of the mean $\pm$ standard error. The standard error is the standard deviation of the sample mean and is computed as the standard deviation divided by the square root of the sample size. The horizontal bars represent the mean $\pm$ standard deviation. The standard deviation is the positive square root of the variance. The variance is the sum of the squared deviations from the mean divided by the sample size minus one.

An analysis was performed to determine if there was a correlation between total PLFA and occurrence of specific fatty acids. It was found that higher total PLFA was positively correlated to the presence of C15:0 (R^2=0.81), Cy17:0 (R^2=0.91), C17:0 (R^2=0.78), Cy19:0 (R^2=0.85), ratio of Cy17:0 to C16:1w7c (R^2=0.82), and the ratio of Cy19:0 to C18:1w7c (R^2=0.83). The correlation of total PLFA to cyclopropyl fatty acids was expected because as cells age and enter stationary phase, the cyclopropyl fatty acids increase (Guckert and White 1988; Casadei and others 2002).

Table 3.1. Comparison of total PLFA and fatty acid (FA) composition of CIN-adapted or non-adapted cells with or without trans-cinnamaldehyde (CIN) to inoculated controls (+ EtOH) and sterile controls after 2h and 24h incubation.

Treatment/control and incubation hr	Total PLFA (pmol/mg)	C12:0 (%)	C14:0 (%)	C15:0 (%)	C16:1w7c (%)	C16:0 (%)	Cy17:0 (%)	C17:0 (%)	C18:1w9c (%)
2h									
Sterile + CIN	42.9 ^{B}	39.8 ^{A}	29.3 ^{A}	0.0	0.0 ^{C}	18.0 ^{B}	0.0 ^{B}	0.0	0.9
Sterile + EtOH	191.9 ^{B}	35.4 ^{A}	29.0 ^{A}	0.0	0.0 ^{C}	21.6 ^{AB}	0.0 ^{B}	0.0	1.2
Non-adapt + CIN	1817.5 ^{B}	19.4 ^{AB}	16.6 ^{BC}	0.0	5.2 ^{B}	27.3 ^{AB}	2.5 ^{A}	0.0	1.9
Adapt + CIN	635.2 ^{B}	22.1 ^{AB}	19.8 ^{AB}	0.0	2.5 ^{BC}	25.2 ^{AB}	1.1 ^{ABZ}	0.0 ^{Z}	2.3
Non-adapt + EtOH	7625.3 ^{AZ}	0.8 ^{B}	7.8 ^{C}	0.0 ^{Z}	25.7 ^{AZ}	29.5 ^{AZ}	2.3 ^{ABZ}	0.0 ^{Z}	0.5
Adapt + EtOH	3729.1 ^{BZ}	0.9 ^{B}	7.8 ^{BC}	0.0	25.8 ^{AZ}	29.7 ^{ABZ}	2.0 ^{ABZ}	0.0	0.4
24h									
Sterile + CIN	741.6 ^{B}	46.5 ^{A}	27.2 ^{A}	0.0 ^{B}	0.0 ^{C}	16.7 ^{D}	0.0 ^{C}	0.0 ^{C}	1.3 ^{B}
Sterile + EtOH	2396.1 ^{B}	23.7 ^{B}	31.7 ^{A}	0.0 ^{B}	0.0 ^{C}	21.7 ^{C}	0.0 ^{C}	0.0 ^{C}	3.9 ^{A}
Non-adapt + CIN	41495.6 ^{A}	4.8 ^{C}	8.8 ^{B}	0.1 ^{AB}	14.6 ^{AB}	40.8 ^{A}	8.6 ^{B}	0.0 ^{BC}	0.3 ^{B}
Adapt + CIN	53991.8 ^{A}	0.2 ^{C}	5.9 ^{B}	0.1 ^{AB}	16.1 ^{A}	41.9 ^{A}	11.7 ^{AB}	0.1 ^{AB}	0.1 ^{B}
Non-adapt + EtOH	69791.6 ^{A}	0.3 ^{C}	7.7 ^{B}	0.2 ^{A}	13.7 ^{B}	36.7 ^{B}	16.0 ^{A}	0.1 ^{AB}	0.3 ^{B}
Adapt + EtOH	64520.6 ^{A}	0.2 ^{C}	7.4 ^{B}	0.1 ^{AB}	14.0 ^{B}	38.4 ^{AB}	15.3 ^{A}	0.0 ^{BC}	0.2 ^{B}

ABCD: Means in a column with different superscripts within 2h or 24h are different ($p<0.05$).

Z: Means in a column are different from 2h to 24h within a treatment or control group (Bonferroni $p<0.017$).

Table 3.1. Continued.

Treatment/control and incubation hr	C18:1w7c (%)	C18:1w7t (%)	C18:0 (%)	Cy19:0 (%)	Sat. FA (%)	Unsat. FA (%)	Cy17 to C16:1 ratio	Cy19 to C18:1 ratio	SFA to UFA ratio
2-h									
Sterile + CIN	0.6 ^{C}	0.2	11.2 ^{A}	0.0	98.3 ^{A}	1.7 ^{C}	0.0	0.0	18.1 ^{AB}
Sterile + EtOH	0.9 ^{C}	0.2	11.7 ^{A}	0.0	97.7 ^{A}	2.3 ^{C}	0.0	0.0	30.0 ^{A}
Non-adapt + CIN	17.4 ^{ABC}	0.4	9.3 ^{A}	0.0	72.5 ^{B}	27.4 ^{B}	0.5 ^{A}	0.0	4.9 ^{BC}
Adapt + CIN	13.7 ^{BC}	0.3	12.9 ^{AZ}	0.0	80.1 ^{AB}	19.9 ^{BC}	0.2 ^{AB}	0.0	8.7 ^{BC}
Non-adapt + EtOH	32.6 ^{AZ}	0.0	0.9 ^{B}	0.0 ^{Z}	38.9 ^{CZ}	61.1 ^{AZ}	0.1 ^{BZ}	0.0 ^{Z}	0.6 ^{CZ}
Adapt + EtOH	32.4 ^{ABZ}	0.0	1.0 ^{B}	0.0	39.4 ^{CZ}	60.6 ^{AZ}	0.1 ^{BZ}	0.0	0.7 ^{CZ}
24-h									
Sterile + CIN	1.0 ^{B}	0.5 ^{AB}	6.9 ^{B}	0.0 ^{C}	97.2 ^{A}	2.8 ^{C}	0.0 ^{B}	0.0 ^{B}	17.1 ^{A}
Sterile + EtOH	1.5 ^{B}	0.7 ^{A}	16.7 ^{A}	0.0 ^{C}	93.8 ^{A}	6.2 ^{C}	0.0 ^{B}	0.0 ^{B}	15.4 ^{A}
Non-adapt + CIN	19.8 ^{A}	0.0 ^{B}	1.7 ^{B}	0.4 ^{BC}	56.2 ^{B}	43.8 ^{B}	0.6 ^{AB}	0.0 ^{AB}	1.4 ^{B}
Adapt + CIN	22.6 ^{A}	0.0 ^{AB}	0.4 ^{B}	0.9 ^{AB}	48.6 ^{BC}	51.4 ^{AB}	0.7 ^{AB}	0.0 ^{AB}	0.9 ^{B}
Non-adapt + EtOH	23.2 ^{A}	0.0 ^{B}	0.5 ^{B}	1.2 ^{A}	45.6 ^{C}	54.4 ^{A}	1.2 ^{A}	0.1 ^{A}	0.8 ^{B}
Adapt + EtOH	23.0 ^{A}	0.0 ^{B}	0.4 ^{B}	0.8 ^{AB}	46.6 ^{BC}	53.4 ^{AB}	1.1 ^{A}	0.0 ^{AB}	0.9 ^{B}

ABCD: Means in a column with different superscripts within 2h or 24h are different ($p<0.05$).

Z: Means in a column are different from 2h to 24h within a treatment or control group (Bonferroni $p<0.017$) .

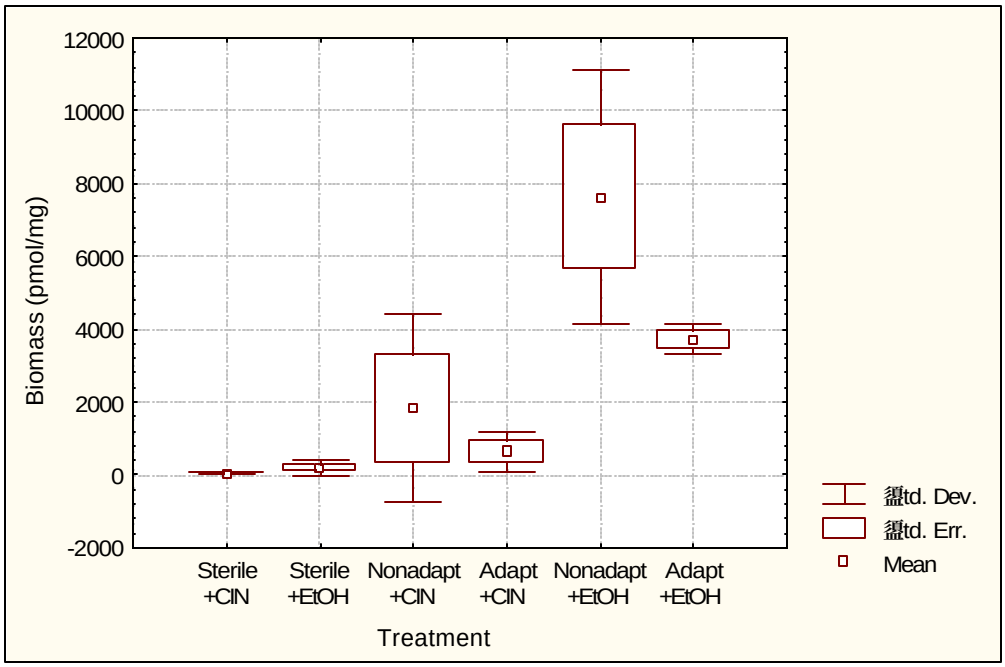


Figure 3.1. Comparison of total PLFA or biomass of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

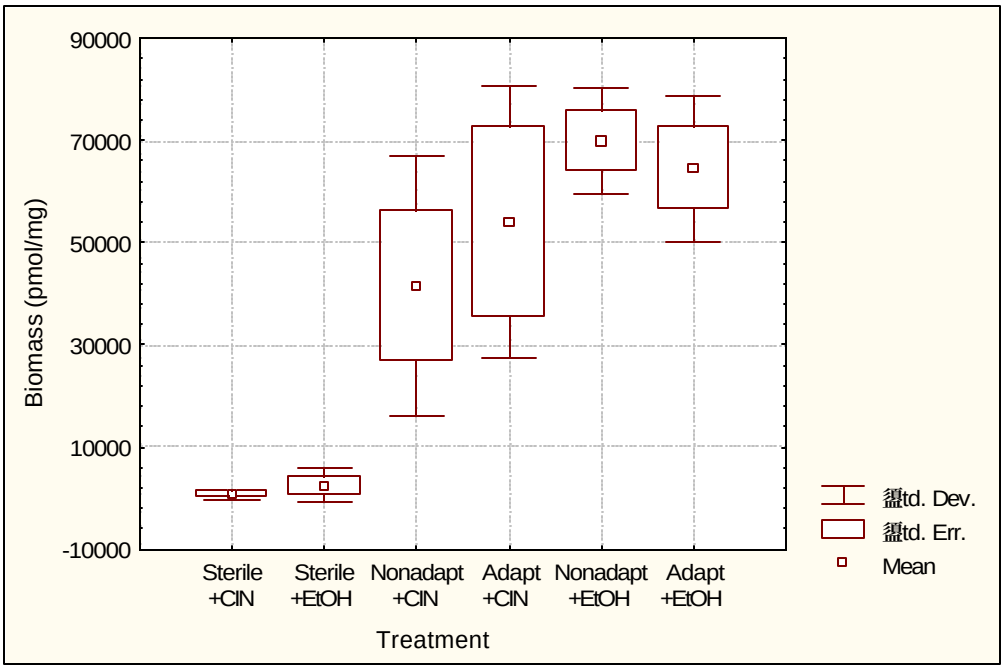


Figure 3.2. Comparison of total PLFA or biomass of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

Comparison of total PLFA and each fatty acid across sterile, treatment, and control groups (refer to Table 3.1 for means).

Total PLFA. Mean total PLFA of treatment groups at 2h is shown in Table 3.1. Two outlying data points were eliminated. One 2h replicate of the adapted control (+ ethanol or EtOH) had a total PLFA of 124.1 pmol/mg. One 24h replicate of the adapted treatment (+ CIN) had a total PLFA of 228.1 pmol/mg. These were eliminated because of their low total PLFA, which indicated very low or no growth and their fatty acid compositions correlated highly with the sterile groups. This replicate of the adapted treatment was likely to have been completely inhibited by CIN since the concentrations used for adaptation and treatment were the dosage at which 25% probability of lethality was predicted.

In the 2-hr samples, the non-adapted *Salmonella* inoculated EtOH control (no CIN) had the highest total PLFA (Figure 3.1 and Table 3.1). The total PLFA of the non-adapted *Salmonella* inoculated EtOH control was highest because the cells were not exposed to CIN and were able to grow more quickly than the other groups even at 2 hours. The total PLFA of all other groups was lower and not different from each other ($p>0.05$).

Although not significantly ($p>0.05$) higher, the adapted control appeared to have more total PLFA than the non-adapted and adapted cells treated with CIN. The bacteria in the adapted and non-adapted EtOH controls seemed to grow faster because they were not inhibited by CIN. The sterile controls contained very low total PLFA because they were not inoculated (Figures 3.1 and 3.2). The

total PLFA found in uninoculated controls was due to the fatty acids present in the culture medium from the manufacturer's formulation of BHI.

At 24h of incubation the total PLFA of all inoculated groups was significantly ($p<0.05$) higher than the sterile groups (Figure 3.2). Only the adapted and non-adapted EtOH controls had a significantly ($p<0.001$ and $p<0.05$, respectively) higher total PLFA at 24h compared to 2h (Table 3.1). The adapted and non-adapted treatment groups had a trend of increasing total PLFA over time, but this was not significantly different ($p>0.05$ and $p>0.02$, respectively). The wide variability within the groups probably obscured the significance of this increase.

C12:0 fatty acid. At 2h of incubation, the C12:0 fatty acid detected in the sterile groups was from the culture medium (Figure 3.3). The sterile groups had more C12:0 fatty acid than the non-adapted and adapted controls. This difference indicates that even at 2h the bacteria in the controls were growing and using C12:0, and therefore decreasing the relative percentage of C12:0. The abundance of C12:0 in the non-adapted and adapted treatment groups did not differ from each other and the sterile controls. The growth of bacterial cells in the treatment groups was inhibited by CIN so lipid biosynthesis by the anaerobic pathway was decelerated. They incorporated C12:0 from the medium but could not oxidize and convert it to other fatty acids in the presence of CIN.

At 24h all four inoculated groups contained significantly ($p<0.05$) less C12:0 than sterile groups (Figure 3.4). This reflects the difference in total PLFA at 24h and utilization of C12:0. The bacteria in the inoculated groups had more

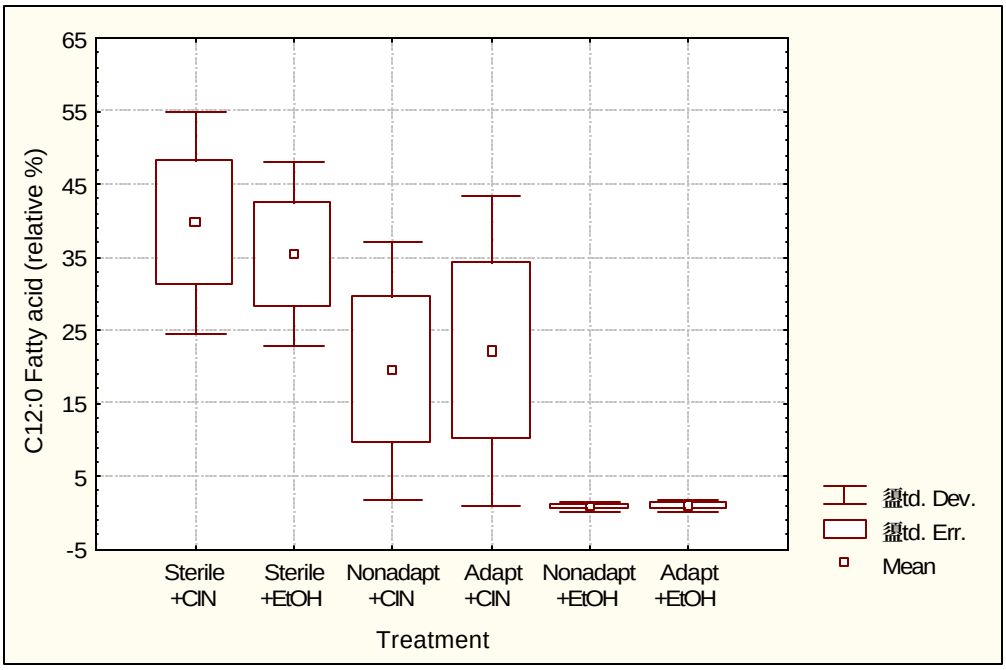


Figure 3.3. Comparison of C12:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

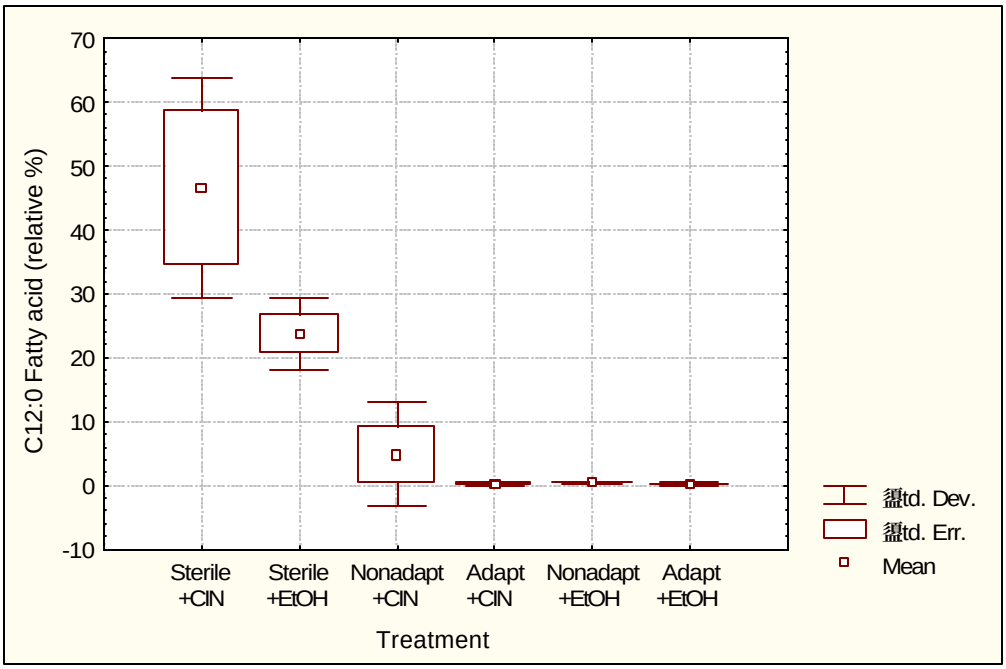


Figure 3.4. Comparison of C12:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

time to grow and utilize 12:0. There were no significant ($p>0.05$) differences among the inoculated groups because the bacteria in the treatment groups were able to grow and convert 12:0 to another fatty acid.

However, the wider standard error of the non-adapted cells treated with CIN implies that some *Salmonella* cells in a culture were less able to decrease the proportion 12:0 than others because they were more inhibited due to CIN exposure. This wider range of response indicates that a population of non-adapted cells can display a range of sensitivity to CIN and therefore a range in the ability to grow and decrease the percentage of C12:0.

In contrast, the adapted cells group seem to be less inhibited than its non-adapted counterpart because they were able to decrease C12:0 percentage more readily with a narrower range of error. The population of adapted cells seems to demonstrate more similar sensitivity to CIN than non-adapted cells. The groups did not have more C12:0 fatty acid at 24h compared to 2h.

C14:0 fatty acid. At 2 and 24h, the sterile groups had similar percentages of C14:0 (Figures 3.5 and 3.6). Even at 2h the inoculated controls (+EtOH) were able to grow and convert 14:0, which resulted in significantly ($p<0.05$) lower C14:0 than the sterile groups. The level of C14:0 in the adapted and non-adapted cells treated with CIN was in between that of the sterile and inoculated controls. The growth of bacterial cells in the treatment groups was inhibited by CIN so lipid biosynthesis was decelerated. They incorporated C14:0 from the medium but could not oxidize and convert it to other fatty acids as well as the inoculated controls.

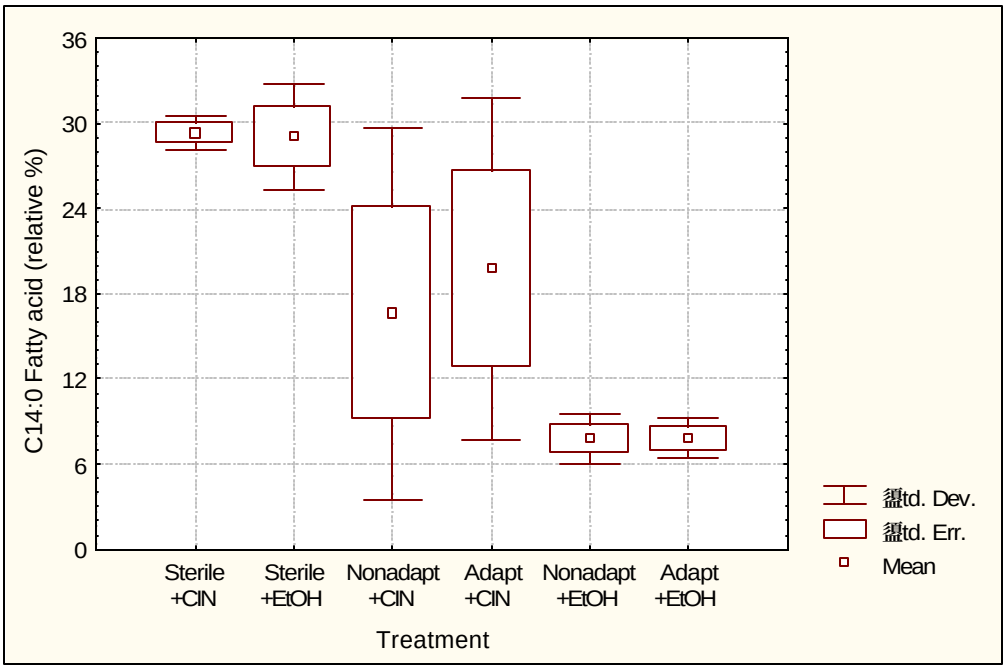


Figure 3.5. Comparison of C14:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

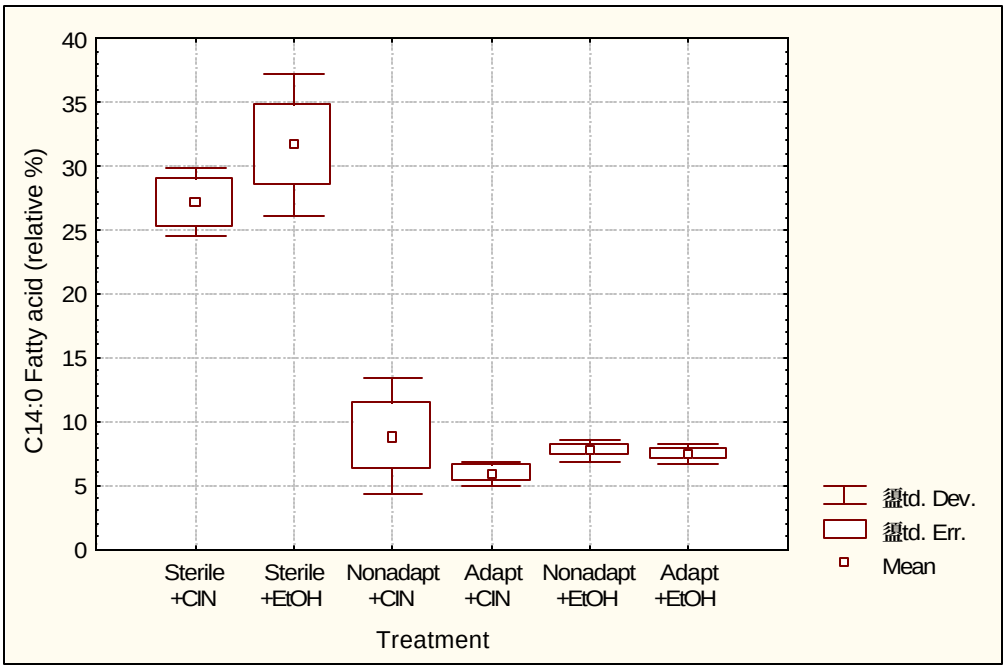


Figure 3.6. Comparison of C14:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

At 24h the bacteria in all four inoculated groups grew and the proportion of C14:0 was significantly ($p<0.05$) lower than the sterile groups (Figure 3.6). The abundance of C14:0 did not differ significantly ($p>0.05$) among inoculated groups because the bacteria in the treatment groups grew and were able to convert 14:0 to another fatty acid. As with C12:0 at 24h, the wider standard error indicates that some non-adapted cells treated with CIN were not as able to lower the percentage of C14:0 as quickly as the adapted cells treated with CIN and inoculated controls since the cells were more inhibited by exposure to CIN. The percentage of C14:0 did not differ from 2h to 24h for all groups.

C15:0 fatty acid. C15:0 was detected at very low percentages at 2h (Figure 3.7). There were no significant ($p<0.05$) differences among the groups. Only the non-adapted control had a significantly ($p<0.$) higher abundance C15:0 at 24h than at 2h (Table 3.1). At 24 hours the bacteria in the non-adapted control produced significantly ($p<0.05$) more C15:0 than the very low level present in the culture medium of the sterile controls (Figure 3.8). Among the inoculated groups there were no significant ($p>0.05$) differences at 24h.

C16:1w7c fatty acid. At 2h the inoculated controls (+EtOH) were able to produce more C16:1w7c than all the other groups since the bacteria were not inhibited by CIN (Figure 3.9). The non-adapted and adapted treatment groups were still inhibited by CIN at 2h and not able to produce as much C16:1w7c. At 24h, the levels of C16:1w7c produced by bacteria in the four inoculated groups was greater ($p<0.05$) than the low amount present in the culture medium of the sterile controls (Figure 3.10). C16:1w7c in the adapted cells treated with CIN

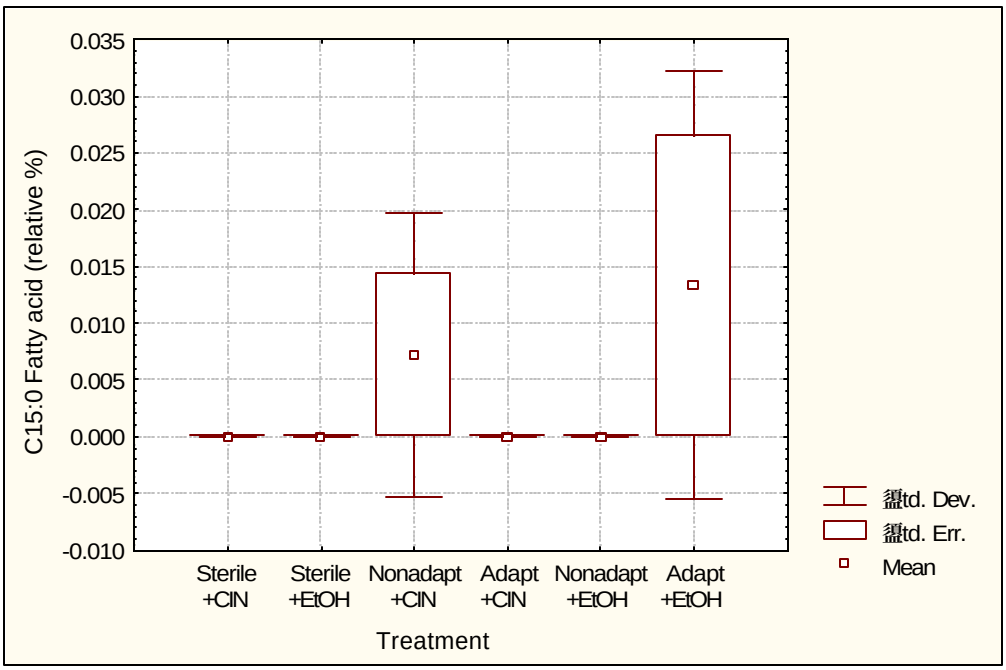


Figure 3.7. Comparison of C15:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

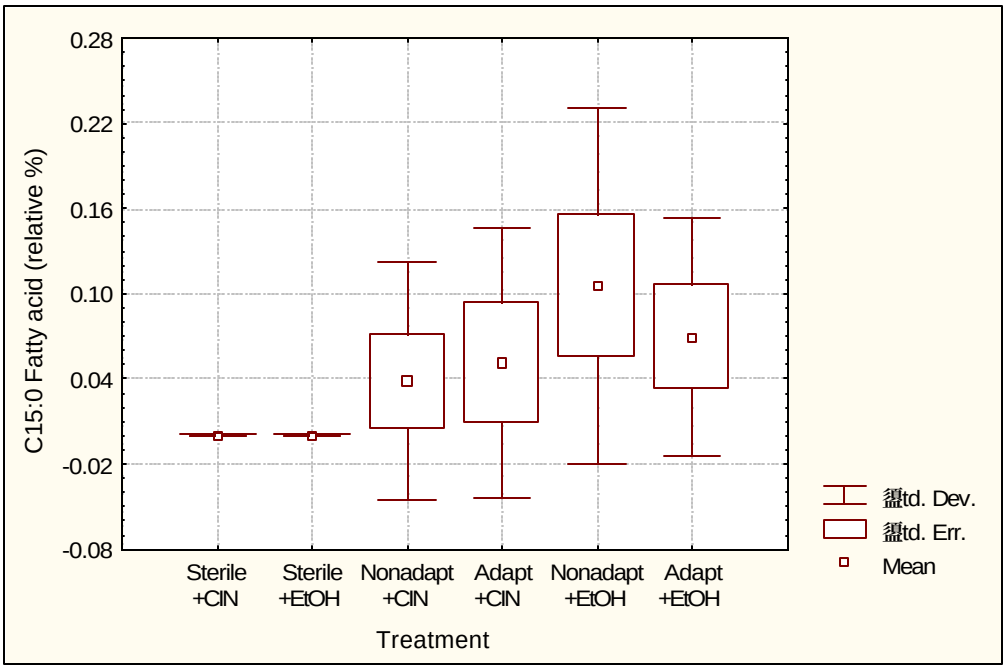


Figure 3.8. Comparison of C15:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

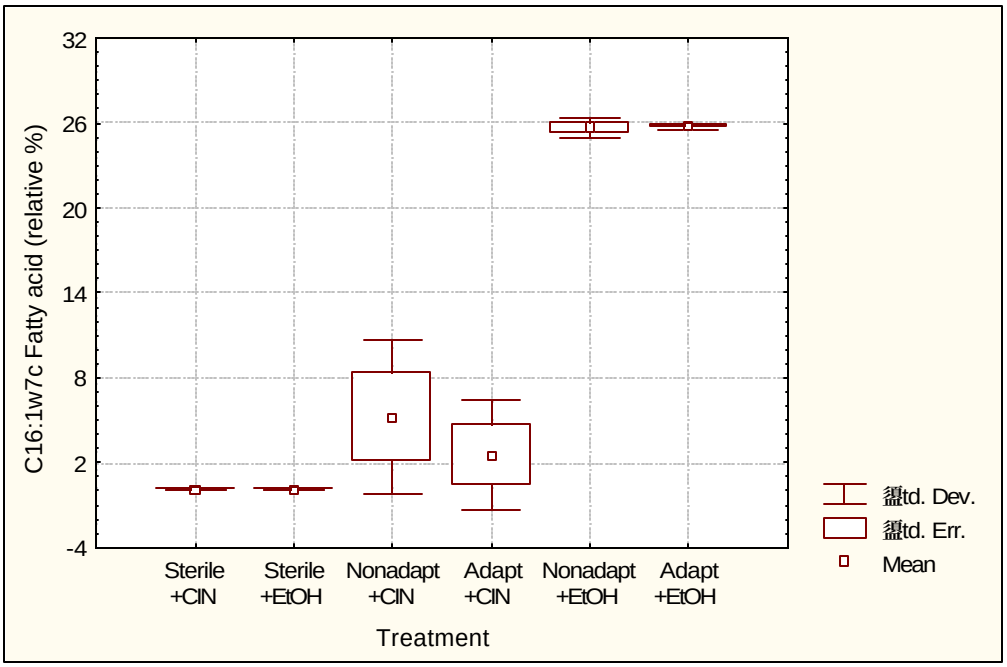


Figure 3.9. Comparison of C16:1w7c fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

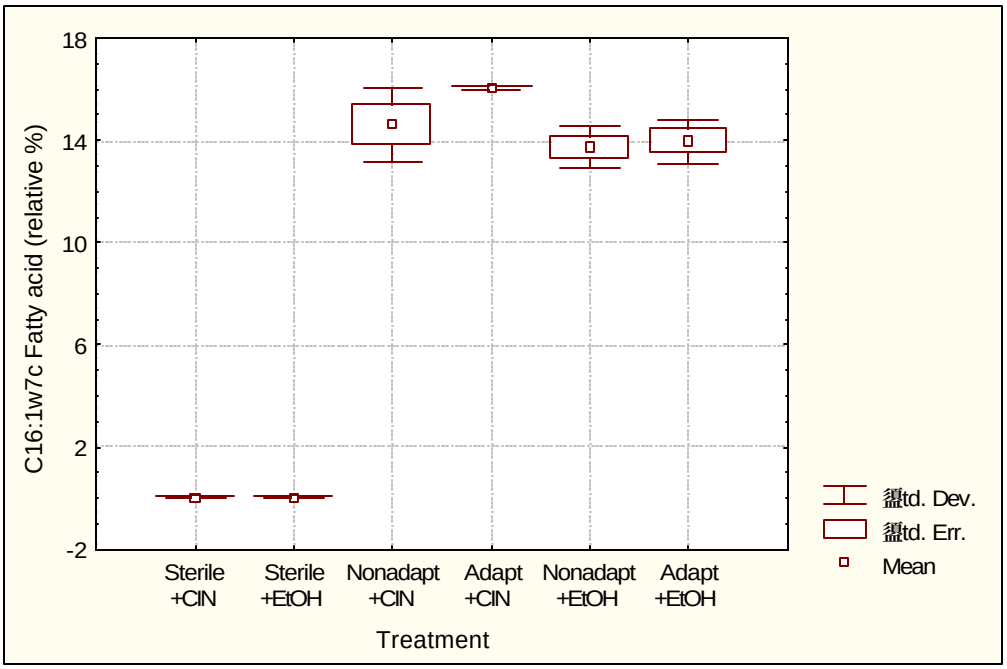


Figure 3.10. Comparison of C16:1w7c fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

were higher than in the inoculated controls. Only the adapted and non-adapted controls significantly ($p<0.0001$ and $p<0.0005$) decreased C16:1w7c from 2h to 24h (Table 3.1). In contrast, there was a trend of increased C16:1w7c at 24h produced by the two treatment groups exposed to CIN, but this was not significant ($p>0.04$ and $p>0.02$, respectively).

C16:0 fatty acid. At 2h there was a broad range of converting C16:0 in the non-adapted and adapted cells treated with CIN (Figure 3.11). The broad range reflected the different CIN sensitivities of cells in the population. In contrast, the inoculated controls had a narrower range. Overall, the sterile groups did not differ from inoculated groups in percentage of C16:0.

At 24h, the percentage of C16:0 in the inoculated groups was significantly ($p<0.05$) higher than the sterile groups (Figure 3.12). The non-adapted and adapted cells treated with CIN produced more C16:0 than the non-adapted control. Only the non-adapted and adapted controls produced more C16:0 at 24h than at 2h ($p<0.001$ and $p<0.01$, respectively) (Table 3.1). The non-adapted treatment group seemed to synthesize more C16:0 at 24h, but this was not significant ($p>0.04$), and perhaps obscured by variation within the group.

Cy17:0 fatty acid. The percentage of Cy17:0 did not differ among the four inoculated groups at 2h (Figure 3.13). The non-adapted cells treated with CIN had a significantly ($p<0.05$) higher percentage of Cy17:0 than the steriles. Again, there is a wide range of response from the non-adapted cells treated with CIN.

At 24h Cy17:0 was more abundant in the control than in the sterile groups and non-adapted treatment group, which was inhibited by CIN (Figure 3.14).

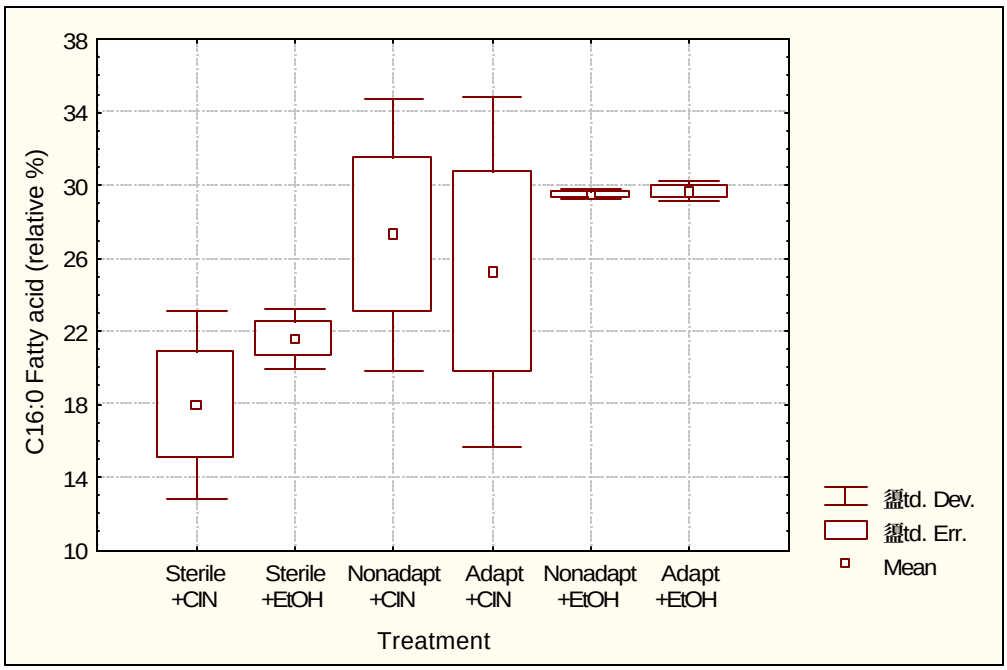


Figure 3.11. Comparison of C16:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

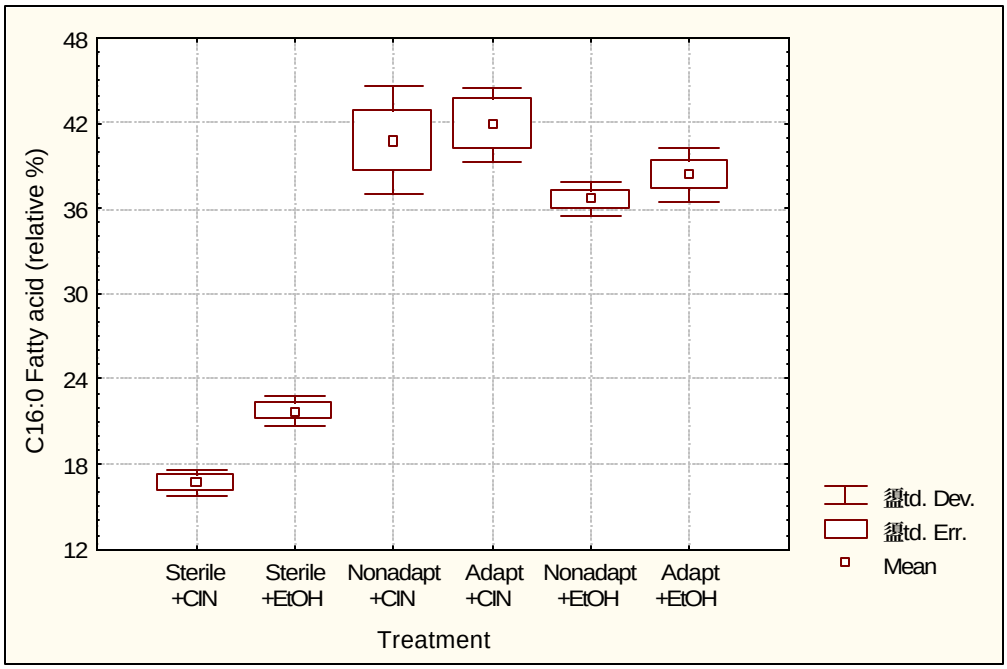


Figure 3.12. Comparison of C16:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

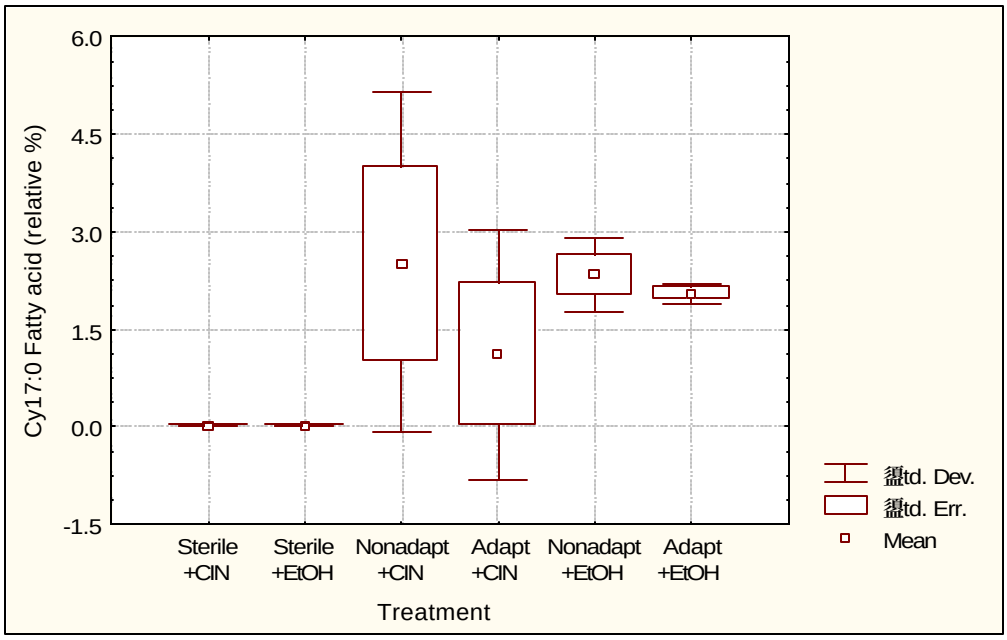


Figure 3.13. Comparison of Cy17:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

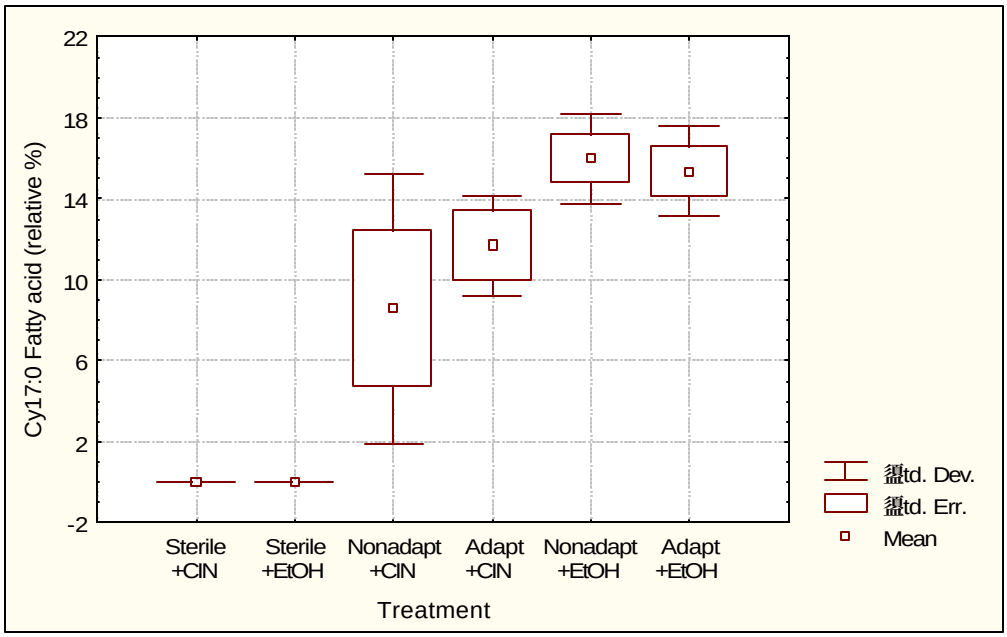


Figure 3.14. Comparison of Cy17:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

This indicated that the controls reached stationary phase sooner since they were not inhibited. The adapted cells treated with CIN did not differ from the non-adapted treated cells and the controls. The adapted treatment and control groups formed significantly ($p<0.05$ and $p<0.005$, respectively) more Cy17:0 at 24h than at 2h (Table 3.1).

C17:0 fatty acid. At 2h, there were no differences ($p>0.05$) in C17:0 among all the groups (Figure 3.15). The pattern of wider standard errors in the non-adapted treatment group and adapted control group resembled that of C15:0 at 2h. At 24h the non-adapted control produced more C17:0 than the sterile groups, adapted control, and non-adapted cells treated with CIN (Figure 3.16). Compared to 2h, C17:0 was significantly more abundant in the adapted treatment and non-adapted control groups at 24h ($p<0.0005$ and $p<0.005$, respectively) (Table 3.1).

C18:1w9c fatty acid. The percentages of C18:1w9c among all six groups were not significantly ($p>0.05$) different at 2h (Figure 3.17). The four inoculated groups did not differ from the sterile + CIN group in this fatty acid at 24h (Figure 3.18). The sterile + EtOH group had more C18:1w9c than all other groups. The abundance of this fatty acid did not differ from 2h to 24h within each group, except for a slight, but insignificant ($p>0.02$) decrease in the adapted cells treated with CIN.

C18:1w7c fatty acid. The non-adapted control had higher ($p<0.05$) levels of C18:1w7c than the sterile groups at 2 and 24h (Figures 3.19 and 3.20). This

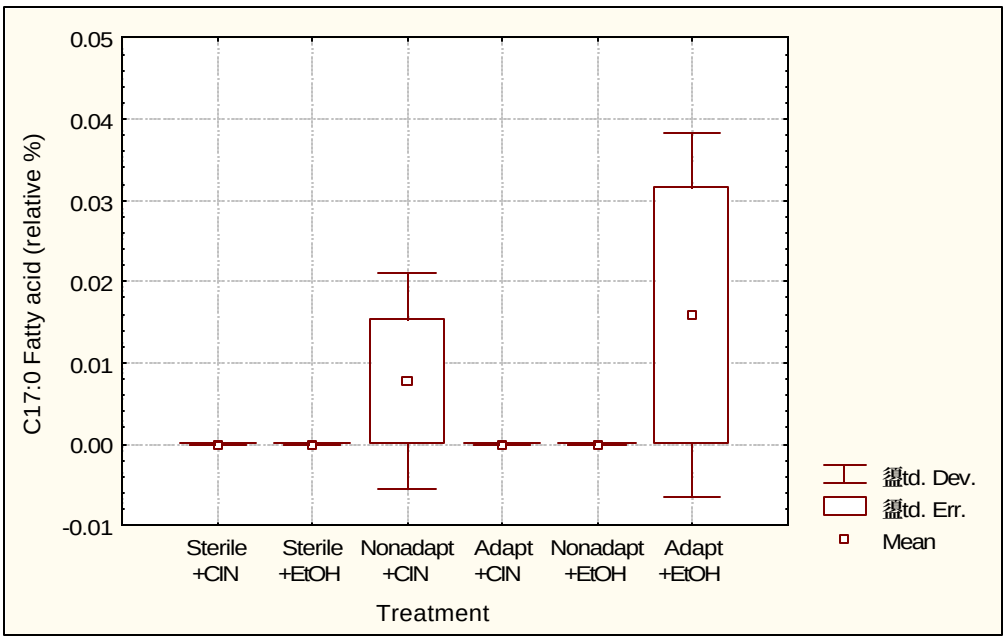


Figure 3.15. Comparison of C17:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

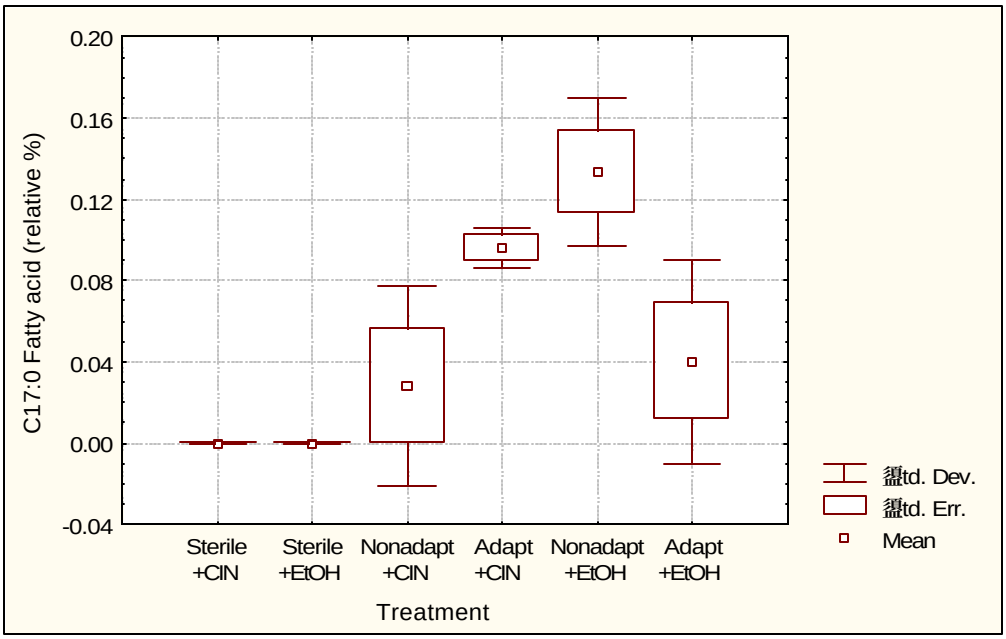


Figure 3.16. Comparison of C17:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

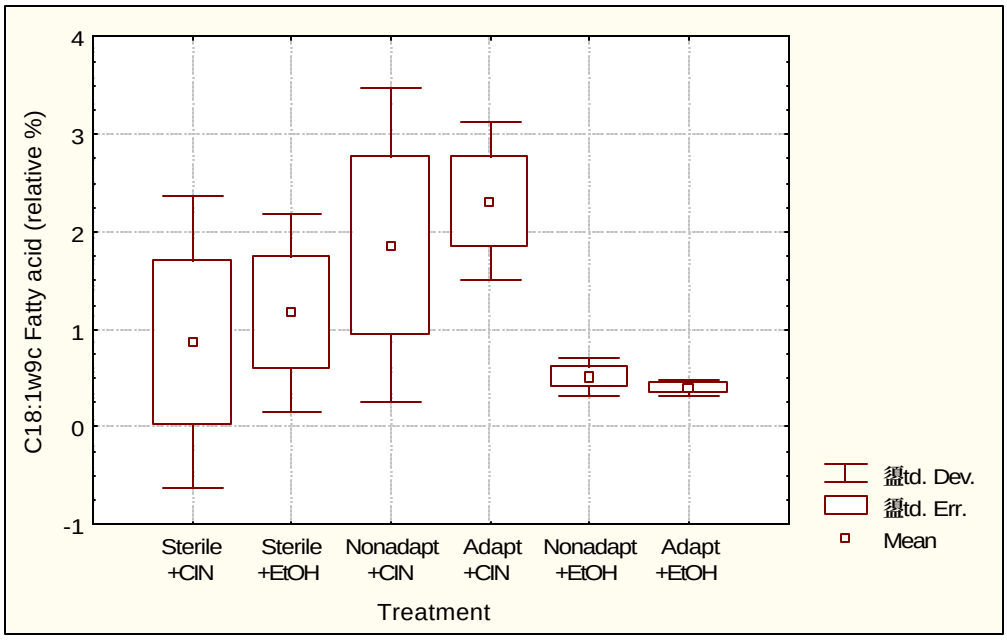


Figure 3.17. Comparison of C18:1w9c fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

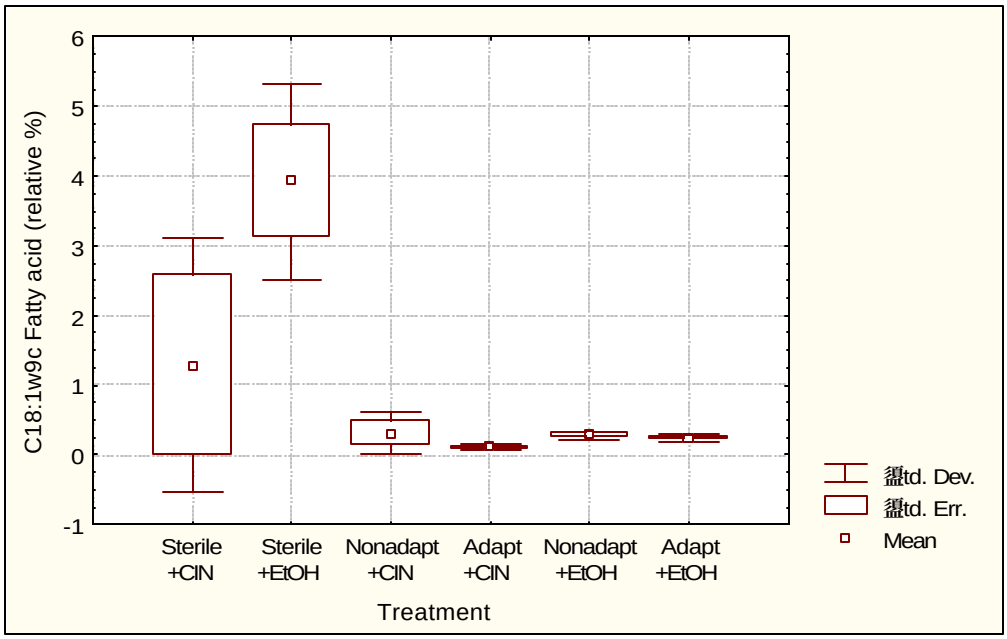


Figure 3.18. Comparison of C18:1w9c fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

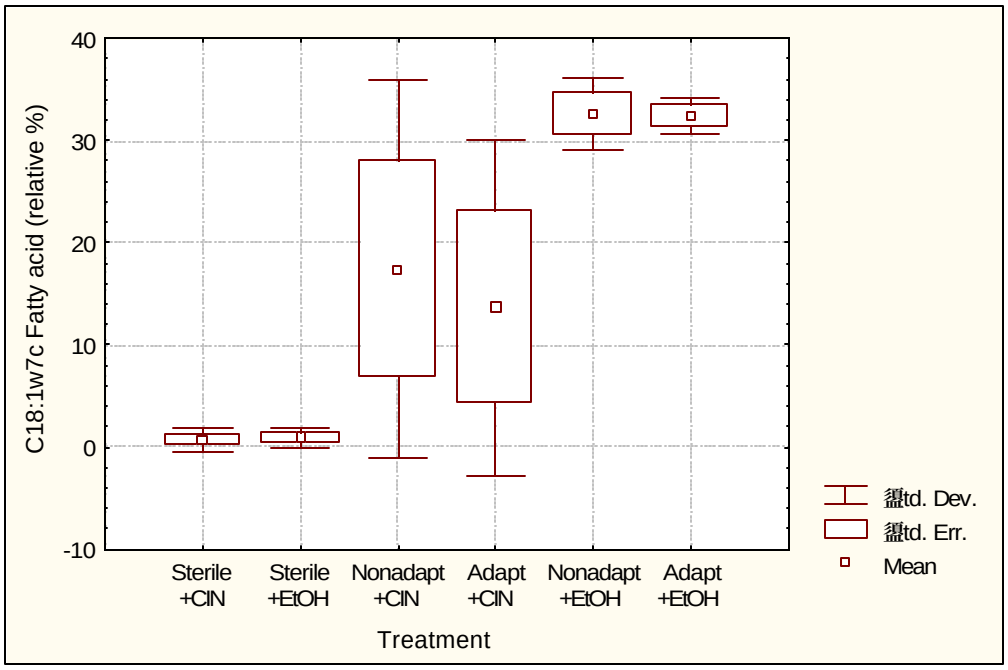


Figure 3.19. Comparison of C18:1w7c fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

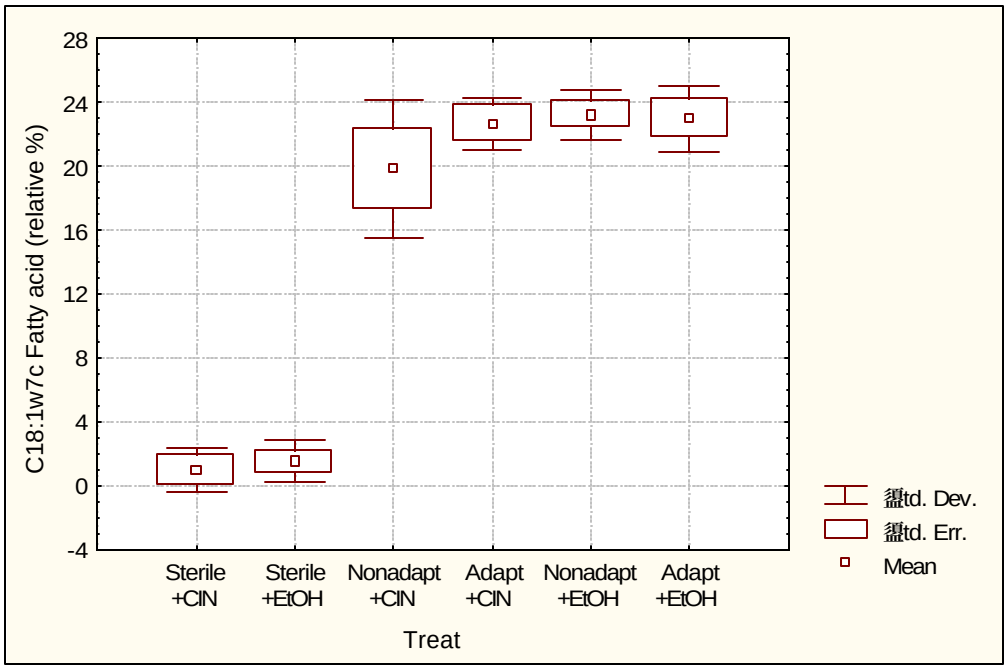


Figure 3.20. Comparison of C18:1w7c fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

fatty acid was not detected in the medium. As seen with other fatty acids, there was a wide standard error of C18:1w7c produced in the non-adapted and adapted cells treated with CIN, which reflected the range of sensitivity of cells to CIN. At 24h, these cells were able to grow and synthesize at proportions similar to the control groups because the percentage of C18:1w7c was not different (Figure 3.20). All four inoculated groups produced more of this fatty acid than the sterile groups. Compared across time, only the controls significantly ($p<0.02$) decreased C18:1w7c from 2h to 24h (Table 3.1).

C18:1w7t fatty acid. The abundance of C18:1w7t at 2h did not differ among the groups (Figure 3.21). It was only present in the sterile groups at 2 and 24h and in the treatment groups at 2h, but depleted at 24h. It was only present in the treatment groups because the bacteria were inhibited by CIN and had not yet metabolized it. There were no significant changes in C18:1w7t from 2h to 24h and no differences among groups at 24h (Figure 3.22).

C18:0 fatty acid. At 2h, the non-adapted and adapted controls lowered the proportion of C18:0 because they were able to grow (Figure 3.23). The non-adapted and adapted cells treated with CIN had percentages of C18:0 similar to those of the sterile groups. They had incorporated C18:0 from the medium. The growth of bacterial cells in the treatment groups was inhibited by CIN so lipid biosynthesis was decelerated. They incorporated C18:0 from the medium but could not oxidize and convert it to other fatty acids as well as the inoculated controls, but this changed at 24h (Figure 3.24).

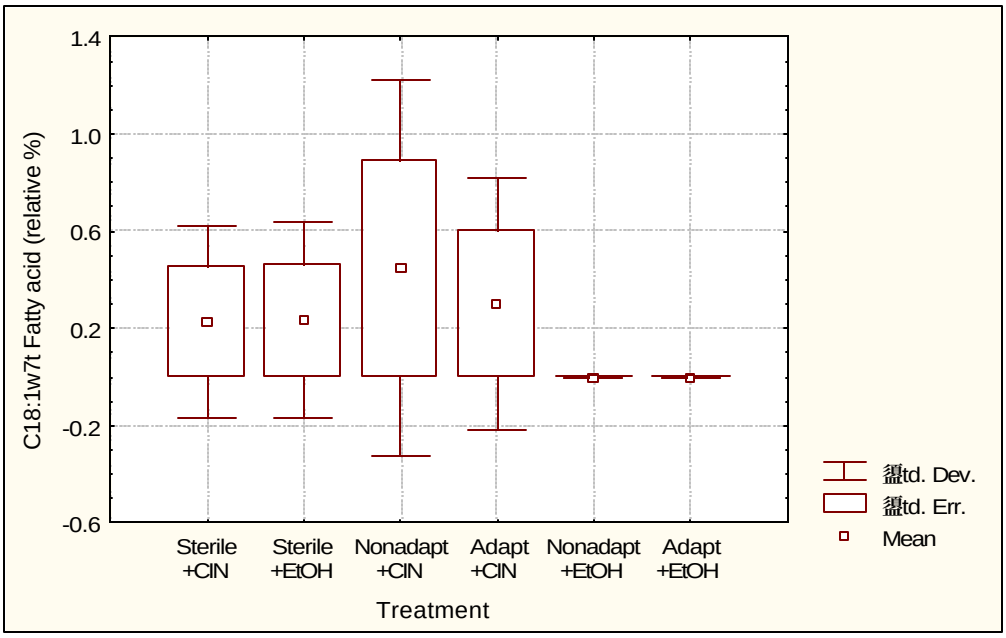


Figure 3.21. Comparison of C18:1w7t fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

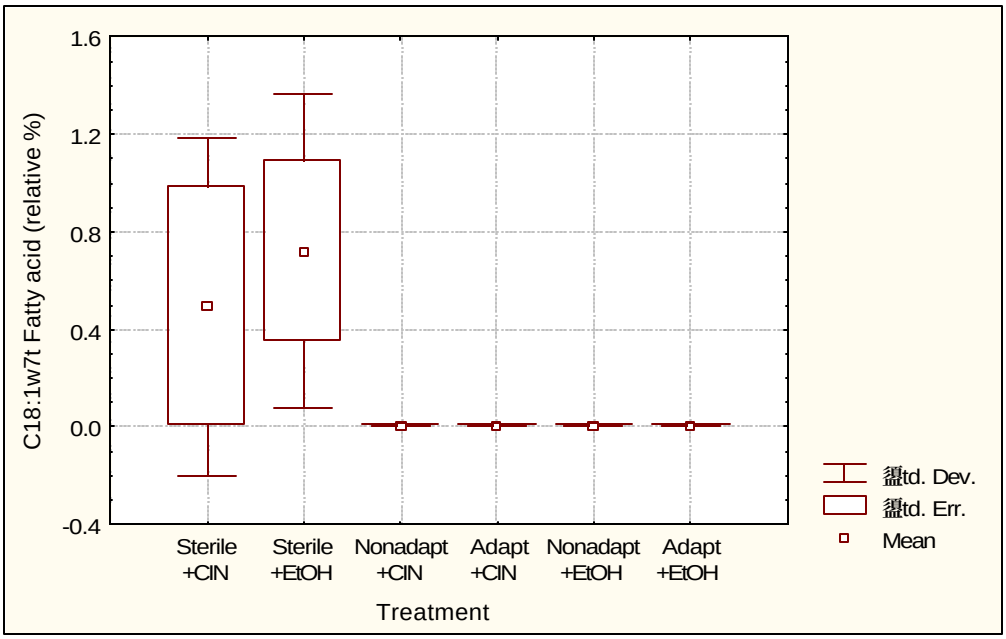


Figure 3.22. Comparison of C18:1w7t fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

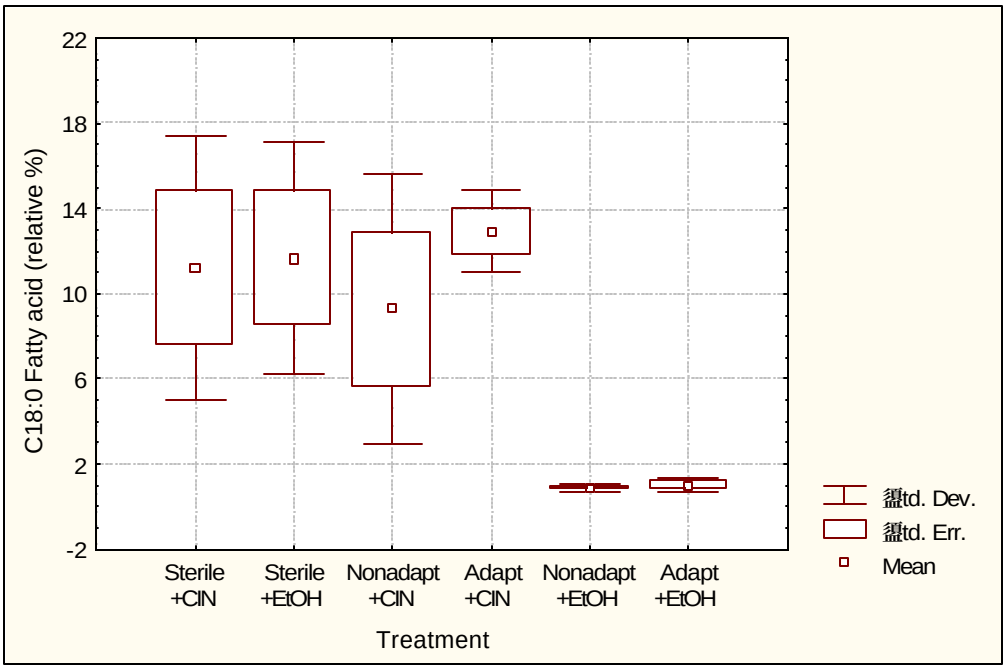


Figure 3.23. Comparison of C18:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

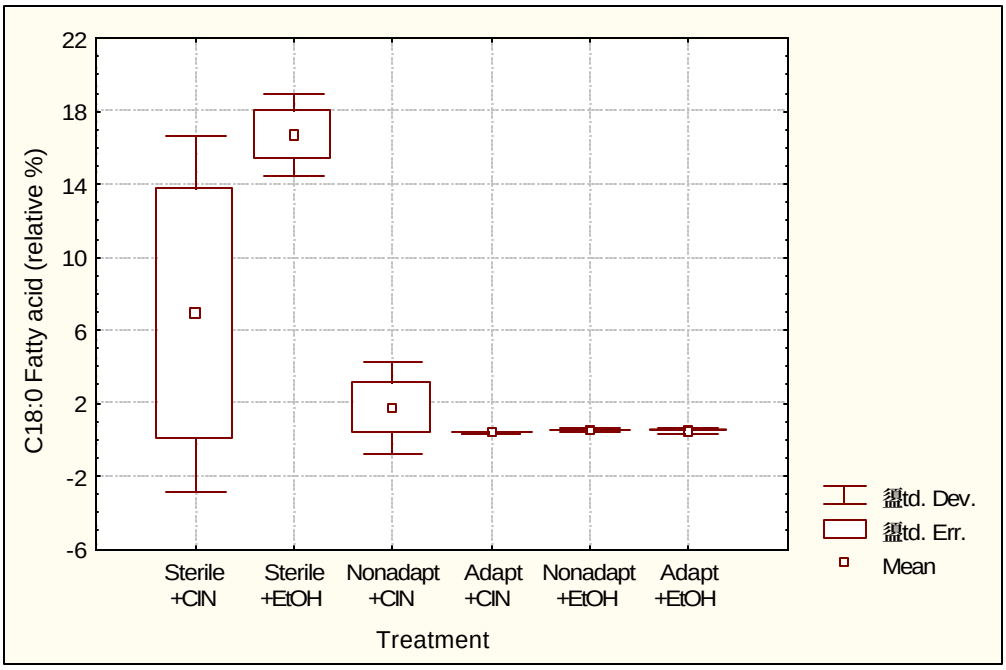


Figure 3.24. Comparison of C18:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

At 24h, as the cells grew in the non-adapted and adapted treatment groups, they were able to convert C18:0 to another fatty acid and therefore decrease the percentage of C18:0 (Figure 3.24). The percentage of C18:0 significantly ($p<0.005$) decreased from 2h to 24h in adapted cells treated with CIN (Table 3.1). Therefore, the abundance of C18:0 did not differ among the inoculated groups. There was a pattern of decreased fatty acid over time in the non-adapted control, but this was not significant.

Cy19:0 fatty acid. Cy19:0 was not detected in any group at 2h (Figure 3.25). However, at 24h the non-adapted control had formed more Cy19:0 than the non-adapted treatment because it reached stationary phase sooner (Figure 3.26). Since the treatment group had a longer lag phase it did not produce as much Cy19:0. There were no differences among the treatment groups and the adapted control at 24h. The non-adapted control formed significantly ($p<0.01$) more Cy19:0 at 24h than at 2h (Table 3.1). The same trend occurred with the adapted treatment group, but this was not significant.

Saturated fatty acids. At 2h, the control groups had less SFA than the other groups (Figure 3.27). At 24h, the treatment groups were able to decrease the percentage of SFA to similar levels as the controls (Figure 3.28). These fatty acids were highest in the sterile groups. There was wide variation in the non-adapted treatment group and more SFA than in the non-adapted control. From 2h to 24h, the controls significantly ($p<0.02$) produced more SFA (Table 3.1).

Monounsaturated fatty acids. The controls synthesized more UFA than the other groups at 2h (Figure 3.29). At 24h (Figure 3.30) the treatment groups

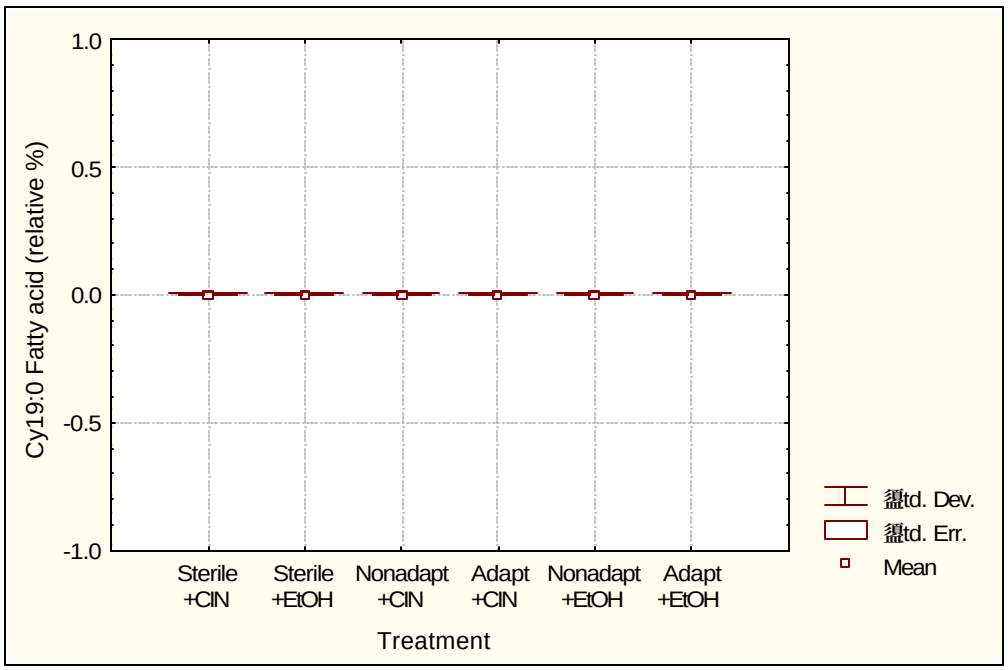


Figure 3.25. Comparison of Cy19:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

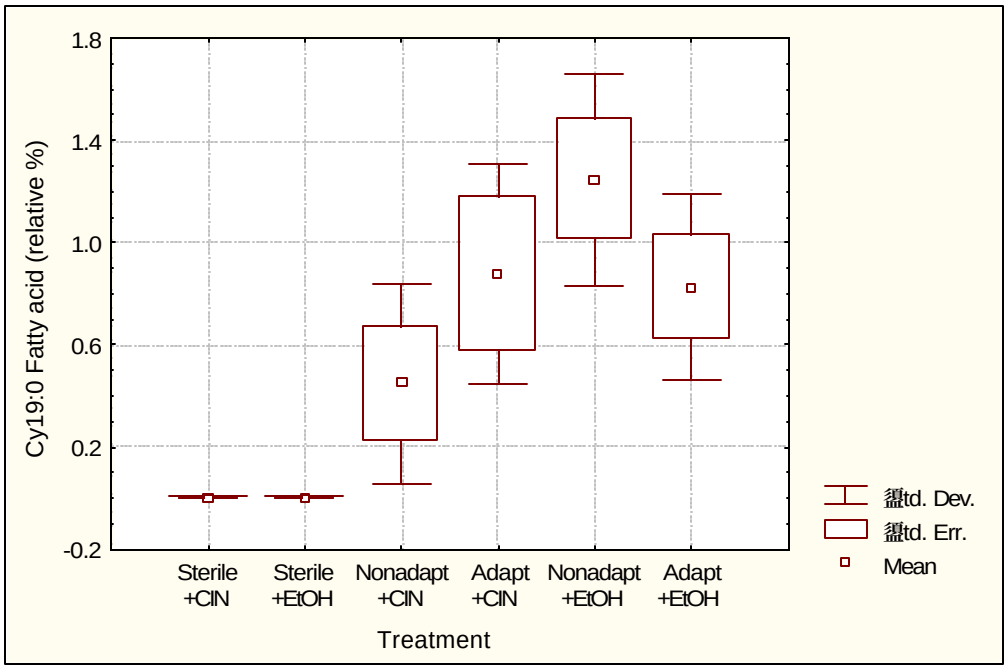


Figure 3.26. Comparison of Cy19:0 fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

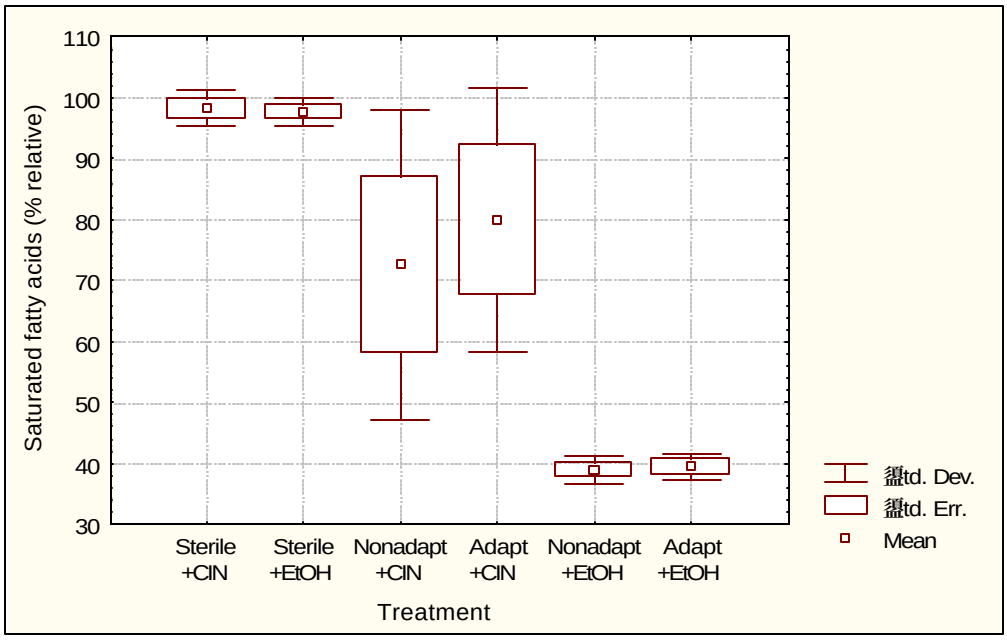


Figure 3.27. Comparison of saturated fatty acids of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

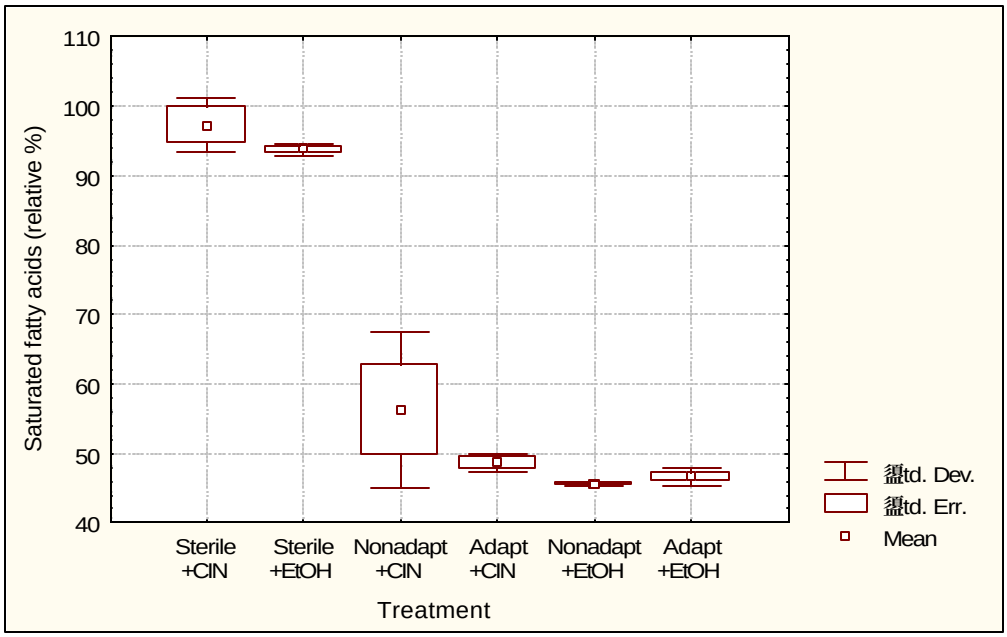


Figure 3.28. Comparison of saturated fatty acids of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

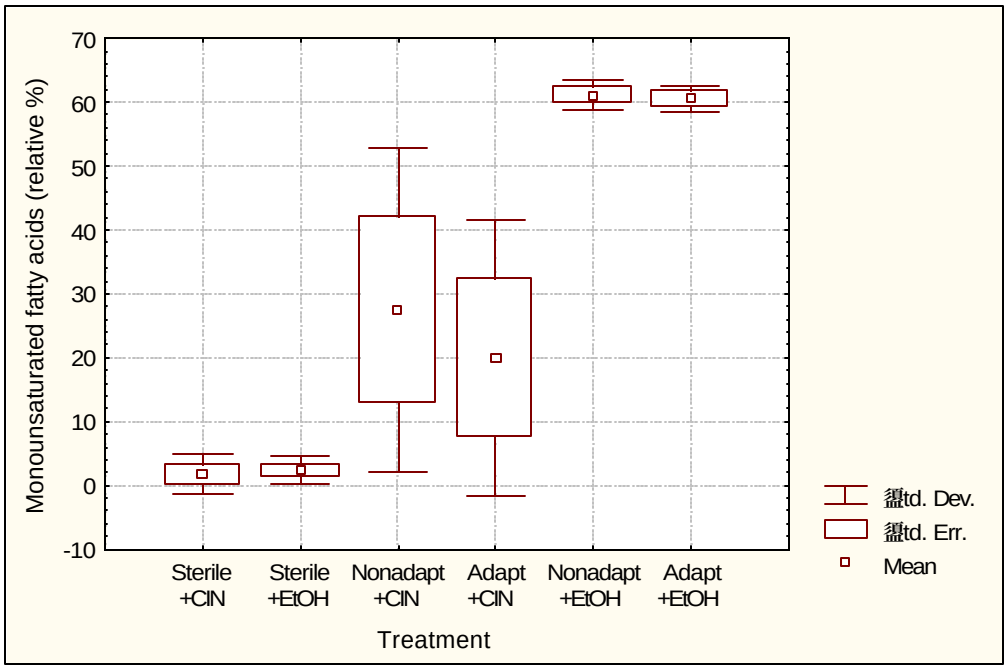


Figure 3.29. Comparison of monounsaturated fatty acids of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

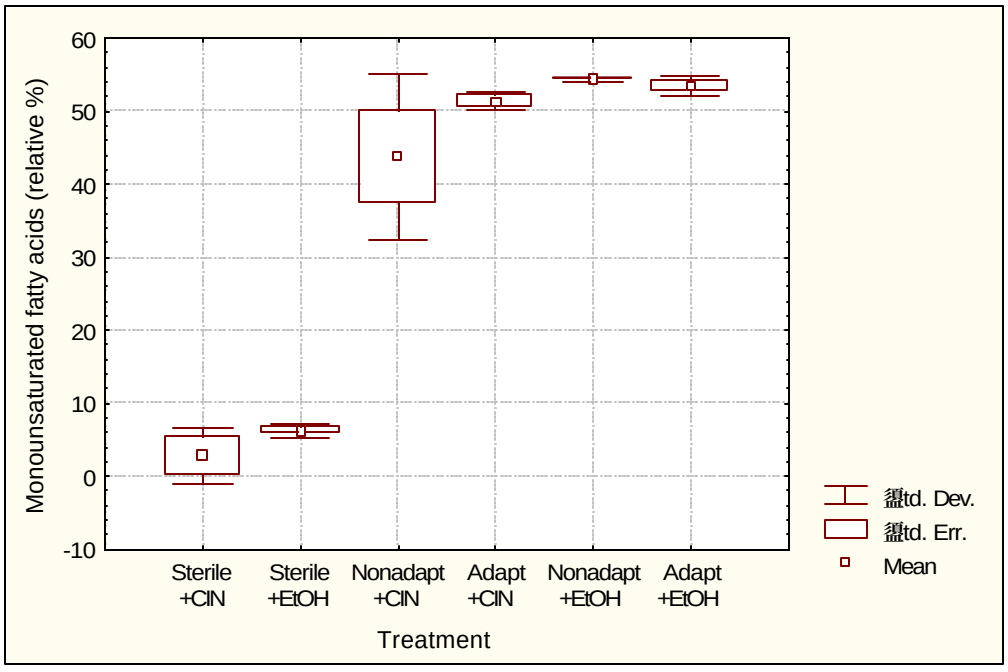


Figure 3.30. Comparison of monounsaturated fatty acids of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

produced more UFA to levels similar to the controls. These fatty acids were higher in the non-adapted control than in the non-adapted treatment. The controls significantly ($p<0.02$) decreased UFA from 2h to 24h (Table 3.1).

Ratio of Cy17:0 to C16:1w7c. C16:1w7c is the precursor to Cy17:0. There were no values for this ratio in the sterile groups because no Cy17:0 was present in the culture medium nor bacterial cells to form it (Figures 3.31 and 3.32). The ratio did not differ between the treatments groups or between the control groups at 2h. The non-adapted treatment group had a higher ratio than the control groups. At 24h, there were no differences among the inoculated groups, although the ratio appears to be higher in the control groups, suggesting earlier entry into the stationary phase (Figure 3.32). From 2h to 24h, the ratio increased significantly ($p<0.01$) for the control groups (Table 3.1). The ratio increases in the treatment groups were not significant, but probably obscured by the wide variation at 2h.

Ratio of Cy19:0 to C18:1w7c. C18:1w7c is the precursor to Cy19:0. The ratios of Cy19:0 to C18:1w7c were zero at 2h since no Cy19:0 was detected (Figure 3.33). At 24h, the ratios were not different among the inoculated groups (Figure 3.34). The ratio increased from 2 to 24h for the non-adapted control group only ($p<0.02$) (Table 3.1). There were slight increases in the adapted treatment and adapted control groups, but these were not significant.

Ratio of saturated to unsaturated fatty acids. At 2 and 24h, the sterile groups had a higher ratio because of the medium (Figures 3.35 and 3.36). It seemed that that these ratios were varied more in treatment groups. This

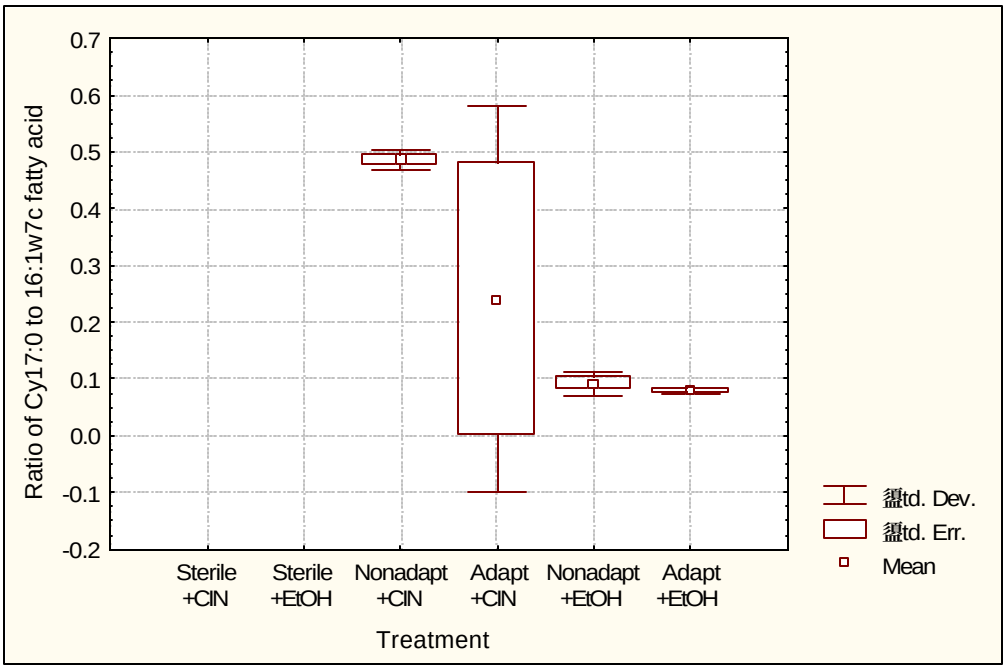


Figure 3.31. Comparison of ratio of Cy17:0 to 16:1w7c fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

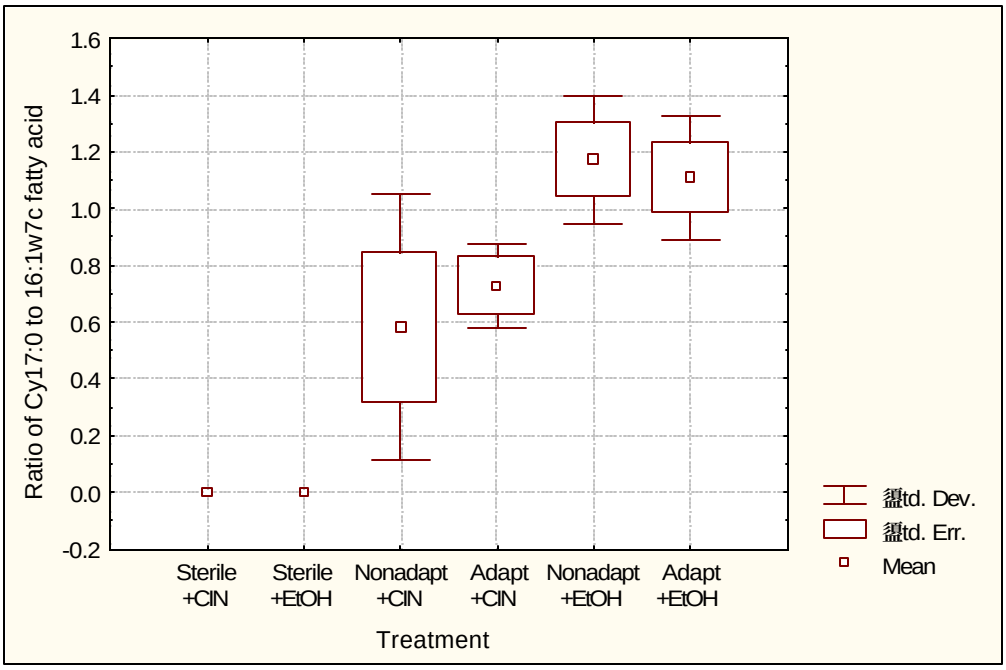


Figure 3.32. Comparison of ratio of Cy17:0 to 16:1w7c fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

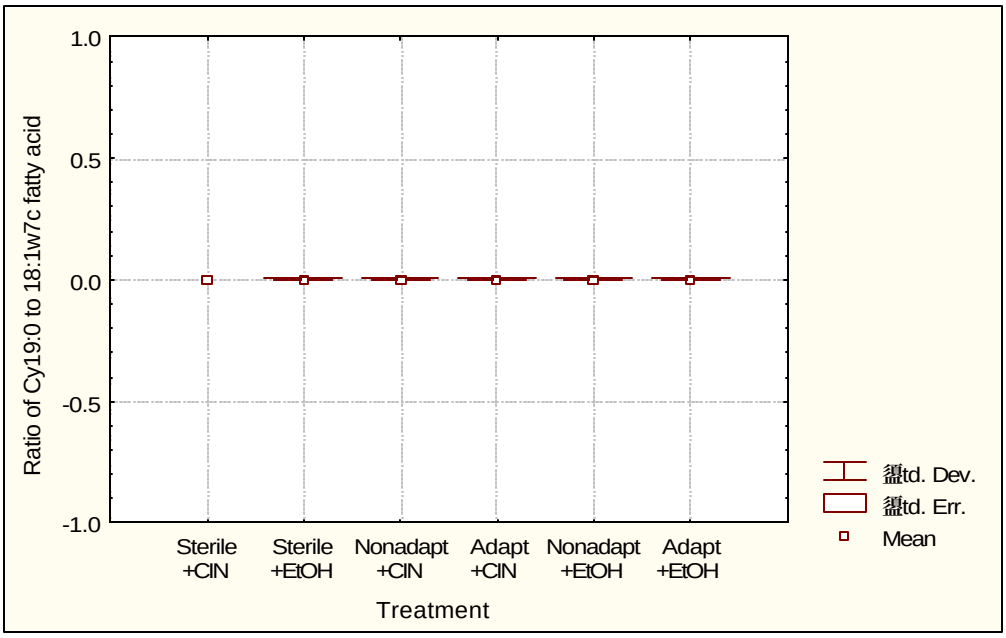


Figure 3.33. Comparison of ratio of Cy19:0 to 18:1w7c fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

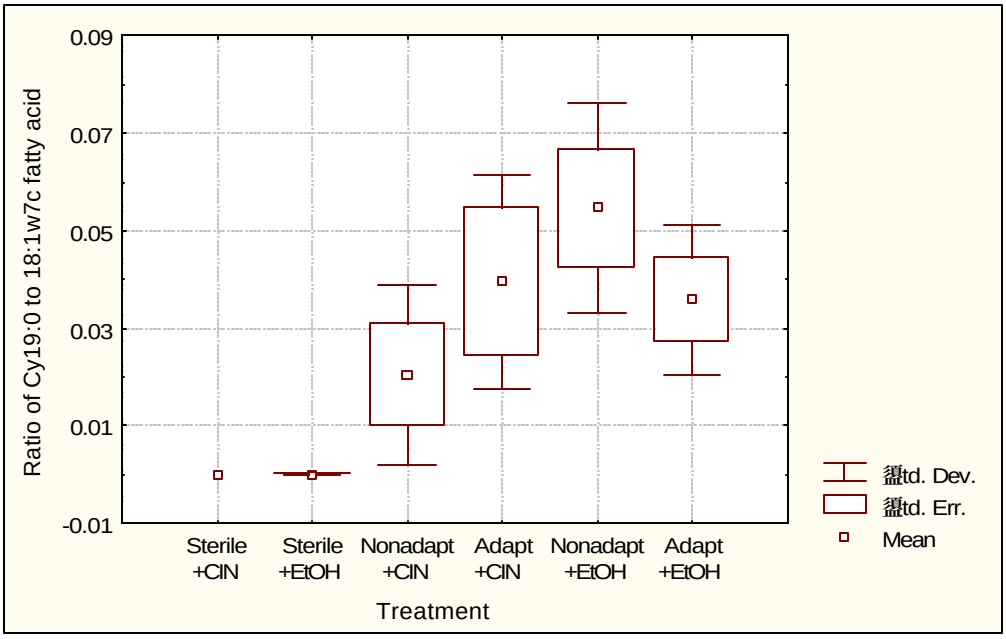


Figure 3.34. Comparison of ratio of Cy19:0 to 18:1w7c fatty acid of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

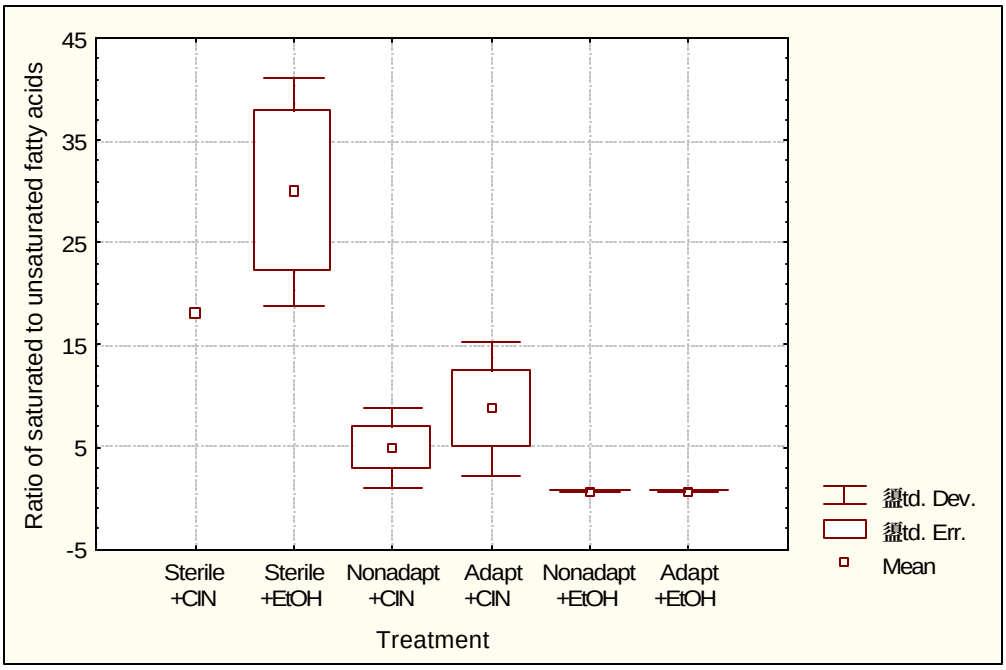


Figure 3.35. Comparison of ratio of saturated to unsaturated fatty acids of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

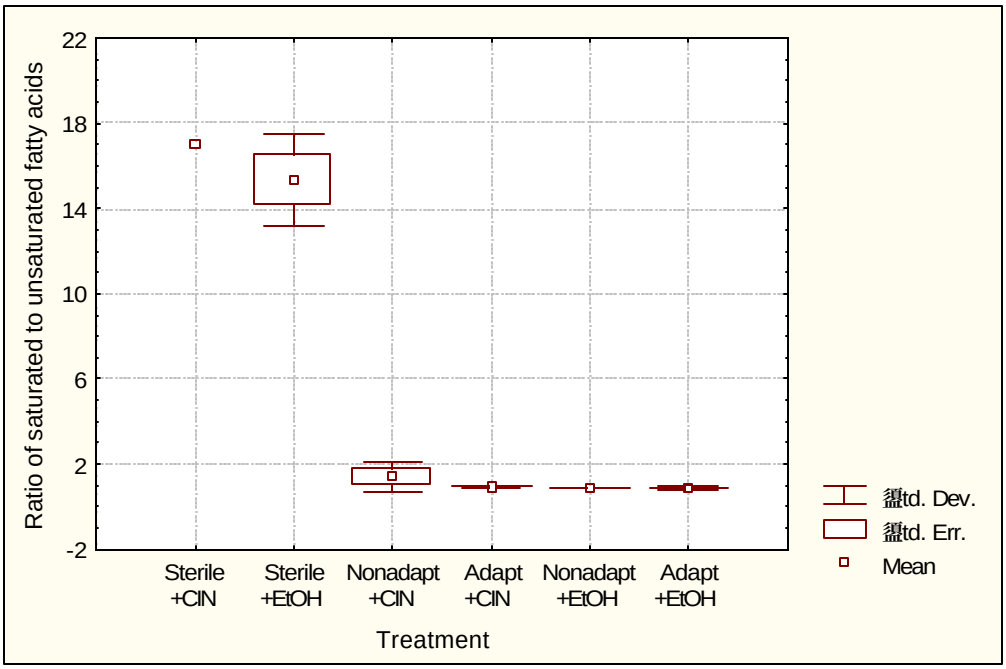


Figure 3.36. Comparison of ratio of saturated to unsaturated fatty acids of CIN-adapted or non-adapted cells with or without CIN to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

indicated that the groups were more inhibited by CIN and the cells differed in their sensitivity to CIN. Their ratios were numerically greater than in the controls. They were less able to decrease the ratio of SFA to UFA than the controls because they were inhibited. However, the ratios of the four inoculated groups did not differ significantly ($p>0.05$) from each other.

At 24h, all four inoculated groups had lower ratios than the sterile groups (Figure 3.36). From 2h to 24h, the ratio increased significantly ($p<0.02$) only in the controls (Table 3.1). Numerically, it seemed that the ratio decreased for the treatment groups. However, this was not significant ($p>0.1$) and the difference may have been obscured by the variability at 2h.

Discussion

Anaerobes, facultative anaerobes, and also some aerobic bacteria synthesize lipids via the anaerobic pathway, which has been studied in *E. coli*. The starting reactants are acetyl-CoA and 3 malonyl-ACP. A trans double bond is produced during elongation. From that point, it can be reduced to form a saturated fatty acid or isomerized into a cis-monoenoic fatty acid. This cis fatty acid cannot be reduced and remain unchanged with further elongation. These saturated and cis-unsaturated chains are incorporated into phospholipids of the membrane. Since the degree of saturation cannot change after synthesis, the ratio of SFA to UFA cannot be changed in cells that cease growth. Phospholipid biosynthesis quickly decelerates if growth stops. However, post-synthesis

modification of cis-monoenoic fatty acids to their cyclopropyl derivatives and trans-isomers can still occur (Diefenbach and others 1992; Keweloh and Heipieper 1996).

The uptake of fatty acids from the culture medium can occur by acyl-chain unspecific or active or passive transport. They are oxidized or incorporated in membrane lipids, which is regulated. Biosynthesis is regulated as well (Diefenbach and others 1992; Keweloh and Heipieper 1996).

Membrane fluidity has been correlated with changes in fatty acid composition (Mazzotta and Montville 1997). Certain types of lipid may have a greater influence on the fluidity than other. For example, converting a cis-monoenoic acid to its trans-isomer greatly reduces fluidity, but not as significant as a change in cis to saturated fatty acids (Keweloh and Heipieper 1996).

The melting points of unsaturated fatty acid (UFA) and branched chain fatty acids are lower than that of saturated fatty acids (SFA). There are hydrophobic interactions among acyl chains of SFA. In membranes rich with SFA the membrane is packed and rigid. The chain can extend at its maximum if the saturated chain rotates into a trans-conformation (Keweloh and Heipieper 1996).

In contrast, the double bond in cis-UFA creates a 30° angle in the acyl chain. This kink disrupts the ordered packing of the acyl chains in the membrane (Keweloh and Heipieper 1996). Therefore, an increase in UFA causes a decrease in the temperature for the transition from the gel to the liquid-crystal

membrane phase and an increase in fluidity (Keweloh and Heipieper 1996; Casadei and others 2002).

A shortening in the acyl chain length will also lower the melting temperature and increase fluidity. A change in the degree of saturation is thought to be more effective than a change in the chain length (Keweloh and others 1991). Cyclopropyl fatty acids (CFA) are more physically similar to cis-monoenoic fatty acids, which are precursors of CFA. Trans-fatty acids are more stable and resemble SFA (Guckert and others 1986). Trans-UFA acyl chains are long and extended, packing more rigidly and occupying lower molar volume in the membrane to significantly increase the phase transition temperature (Diefenbach and others 1992; Keweloh and Heipieper 1996).

The ratio of UFA to SFA indicates membrane fluidity. Casadei and colleagues (2002) developed a fluidity index that was calculated as the ratio of UFA plus cyclopropane fatty acids (CFA) in stationary phase to SFA. They extracted phospholipids from *E. coli* grown at different temperatures and measured the phase transition temperatures by DSC.

Casadei and colleagues (2002) confirmed the validity of the fluidity index as an indicator of membrane fluidity. They found that there was a direct linear relationship between the phase transition temperature and growth temperature and then an inverse linear relationship between the phase transition temperature and the fluidity index of phospholipids. The proportion of SFA increased while the proportion of UFA decreased with increasing growth temperature in stationary and exponential phase cells. The fluidity index was slightly greater in

exponential than stationary phase cells. They reported that the abundance of CFA increased and C18:1 and C16:1 decreased in stationary phase cells. Since most of the treatment groups in this present data contained mostly SFA, a ratio was calculated of SFA to UFA plus CFA.

The measurement of the ester-linked phospholipid fatty acids (PLFA) is useful. The PLFA content quantitatively indicates the viable biomass since they have a high turnover rate and are present in consistent abundance in cell membranes. When cells die or lyse, hydrolysis of phospholipids quickly occur and the polar head group and diglyceride remain (White and Ringelberg 1997).

In addition to providing an estimate of the viable biomass, PLFAs can indicate nutritional and physiological status using the ratio of trans-monoenoic fatty acids to their cis homologues and the ratio of cyclopropyl derivatives to their monoenoic fatty acid precursors (White and Ringelberg 1997). During starvation of cultures of *Vibrio cholerae*, the trans/cis ratio increased. The cis-monoenoic fatty acids (such as 16:1w7c and 18:1w7c) decreased and the SFA, CFA, and trans-monoenoic fatty acids increased. During starvation, membrane fluidity decreased overall (Guckert and others 1986).

When loss of phospholipid occurred during starvation, cis-monoenoic acids were preferentially utilized to possibly enhance survival. Cis fatty acids have faster turnover than saturated fatty acids and are more easily metabolized. During starvation, bacteria may synthesize trans-monoenoic acids, which bacteria do not readily metabolize, or transmethylate unstable cis- monoenoic

fatty acids to form their cyclopropyl derivatives to survive (Guckert and others 1986).

It has been suggested that CFA are more stable to turnover and degradation (Guckert and others 1986). CFAs were formed (with a decline in their cis-fatty acid precursors) in *Vibrio cholerae* and *Pseudomonas putida* as cells aged and entered the stationary phase (Guckert and others 1986; Diefenbach and others 1992; Loffeld and Keweloh 1996). It has been hypothesized that CFA are formed under stress when growth stops (Guckert and others 1986).

The objectives in our study were to:

1) Determine the effect of exposure to CIN on the fatty acid composition of the bacterial membrane of *Salmonella*.

At 2h the non-adapted treatment group had significantly ($p<0.05$) lower total PLFA and lower ($p<0.05$) C16:1w7c than the non-adapted control (+ EtOH), which contributed to lower ($p<0.05$) UFA and a higher ($p<0.05$) Cy17:0 to C16:1w7c ratio. C18:0 was also higher ($p<0.05$), which increased the abundance of the saturated fatty acids. CIN decreased the fluidity of the membrane within 2h of exposure and inhibited growth. At 2h, the non-adapted + CIN group had a numerically higher SFA to UFA ratio than the non-adapted + EtOH group, which also suggested that CIN decreased fluidity. However, this difference lessened at 24h.

At 24h the non-adapted treatment group had significantly ($p<0.05$) higher C16:0, but lower Cy17:0 and Cy19:0 than the non-adapted control. Similar to

results at 2h, CIN decreased the fluidity. SFA was significantly ($p<0.05$) higher and UFA was significantly ($p<0.05$) lower in the non-adapted treatment group than in the non-adapted control. The total PLFA of the non-adapted treatment group was lower than the control group, which indicated lower lipid biosynthesis and growth. The non-adapted treatment group had a numerically higher SFA to UFA ratio than the non-adapted control group, which indicated that long-term CIN exposure reduced fluidity.

2) Determine whether changes in the fatty acid composition of the bacterial membrane of *Salmonella* occur due to growth in the presence of CIN as a possible adaptive response to CIN.

At 2h and 24h there were no significant ($p>0.02$) differences in the total PLFA or fatty acids between the treatment groups as a function of the incubation time (Table 3.1). However, at 2h the SFA to UFA ratio of the adapted treatment group was numerically higher than that of non-adapted group. This suggested that adaptation decreased membrane fluidity.

At 24h, the SFA to UFA ratio was smaller in the adapted than non-adapted group, perhaps because the numeric increase in C16:1w7c in the adapted treatment was more drastic than in the non-adapted group. At 24h the difference in the SFA to UFA ratio between the groups is considerably smaller, perhaps because the 24h growth allowed adaptation of the non-adapted treatment group, which allowed it to synthesize lipid similarly to the adapted treatment group.

3) Determine whether reversal of these changes in fatty acid composition occurs when the stressor, CIN, is removed.

At 2h and 24h there were no significant ($p>0.05$) differences in the fatty acids between the groups. Even the SFA to UFA ratios were similar. This indicated that any changes during adaptation (in the absence of subsequent exposure) were not maintained. At 24h, the total PLFA of the non-adapted control was numerically higher than that of the adapted control, which may indicate that the initial adaptation caused some growth inhibition.

4) Determine whether any adaptive changes in the fatty acid composition are maintained after subsequent exposure to the CIN.

At 2h the adapted treatment group had less C16:1w7c than the adapted control, which decreased the overall UFA, and more C18:0 which increased the abundance of SFA. The SFA to UFA ratio in the adapted treatment was also numerically greater. CIN adaptation and subsequent exposure decreased the fluidity within 2h, and also inhibited growth as shown in the numerically lower total PLFA.

At 24h the only significant ($p<0.05$) difference was that the adapted treatment group produced more C16:1w7c. The adapted treatment group had a numerically higher SFA to UFA ratio than the adapted control at 24h, but not as higher as it was at 2h. Therefore, at 24h the membrane was less fluid in the adapted control.

Adaptation, in terms of decreased fluidity, seems to have been maintained with subsequent exposure for 2 and 24h.

5) Determine the effect of growth in the presence of CIN (adaptation) and subsequent CIN treatment on affect fatty acid composition.

Overall, most of the changes were in the longer chain fatty acids than in the shorter chain fatty acids such as C12:0, 14:0, and 15:0. At 2h compared to the non-adapted control, the adapted treatment group had lower total PLFA, C16:1w7c, C18:1w7c, and monounsaturated fatty acids and higher C14:0, C18:0, and SFA. Therefore, with adaptation and subsequent exposure, CIN caused a decrease in fluidity. At 24h, the group had higher C16:1w7c and C16:0 than the non-adapted control. The SFA to UFA ratio of the adapted treatment was much greater than the control at 2h, but that magnitude of difference declined at 24h.

It appears that in both treatment groups the membrane became more fluid and in the control groups less fluid with time. At 24h differences between the treatments and controls had decreased compared to 2h in terms of the SFA to UFA ratio. With only 2h of exposure to CIN, both treatment groups synthesized a numerically higher proportion of SFA (compared to the non-adapted control) in response to CIN. Perhaps this response to 2h incubation was more similar to an adaptation. The length of time needed for an adaptation to develop to CIN is not known.

Significant ($p<0.02$) changes from 2h to 24h occurred mostly with the control groups and occasionally with the adapted treatment group. However, significant differences may not have been detected due to variability within these treatment groups. At 2h, the proportions of C12:0, C14:0 and C18:0 of the treatment groups were more similar to that sterile controls or in between that of the sterile controls and inoculated controls. It is hypothesized that the bacteria of the treatment groups had incorporated these fatty acids from the medium but

were not able to convert them to other fatty acids because growth was inhibited by CIN treatment. With time, the non-adapted treatment overcame the inhibition and was able to grow and synthesize phospholipids (as evidenced by the numerical increase in total PLFA and decrease in these fatty acids) for a more favorable proportion of SFA to UFA ratio that is closer to homeostasis such as in the non-adapted + EtOH group.

The concentration of membrane fatty acids in the controls was expected to increase in the SFA to UFA ratio because C16:1w7c and C18:1w7c typically decline and CFA increase in stationary phase (Casadei and others 2002). These significant ($p<0.02$) changes occurred in the controls from 2 to 24h. Over time, SFA increased in the controls, which was a sign of starvation, along with the decrease in C16:1w7c and C18:1w7c and increase in CFA (Guckert and others 1986). The treatments did not seem to have entered stationary phase over time because there were numerical increases in C16:1w7c and C18:1w7c. However, there was also a significant increase of Cy17:0 in the adapted treatment and a numerical increase in the non-adapted treatment as well as numeric increases in Cy19:0.

Since lipophilic compounds accumulate and act on the membrane, it was expected that membranes would respond to CIN, which is a lipophilic, aromatic aldehyde. Carvacrol, which is a lipophilic and phenolic essential oil component, was found to expand the liposomal membrane using fluorescent probes. This suggested an increased fluidity (Ultee and others 2002). If the membranes

become too fluid, additional carvacrol can continue to permeate the membrane, accumulate, and cause more damage (Ultee and others 1998).

It is believed that this swelling leads to a destabilized membrane and therefore a leakage of ions (Ultee and others 2002). During exposure to carvacrol (CRV), an influx of protons and efflux of potassium ions in *Bacillus cereus* was reported (Ultee and others 1999). This leakage was also evident in the decrease of the pH gradient across the membrane (Ultee and others 2002). The leakage of ions leads to a drop in the membrane potential and depletion of the intracellular ATP pool (Ultee and others 2002). It was found that 300 mg/L of CRV reduced viable count of *Bacillus cereus* cells in 30 min and depleted intracellular ATP within 7 min without a proportional increase in extracellular ATP (Ultee and others 1999).

There was no literature that has determined the precise mechanism of action of CIN. Helander and others (1998), who reported that carvacrol at 300 mg/L (MIC = 451 mg/L) increased the uptake of hydrophobic fluorescent probe NPN in *E. coli* O157:H7 and S. Typhimurium and decreased intracellular ATP in *E. coli* O157:H7, but with a smaller increase in extracellular ATP. Uptake of NPN occurred because the outer membrane was no longer acting as a strong permeability barrier. These effects indicated that CRV disrupted the outer membrane. In contrast, it was suggested that CIN did not disrupt the outer membrane of *E. coli* O157:H7 and *S.* Typhimurium because at 264 mg/L it did not increase the uptake of NPN or influence intra- or extracellular ATP pools of (Helander and others 1998).

No literature has been published that has investigated the adaptation to CIN. There was a study that examined the adaptive response of *Bacillus cereus* to CRV (Ultee and others 2000). They reported that after CRV was added to exponentially growing, non-adapted cells there was a decrease in the melting temperature (T_m) of the membrane (20.5°C to 12.6°C). This disagreed with the finding in this present study that exposure to CIN decreased membrane fluidity as reflected by the higher SFA to UFA ratio. Ultee and others (2000) reported that compared to non-adapted cells, cells adapted to 60 mg/L exhibited a less fluid membrane because the T_m increased from 20.5°C to 28.3°C. *Iso*-C13:0, C14:0 and iso-C15:0 fatty acids (which are higher melting) increased in the membrane lipid composition of *B. cereus* cells incubated with 60 mg/L carvacrol. Similarly, this study found a higher SFA to UFA ratio with adaptation, which indicated decreased fluidity.

Greater saturation found with adaptation to phenol was suggested to result in a more rigid membrane that compensated for an increase in fluidity and permeability caused by phenol. Saturated fatty acids have acyl chains that are packed more closely, which could limit the entry of phenol and therefore, its toxic effect (Keweloh and others 1991). In the same way, the increase of saturation upon exposure and adaptation to CIN could reduce its accessibility into or its impact on the cell.

CIN did not disrupt the outer membrane because it did not increase the uptake of a hydrophobic probe or affect ATP pools according to Helander and others (1998). Therefore, it is reasonable that an increase in fluidity upon

exposure or adaptation was not observed in this present study. It appears that the mechanism of action of CIN is not an increase in permeability. Its mechanism is unlikely to be related to an interaction with acyl chains as with CRV. The carbonyl group of aldehydes can bind to metal ions, sulfhydryl groups, amino acids, and proteins (Bowles and Miller 1993). CIN was found to be an effective inhibitor of histidine decarboxylase activity of *Enterobacter aerogenes* and its carbonyl group was suggested as the functional group (Wendakoon and Sakaguchi 1995).

Significant findings

After 2h of exposure to CIN, the non-adapted treatment group had significantly ($p<0.05$) lower total phospholipid fatty acid (PLFA), lower C16:1w7c and unsaturated fatty acid (UFA) and higher ($p<0.05$) C18:0, saturated fatty acid (SFA), and Cy17:0/C16:1w7c ratio than the non-adapted control. After 24h of exposure to CIN, the non-adapted treatment group had significantly ($p<0.05$) lower Cy17:0, Cy19:0, and UFA than the non-adapted control and higher ($p<0.05$) C16:0 and SFA. At 2h, the SFA/UFA ratio in the membranes of adapted cells exposed to CIN was numerically higher than in the non-adapted cells treated with CIN. Although there were no significant differences in fatty acids as a function of incubation time, at 24h the SFA /UFA ratios of the adapted and non-adapted *Salmonella* were similar. Since the fatty acid composition and total PLFA of the non-adapted control and adapted controls were similar at 2 and 24h,

the adaptive response had been reversed since the stressor, CIN, was not used to treat the adapted controls.

Most of the changes from 2 to 24h occurred in the membrane of the non-adapted and adapted control *Salmonella*. Over time there was a decrease ($p<0.01$) in C16:1w7c, C18:1w7c, and UFA and an increase ($p<0.01$) in total PLFA, C16:0, Cy17:0, SFA, Cy17:0/ C16:1w7c ratio, and SFA/UFA ratio in the control groups. In the adapted and non-adapted treated *Salmonella* there was a numerical decrease in SFA/UFA ratio from 2 to 24h

Since fatty acid composition is one of the factors that affect membrane fluidity it appears that short- and long-term treatment with CIN at sub-lethal concentrations decreased the fluidity of the *Salmonella* membrane by significantly increasing the proportion of saturated fatty acids. This decreased fluidity may prevent additional CIN from permeating. The effect of bacterial growth in the presence of CIN on the fatty acid composition was much smaller. Determination of any changes in the phospholipid head groups would be useful because of their affect on fluidity. Measurement of the membrane transition temperature from gel-to-liquid-crystalline phase by Fourier-transformed infrared spectrometry would indicate the interaction between their acyl chains and therefore fluidity. A nuclear magnetic resonance spectrometer or a Langmuir-Blodgett trough can also measure fluidity. Investigation about the target site that CIN acts upon and the specific interactions of CIN with membrane lipids and proteins using differential scanning calorimetry would contribute to the understanding of its mechanism of action and the bacterial response.

REFERENCES

Bowles BL, Miller AJ. 1993. Antibotulinal properties of selected aromatic and aliphatic aldehydes. Journal of Food Protection 56:788-794.

Brul S, Coote P. 1999. Preservative agents in foods: Mode of action and microbial resistance mechanisms. International Journal of Food Microbiology 50:1-17.

Casadei MA, Manas P, Niven G, Needs E, Mackey BM. 2002. Role of membrane fluidity in pressure resistance of *Escherichia coli* NCTC 8164. Applied and Environmental Microbiology 68:5965-5972.

Denich TJ, Beaudette LA, Lee H, Trevors JT. 2003. Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. Journal of Microbiological Methods 52:149-182.

Diefenbach R, Heipieper HJ, Keweloh H. 1992. The conversion of cis into trans unsaturated fatty acids in *Pseudomonas putida P8*: Evidence for a role in the regulation of membrane fluidity. Applied Microbiology and Biotechnology 38:382-387.

Friedman M, Henika PR, Mandrell RE. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes,* and *Salmonella enterica*. Journal of Food Protection 65:1545-1560.

Guckert JB, White DC *Proceedings of the Fourth International Symposium of Microbial Ecology*, p pp 455-459.

Guckert JB, Hood MA, White DC. 1986. Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio Cholerae*: increases in the trans/cis ratio and proportions of cyclopropyl fatty acids. Applied and Environmental microbiology 52:794-801.

Guckert JB, Antworth CP, Nichols PD, D.C. W. 1985. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. FEMS Microbiology Ecology 31:147-158.

Helander I, Alakomi H, Latva KK, Mattila ST, Pol I, Smid E. 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. Journal of Agricultural and Food Chemistry 46:3590-3595.

Juneja VK, Davidson PM. 1993. Influence of altered fatty acid composition on resistance of *Listeria monocytogenes* to antimicrobials. Journal of Food Protection 56:302-305.

Keweloh H, Heipieper HJ. 1996. Trans unsaturated fatty acids in bacteria. Lipids 31:129-137.

Keweloh H, Diefenbach R, Rehm HJ. 1991. Increase of phenol tolerance of *Escherichia coli* by alterations of the fatty acid composition of the membrane lipids. Archives of Microbiology 157:49-53.

Kim JM, Marshall MR, Cornell JA, Preston JF, Wei CI. 1995. Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella typhimurium* in culture medium and on fish cubes. Journal of Food Science 60:1364-1368.

Kwon JA, Yu CB, Park HD. 2003. Bacteriocidal effects and inhibition of cell separation of cinnamic aldehyde on *Bacillus cereus*. Letters in Applied Microbiology:61-65.

Lis-Balchin M, Deans SG. 1997. Bioactivity of selected plant essential oils against *Listeria monocytogenes*. Journal of Applied Microbiology 82:759-762.

Loffeld B, Keweloh H. 1996. Cis/trans isomerization of unsaturated fatty acids as possible control mechanism of membrane fluidity in *Pseudomonas putida P8*. Lipids 31:811-815.

Mazzotta AS, Montville TJ. 1997. Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10 degree C and 30 degree C. Journal of Applied Microbiology 82:32-38.

Russell NJ, Evans RI, ter Steeg PF, Hellemons J, Verheul A, Abee T. 1995. Membranes as a target for stress adaptation. International Journal of Food Microbiology 28:255-261.

Seltmann G, Holst O. 2002. The bacterial cell wall. Berlin: Springer-Verlag.

Sikkema J, de Bont JAM, Poolman B. 1994. Interactions of cyclic hydrocarbons with biological membranes. Journal of Biological Chemistry 269:8022-8028.

Sikkema J, de Bont JAM, Poolman B. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews 59:201-222.

Ultee A, Gorris LG, Smid EJ. 1998. Bactericidal action of carvacrol towards the food-borne pathogen *Bacillus cereus*. Journal of Applied Bacteriology 85:211-218.

Ultee A, Kets EPW, Smid EJ. 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 65:4606-4610.

Ultee A, Bennik MHJ, Moezelaar R. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 68:1561-1568.

Ultee A, Kets EPW, Alberda M, Hoekstra FA, Smid EJ. 2000. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. Archives of Microbiology 174:233-238.

Valero M, Salmerón MC. 2003. Antibacterial activity of 11 essential oils against *Bacillus cereus* in tyndallized carrot broth. Journal of Food Microbiology 85:73-81.

Wendakoon CN, Sakaguchi M. 1995. Inhibition of amino acid decarboxylase activity of *Enterobacter aerogenes* by active components in spices. Journal of Food Protection 58:280-283.

White DC, Ringelberg DB. 1997. Utility of the signature lipid biomarker analysis in determining the in situ viable biomass, community structure, and nutritional/physiologic status of deep subsurface microbiota. In: P. S. Amy and D. L. Haldeman. The microbiology of the terrestrial deep subsurfarce. Boca Raton: CRC Lewis Publishers.

Yousef AE, Courtney PD. 2003. Basics of stress adaptation and implications in new-generation foods. In: A. E. Yousef and V. K. Juneja. Microbial stress adaptation and food safety. Boca Raton: CRC Press. 31-54.

PART 4. CHANGES IN FATTY ACID COMPOSITION ASSOCIATED WITH EXPOSURE AND ADAPTATION TO CARVACROL

Abstract

Carvacrol (CRV) is a component found in the essential oil fraction of oregano oil and is inhibitory to various pathogens. The objective of this study was to evaluate changes in bacterial membrane composition by observing changes in total phospholipid fatty acid (PLFA) and fatty acid composition associated with exposure and adaptation to CRV. *Salmonella* Typhimurium DT104 cells were grown for 24h at 35°C with or without 871 mg/L CRV in 9-ml ml brain heart infusion broth. Log 5.7 CFU/ml of *S.* Typhimurium was inoculated into 200ml broth containing 871 mg/L CRV. At 2h and 24h, samples were centrifuged, washed, lyophilized, and extracted for lipids. Fatty acid methyl esters prepared from phospholipids were quantified by GC-MS. Compared to the non-adapted *Salmonella* at 2h, the non-adapted treatment had lower ($p<0.05$) C16:1w7c, Cy17:0, C18:1w7c, and unsaturated fatty acid (UFA) and higher ($p<0.05$) C14:0, C18:1w9c, C18:0, saturated fatty acid (SFA), and SFA/UFA ratio. At 24h, the non-adapted treatment had lower ($p<0.05$) total phospholipid fatty acid (PLFA), lower ($p<0.05$) Cy17:0, C17:0, C18:1w7c, and UFA than the non-adapted control and higher ($p<0.05$) C16:1w7c and SFA. At 2h there were no significant differences between the fatty acid compositions of adapted *Salmonella* treated with CRV and non-adapted cells treated with CRV. At 24h, the adapted cells treated with CRV had lower ($p<0.05$) C16:1w7c and UFA and higher ($p<0.05$) SFA than non-adapted cells treated with CRV. The SFA/UFA ratio was numerically higher in the adapted *Salmonella* than the non-adapted

cells at 24h. Since the fatty acid composition and total PLFA of the non-adapted control and adapted controls were similar, the adaptive response had been reversed since the stressor, CRV, was not used to treat the adapted controls. It appears that CRV treatment at sub-lethal concentrations decreased the fluidity of the *Salmonella* membrane by increasing SFA. This decreased fluidity may prevent additional CRV from permeating. The effect of bacterial growth in the presence of CRV on the fatty acid composition was much smaller. Measurement of the membrane transition temperature from gel-to-liquid-crystalline phase would indicate fluidity. Investigation about the target site that CRV acts upon and the interactions of CRV with membrane lipids and proteins would contribute to the understanding of its mechanism of action and the bacterial response.

Introduction

Consumers desire high-quality, minimally processed foods that have fewer preservatives and an extended shelf-life (Brul and Coote 1999). Studies have reported oregano essential oil to be highly active against various microorganisms (Lis-Balchin and Deans 1997; Hammer and others 1999 ; Friedman and others 2002; Valero and Salmerón 2003). Carvacrol (CRV), the bioactive component found in oregano and thyme essential oils, is used in baked goods, nonalcoholic beverages, and chewing gum (Ultee and others 1999). In-vitro studies have reported the antimicrobial activity of CRV against *E. coli* O157:H7, *S*. enterica Hadar, *S.* Typhimurium, and *L. monocytogenes* were (Kim and others 1995a;

Helander and others 1998; Friedman and others 2002). It has been tested in fish cubes (Kim and others 1995b) and rice (Ultee and others 2000a).

Concentrations that are lethal to bacteria are much higher than tolerable taste thresholds. Therefore, it is preferable to use them at inhibitory concentrations rather than at lethal concentrations (Brul and Coote 1999). Knowledge about the mechanisms of action of antimicrobials is needed to develop effective and economical combinations of antimicrobial additives and formulations to protect foods from spoilage and pathogenic microorganisms without sacrificing organoleptic qualities.

Compounds from essential oils are believed to disrupt the membrane (Brul and Coote 1999), however, there are diverse chemical families representing essential oil components. Lipophilic compounds, such as essential oil components, have been shown to accumulate and act on the cell membrane (Sikkema and others 1994, 1995). As they interact with the phospholipid bilayer of the cell membrane, they may increase membrane permeability and cause cellular constituents to leak (Sikkema and others 1994; Ultee and others 1998).

Although a several studies have investigated the efficacy of CRV, only a few have examined its mechanism of inhibition. It was suggested that CRV disrupts the outer membrane because it decreases the intracellular ATP and increases extracellular ATP in *E. coli* O157:H7 (Helander and others 1998). Due to its hydroxyl group, it caused potassium ions to leak, reduced intracellular pH, dissipated the membrane potential, and depleted the intracellular ATP of *B.*

cereus cells, which would result in lethality (Ultee and others 1999; Ultee and others 2002).

When a microorganism is exposed to sublethal doses of a chemical that negatively influences bacterial growth or survival, it may respond to protect itself, or adapt, to increase its tolerance. A lipid can act as a sensor of stress and cause a change the synthesis of stress-related proteins, which alter cell physiology to increase tolerance to stress. A change in fluidity or membrane structure can be detected and the cell may respond with alterations in membrane composition (Yousef and Courtney 2003).

Changes in bacterial lipids in the membrane are a defense mechanism used to maintain optimal fluidity in the liquid-crystalline phase for important cell processes. Membrane fluidity is the "combined expression of the effects of [fatty acid] chain conformation, lateral and rotational diffusion [of the lipids within the membrane] and the resistance of the membrane to sheer forces" (Denich and others 2003). The fluidity of the membrane affects the growth of the cell and the proper functioning and structural integrity of the membrane (Denich and others 2003). For example, the percentage of unsaturated fatty acids in *E. coli* phospholipids is usually about 50%. This percentage can decrease to 30% without ceasing bacterial growth. If the percentage of saturated fatty acids decreases to less than 15% the membrane permeability increases (Seltmann and Holst 2002). Lipids directly affect passive permeability and indirectly influence cell processes because of their interactions with membrane proteins (Russell and others 1995).

Membrane fluidity is determined by the effect of changes in the fatty acid and phospholipid head-group composition, which modify the lipid membrane melting point (Keweloh and Heipieper 1996). As melting point decreases, fluidity increases (Casadei and others 2002). For example, fatty acids with high melting points, such as saturated, long, and straight-chain fatty acids, decrease membrane fluidity (Juneja and Davidson 1993).

Since CRV is lipophilic it accumulated and expanded liposomal membranes. This swelling was suggested to cause a destabilized membrane in *B. cereus*, which increased fluidity as shown by a decrease in the membrane transition temperature from gel to liquid-crystalline phase (T_m). This led to increased passive permeability and therefore a leakage of ions. Adaptation to CRV of *B. cereus* cells caused an increase in T_m and fatty acid acids with higher melting points, which resulted in decreased sensitivity. This reduction in fluidity was an adaptive response to the compensate for the fluidizing and permeabilizing effect of CRV (Ultee and others 1999; Ultee and others 2000b).

The objectives of this study were to determine:

1) the effect of exposure to CIN on the fatty acid composition of the bacterial membrane of *Salmonella*,

2) whether changes in the fatty acid composition of the bacterial membrane of *Salmonella* occur due to growth in the presence of CIN as a possible adaptive response to CIN, and

3) whether reversal of these changes in fatty acid composition occur when the stressor, CIN, was removed.

Materials and methods

Essential oil component

Stock solutions of CRV (Aldrich, Milwaukee, WI) were prepared in ethanol, absolute (Aldrich, St. Louis, MO). Sterile water dissolved into ethanol served as the control stock solution. Aliquots of CRV stock solution were added into 8 ml brain heart infusion broth, modified (BHI, BBL, Sparks MD) with 2% Tween 20 (Fisher Scientific, Fairlawn, NJ) with glass beads. The final concentration of Tween was 1% and ethanol was 0.5% during adaptation and treatment. Tween and glass beads were used to aid with dispersion of CRV in BHI. All BHI was prepared with phosphate buffer (0.1M, pH 7.2 $\pm$ 0.1) instead of water to prevent pH changes, which could affect the fatty acid composition of bacterial membranes. The pH of BHI was adjusted to 7.35 ($\pm$ 0.1) prior to autoclaving, after which the final pH was pH 7.2 ($\pm$ 0.1). Glass tubes containing the stock and BHI were shaken vigorously forty times to disperse the oil-based components. Dispersions became cloudy-white.

Bacterial culture preparation

Salmonella Typhimurium DT104 ATCC BAA-186 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Experimental cultures were maintained on Tryptic Soy Agar slants. A loopful of culture was used to inoculate 9 ml of BHI with *S.* Typhimurium. The BHI tube was incubated for 24 h at 35℃. Then a loopful was transferred into 9 ml BHI and incubated for 21 h at 35℃.

Adaptation

In our study adaptation was defined as growth in sublethal concentrations of CRV for 24h. On the day of adapting cells to antimicrobials, one ml of the 21-h culture was serially diluted into two sets of three tubes of 9 ml BHI. The diluted cultures from the two tubes of the 10^{-3} dilution were combined in a large tube. Five ml of the diluted culture were used to inoculate a 5-ml BHI tube containing CRV and a 5-ml BHI tube containing 0.5% ethanol control. Both tubes had 1% Tween.

The stock and 5-ml CRV dispersions in BHI were prepared the day before, refrigerated, and allowed to warm to room temperature for 30 min before the 5-ml diluted culture was added. This inoculation halved the concentrations and the final inoculum was approximately log 5.7 CFU/ml. The final concentration of Tween was 1% and ethanol was 0.5%. The two inoculated BHI tubes containing CRV or ethanol control were vortexed and the cells were allowed to adapt to CRV or ethanol (non-adapted) by incubating in a shaking waterbath at 125 rpm for 24 h at 35°C.

Extrapolating from dose-response curves, the concentrations that would achieve a 25% probability of lethality were initially used to adapt cells. This low probability of lethality was chosen to induce changes in the membrane lipid composition yet also allow enough growth for harvesting cells for lipid analysis. This 25% probability of lethality corresponded to 1023 mg/L CRV. After a 24-hour growth in or adaptation to 1023 mg/L CRV, adapted cells would be inoculated into 200 ml BHI in bottles to treat adapted cells to sublethal

concentrations of CRV for 2 and 24 hours. After inoculation, the desired inoculum in these experiments was about log 5.7 CFU/ml to adhere to the same inoculum size as in the adaptation procedure.

The population of cells resulting after 24-h adaptation was tested since these cells would be used to inoculate the bottles. The population of cells exposed to CRV was log 5 CFU/ml. To inoculate several bottles to a final inoculum of log 5.7 CFU/ml with a log 5 CFU/ml culture would be impossible. Then cells were exposed to CRV concentrations corresponding to 15%, 10%, and 5%, after which the cell count was about log 6 CFU/ml. To inoculate bottles with 200 ml BHI would have required a very large inoculum volume of "adapted" cells. There was concern that these were new non-adapted cells that survived and were able to grow under stressed conditions with no physiological changes. Finally after three repetitions, the count of cells exposed 871 mg/L CRV, which corresponded to 2.5% probability of lethality, was log 8.1 CFU/ml. The control population "adapted" to 0.5% ethanol was log 8.8 CFU/ml (average).

Treatment of adapted cells

A total of twelve screw-capped glass bottles containing 190 ml BHI were prepared for two and twenty-four hour exposures to CRV. Ten ml CRV dispersion were added to six bottles for a final concentration of 871 mg/L. *Salmonella* adapted to CRV would be added to two bottles. *Salmonella* "adapted" only to ethanol would be added to two bottles. Two bottles would not be inoculated and were designated as sterile CRV controls since the BHI broth media contained fatty acids. Ten ml of ethanol solution were added to six bottles

(with no CRV). *Salmonella* adapted to CRV would be added to two bottles. *Salmonella* "adapted" only to ethanol would be added to two bottles. Two bottles were not to be inoculated and were designated as sterile ethanol controls since the BHI broth media contained fatty acids from the manufacturer formulation. The final concentrations would contain about 1% Tween and 0.5% ethanol, similar to those used to adapt cells. These twelve bottles containing CRV or ethanol were shaken and stored at 4°C until two hours before use, at which time they were allowed to warm to 25°C. Before adding adapted *Salmonella*, bottles were shaken. The treatments for 2 and 24h incubation were: Sterile controls: sterile + CRV, sterile + ethanol, treatments: non-adapted *Salmonella* + CRV, adapted *Salmonella* + CRV, inoculated controls: non-adapted + ethanol, adapted + ethanol.

Tubes containing adapted cells were vortexed to inoculating *Salmonella* into the designated bottles. Based on the preliminary data of adapted cell counts, 872 µl of cells adapted to CRV and 166 µl of cells exposed to ethanol (non-adapted) were added to designated bottles for a final inoculum size of about log 5.7 CFU/ml. The bottles were shaken and incubated in a shaking waterbath at 125 rpm for either two or twenty-four hours. After incubation the samples were centrifuged on a large volume centrifuge (Beckman J2-HS, Schaumberg, IL) at 12,000 rpm for 30 minutes at 4°C. The pellet was transferred from the centrifuge bottles to centrifuge tubes and washed three times with 0.1 M phosphate buffer (pH 7.2) at 10,000 rpm for 10 minutes at 4°C using a table-top centrifuge (Baxter Biofuge 17 R, VWR Scientific Products, West Chester, PA). The pellet was

suspended in less than 1.5 ml phosphate buffer in small ampoules, wrapped in parafilm and foil and frozen at -30°C. The pellet was lyophilized and ampoules were wrapped until extraction.

Lipid extraction

Lipid extraction and analysis procedures were developed by Dr. David C. White's lab, the University of TN Center for Biomarker Analysis. Lipids from 8-18 mg lyophilized cells were extracted using a modified Bligh/Dyer method (Guckert and others 1985; Guckert and others 1986). The extraction solvent contained 4 ml phosphate buffer, 10 ml methanol, and 5 ml chloroform. After three hours the phases were split with 5 ml water and 5 ml chloroform and allowed to sit overnight for layer separation. The lower chloroform layer was removed and dried under nitrogen. The total lipid extract was suspended in chloroform and separated into the lipid classes (neutral lipids, glycolipids, and polar lipids) with silicic acid (500 mg) columns using three solvents of increasing polarity (5 ml chloroform, 5 ml acetone, and 10 ml methanol) to elute the lipid classes from the silicic acid stationary phase (Guckert and others 1985; Guckert and others 1986).

Analysis of fatty acid methyl esters

To prepare fatty acid methyl esters (FAME) in a mild alkaline methanolic transesterification reaction, 0.5 ml chloroform, 0.5 ml methanol, and 1 ml of methanolic KOH were added to the dried polar lipids and heated at 60°C for 30 minutes. To extract the FAME, 2 ml hexanes, 200 µl glacial acetic acid, and 2 ml nanopure water was added. After centrifuging, the hexane layer was removed.

This was repeated twice. The FAME were dried under nitrogen and transferred three times with 100 µl hexanes to autosampling vials.

The samples were diluted with hexanes containing a 21:0 internal standard (50pmol/µl). The samples and a vial of GC-MIX (mixture of 20 FAME standards) were analyzed on a HP6890 gas chromatograph (GC) coupled to a HP5973 mass spectrophotometer (MS). The GC was equipped with a RJX1 fused silica capillary column (60m, 250µm internal diameter x 25 µm film thickness, Restek, Bellefonte, PA). The column was programmed from an initial temperature of 60℃ for 2 min then raised at a rate of 10℃/min to 150℃. The temperature was increased at 30℃/min to a final temperature of 312℃. The injector port temperature was 230℃ in split-less mode. Helium was used as the carrier gas at a flow rate of 1.41 ml/min. Fatty acids were identified by comparison of retention times to the FAME standards and a mass spectral data library. Fatty acids were quantified based on comparison to the 21:0 internal standard and area under each sample peak and standard peak.

Statistical analysis

Box and whisker plots were generated with Statistica (StatsSoft, Inc., Tulsa, OK) to compare total PLFA and fatty acid composition. The 2 and 24h data were divided and a randomized block design with blocking on each of three replications was used to test the effect of treatments or controls on total PLFA and fatty acids for 2h or 24h. Data were analyzed using SAS (Statistical Analysis System, Cary, NC) to compare differences in total PLFA and fatty acids between treatment and control groups within 2h or 24h with alpha value at 0.05. The

generalized linear model (GLM) was used to test for differences in total PLFA and fatty acids between 2 and 24h within each treatment or control group with a Bonferonni correction factor. This correction minimized Type I error, which increases with the number of statistical tests. The Bonferonni p value was calculated as the alpha value at 0.10 divided by 6 (the number of follow-up tests conducted to test the interaction between the 6 treatments at 2h or 24h), which equals 0.017.

Results

Total phospholipid fatty acid (PLFA)

Total phospholipid fatty acid (PLFA) is the sum of all fatty acids that were hydrolyzed from phospholipids in the membrane. The mean total PLFA is shown in Table 4.1. Depicted in Figures 4.1 and 4.2 are box and whisker plots representing the mean, standard error, and standard deviation of the total PLFA of the sterile groups of *Salmonella* under each of the experimental treatments. The boundaries of the box around the mean consist of the mean $\pm$ standard error. The standard error is the standard deviation of the sample mean and is computed as the standard deviation divided by the square root of the sample size. The horizontal bars represent the mean $\pm$ standard deviation. The standard deviation is the positive square root of the variance. The variance is the

Table 4.1. Comparison of total PLFA and fatty acid (FA) composition of CRV-adapted or non-adapted cells with or without carvacrol (CRV) to inoculated controls (+EtOH) and sterile controls after 2h and 24h incubation.

Treatment/control and incubation hr	Total PLFA (pmol/mg)	C12:0 (%)	C14:0 (%)	C15:0 (%)	C16:1w7c (%)	C16:0 (%)	Cy17:0 (%)	C17:0 (%)	C18:1w9c (%)
2h									
Sterile + CRV	1987.2	34.9^{A}	29.6^{A}	0.0	0.0^{B}	19.1^{C}	0.0^{B}	0.0	2.3^{AB}
Sterile + EtOH	191.9	35.4^{A}	29.0^{A}	0.0	0.0^{B}	21.6^{BC}	0.0^{B}	0.0	1.2^{B}
Non-adapt + CRV	729.3^{Z}	15.0^{BC}	27.3^{A}	0.0	0.3^{BZ}	28.1^{AB}	0.1^{BZ}	0.0	4.3^{A}
Adapt + CRV	7333.2	31.9^{AB}	28.8^{AZ}	0.0	1.7^{BZ}	20.9^{BCZ}	0.0^{B}	0.0	2.2^{AB}
Non-adapt + EtOH	7625.3^{Z}	0.8^{C}	7.8^{B}	0.0^{Z}	25.7^{AZ}	29.5^{AZ}	2.3^{AZ}	0.0^{Z}	0.5^{B}
Adapt + EtOH	2799.4^{Z}	2.2^{C}	8.1^{B}	0.0^{Z}	23.8^{AZ}	31.9^{A}	2.4^{AZ}	0.0^{Z}	0.4^{B}
24h									
Sterile + CRV	2221.6^{B}	36.1^{A}	26.7^{A}	0.0^{B}	0.1^{D}	16.6^{B}	0.0^{C}	0.0^{C}	5.7^{A}
Sterile + EtOH	2396.1^{B}	23.7^{B}	31.7^{A}	0.0^{B}	0.0^{D}	21.7^{B}	0.0^{C}	0.0^{C}	3.9^{A}
Non-adapt + CRV	19541.1^{B}	3.5^{CD}	6.6^{B}	0.1^{B}	22.9^{A}	42.1^{A}	4.0^{BC}	0.0^{C}	0.2^{B}
Adapt + CRV	8524.0^{B}	7.9^{C}	9.2^{B}	0.0^{B}	16.6^{B}	41.3^{A}	4.3^{B}	0.0^{C}	0.3^{B}
Non-adapt + EtOH	69791.6^{A}	0.3^{D}	7.7^{B}	0.2^{A}	13.7^{C}	36.7^{A}	16.0^{A}	0.1^{B}	0.3^{B}
Adapt + EtOH	70051.4^{A}	1.0^{CD}	7.4^{B}	0.2^{A}	11.9^{C}	36.6^{A}	16.6^{A}	0.2^{A}	0.2^{B}

ABCD: Means in a column with different superscripts within 2h or 24h are different ($p<0.05$).
Z: Means in a column are different from 2h to 24h within a treatment or control group (Bonferroni $p<0.017$).

Table 4.1. Continued.

Treatment/control and incubation hr	C18:1w7c (%)	C18:1w7t (%)	C18:0 (%)	Cy19:0 (%)	Sat. FA (%)	Unsat. FA (%)	Cy17 to C16:1 ratio	Cy19 to C18:1 ratio	SFA to UFA ratio
2h									
Sterile + CRV	1.1 ^{B}	0.4	12.6 ^{A}	0.0	96.2 ^{AB}	3.8 ^{BC}	0.0	0.0	20.8 ^{AB}
Sterile + EtOH	0.9 ^{B}	0.2	11.7 ^{AB}	0.0	97.7 ^{A}	2.3 ^{C}	0.0	0.0	30.0 ^{A}
Non-adapt + CRV	4.1 ^{BZ}	0.5	20.2 ^{A}	0.0	90.6 ^{BZ}	9.4 ^{B}	0.4	0.0	12.6 ^{B}
Adapt + CRV	3.6 ^{BZ}	0.7	10.2 ^{ABC}	0.0	91.8 ^{ABZ}	8.2 ^{BCZ}	0.0	0.0	11.9 ^{BZ}
Non-adapt + EtOH	32.6 ^{AZ}	0.0	0.9 ^{C}	0.0 ^{Z}	38.9 ^{CZ}	61.1 ^{AZ}	0.1 ^{Z}	0.0 ^{Z}	0.6 ^{CZ}
Adapt + EtOH	30.0 ^{A}	0.0	1.2 ^{BC}	0.0 ^{Z}	43.4 ^{C}	56.6 ^{AZ}	0.1 ^{Z}	0.0	0.8 ^{C}
24h									
Sterile + CRV	2.6 ^{C}	0.6 ^{AB}	11.6 ^{B}	0.0 ^{B}	90.9 ^{A}	9.1 ^{D}	0.0 ^{B}	0.0 ^{B}	10.2 ^{B}
Sterile + EtOH	1.5 ^{C}	0.7 ^{A}	16.7 ^{A}	0.0 ^{B}	93.8 ^{A}	6.2 ^{D}	0.0 ^{B}	0.0 ^{B}	15.4 ^{A}
Non-adapt + CRV	19.4 ^{B}	0.0 ^{AB}	1.1 ^{C}	0.1 ^{B}	53.5 ^{C}	46.5 ^{B}	0.2 ^{B}	0.0 ^{B}	1.2 ^{C}
Adapt + CRV	17.3 ^{B}	0.0 ^{B}	2.9 ^{C}	0.1 ^{B}	61.3 ^{B}	38.7 ^{C}	0.3 ^{B}	0.0 ^{B}	1.6 ^{C}
Non-adapt + EtOH	23.2 ^{A}	0.0 ^{B}	0.5 ^{C}	1.2 ^{A}	45.6 ^{D}	54.4 ^{A}	1.2 ^{A}	0.1 ^{A}	0.8 ^{C}
Adapt + EtOH	23.6 ^{A}	0.0 ^{B}	0.6 ^{C}	1.7 ^{A}	46.0 ^{D}	54.0 ^{A}	1.4 ^{A}	0.1 ^{A}	0.9 ^{C}

ABCD: Means in a column with different superscripts within 2h or 24h are different ($p<0.05$).

Z: Means in a column are different from 2h to 24h within a treatment or control group (Bonferroni $p<0.017$).

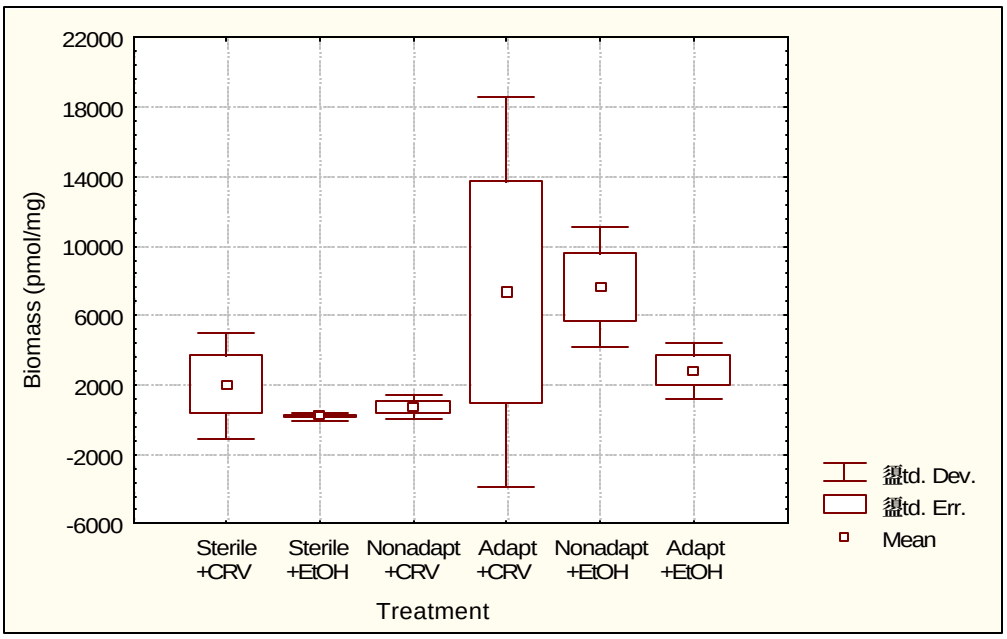


Figure 4.1. Comparison of total PLFA or biomass of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

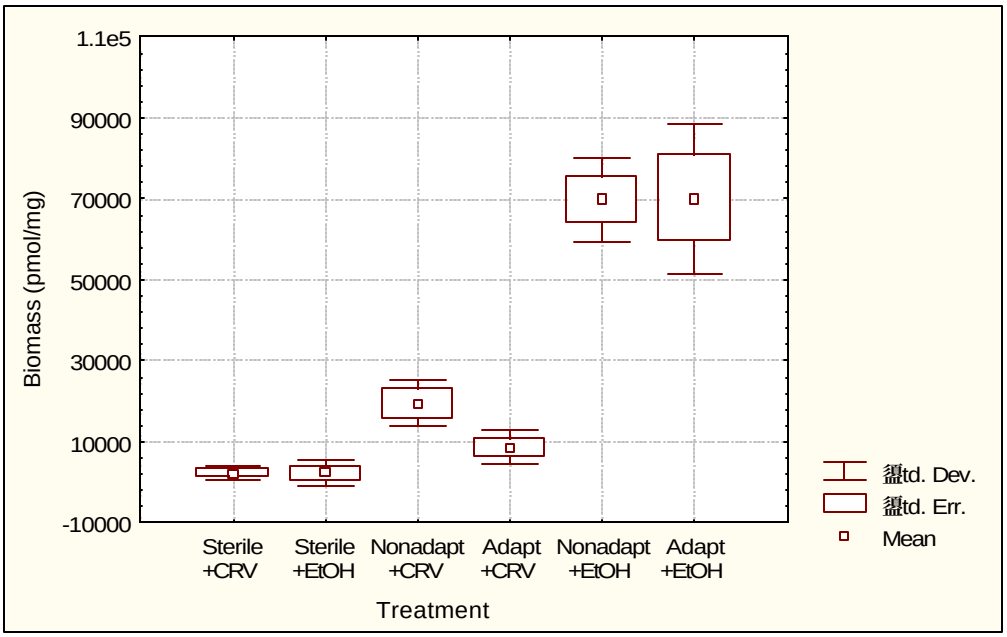


Figure 4.2. Comparison of total PLFA or biomass of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

sum of the squared deviations from the mean divided by the sample size minus one.

An analysis was performed to determine if there was a correlation between total PLFA and occurrence of specific fatty acids. It was found that higher total PLFA was positively correlated to the presence of C15:0 (R^2=0.81), Cy17:0 (R^2=0.91), C17:0 (R^2=0.78), Cy19:0 (R^2=0.85), ratio of Cy17:0 to C16:1w7c (R^2=0.82), and the ratio of Cy19:0 to C18:1w7c (R^2=0.83). The correlation of total PLFA to cyclopropyl fatty acids was expected because as cells age and enter stationary phase, the cyclopropyl fatty acids increase (Guckert and White 1988; Casadei and others 2002).

Comparison of total PLFA and each fatty acid across sterile, treatment, and control groups (refer to Table 4.1 for means).

Total PLFA. Mean total PLFA of treatment groups at 2h is shown in Table 4.1. There were no significant ($p>0.05$) differences among the total PLFA of all groups at 2h (Figure 4.1). The total PLFA found in uninoculated controls was due to the fatty acids present in the culture medium from the manufacturer's formulation of BHI. There was a wide range in total PLFA in the adapted and non-adapted treatment groups. This range may be due to the variation in sensitivity of the cells to CRV. The total PLFA of the adapted control (+EtOH) was numerically lower than the non-adapted control because it may have still been inhibited but the difference was not significant. The total PLFA of the CRV non-adapted *Salmonella* was significantly ($p<0.05$) less than CRV adapted *Salmonella* after 2h indicating a more rapid growth of adapted cells.

At 24h the total PLFA of the *Salmonella* inoculated control groups (no CIN) exceeded those of the sterile and treatment groups because the bacteria were not inhibited by CRV (Figure 4.2). The total PLFA did not differ significantly ($p>0.05$) among sterile and treatment groups. From 2h to 24h the total PLFA increased significantly ($p<0.01$) for the controls and the non-adapted treatment group (+CRV) (Table 4.1).

C12:0 fatty acid. At 2h, the sterile groups contained more C12:0 than the control groups (Figure 4.3). The bacteria in the controls were able to decrease the proportion of this fatty acid within 2h as they grew and used C12:0. The level of C12:0 in the treatment groups was similar to that of the sterile groups. Since bacterial growth was still inhibited by CRV, lipid biosynthesis by the anaerobic pathway was decelerated. They incorporated C12:0 from the medium but could not oxidize and convert it to other fatty in the presence of CRV.

The wider standard error of the non-adapted cells treated with CRV implies that some *Salmonella* cells in a culture were less able to decrease the proportion 12:0 than others because they were more inhibited due to CRV exposure. This wider range of response indicates that a population of non-adapted cells can display a range of sensitivity to CRV and therefore a range in the ability to grow and decrease the percentage of C12:0.

At 24h, all four inoculated groups had significantly ($p<0.05$) less C12:0 than the sterile groups (Figure 4.4). The bacteria in the treatment groups were able to grow and convert 12:0 to another fatty acid. The adapted treatment had a higher percentage of C12:0 than the non-adapted control. The percentage C12:0

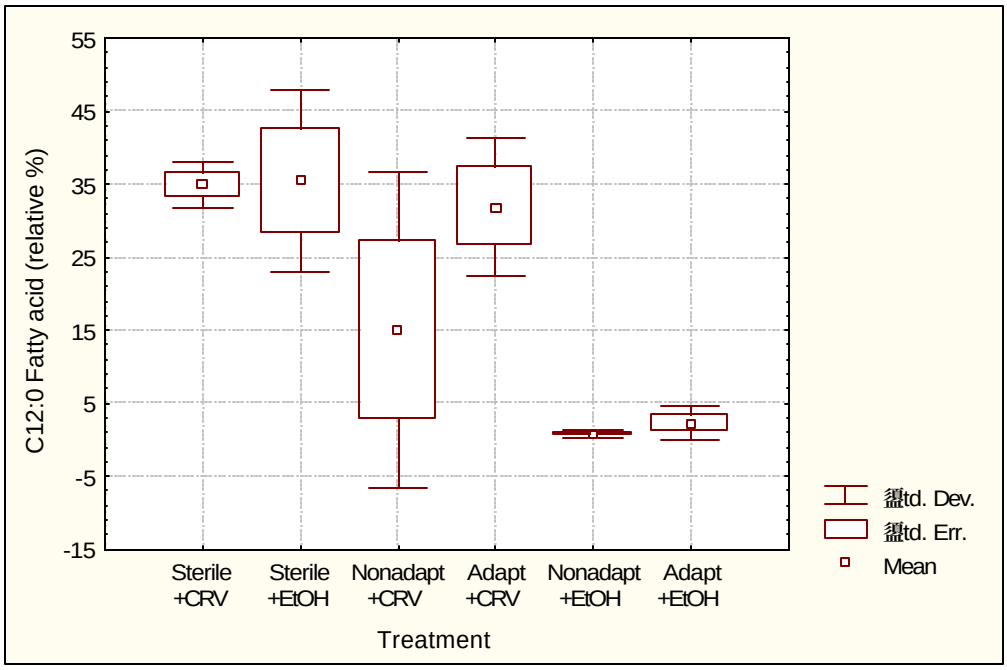


Figure 4.3. Comparison of C12:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

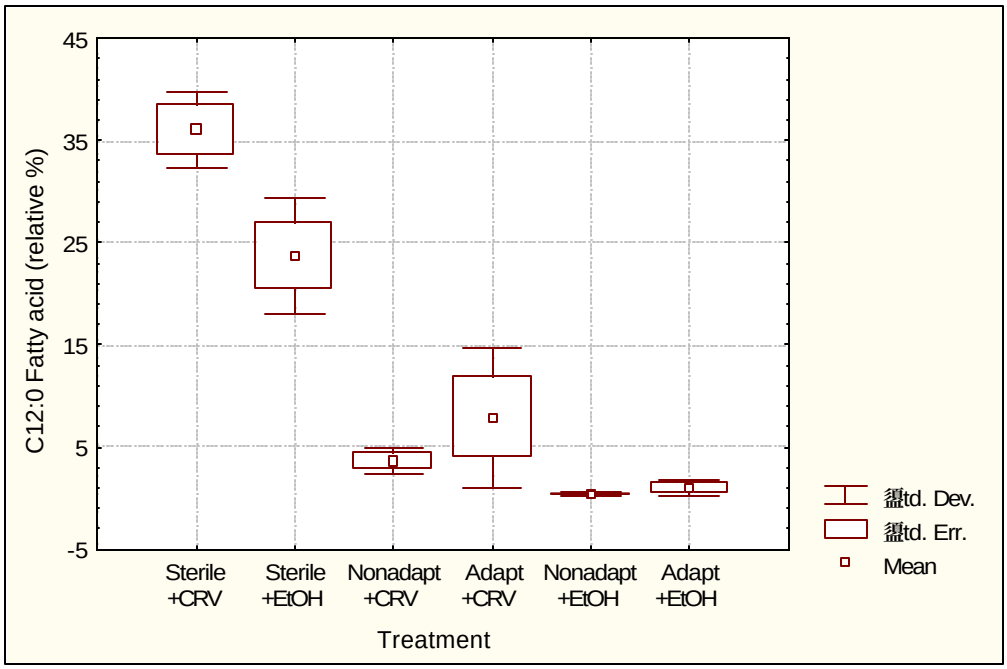


Figure 4.4. Comparison of C12:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

did not significantly ($p>0.05$) change in any group from 2 to 24h, except for a decrease in the adapted treatment group, but this was not significant ($p>0.05$) and may have been obscured by variation within the group.

C14:0 fatty acid. At 2h, the proportion of this fatty acid in the treatment groups did not differ from the sterile groups. They incorporated C12:0 from the medium. Bacterial growth in the treatment groups was still inhibited and could not convert C14:0 to another fatty acid as well as the inoculated controls (Figure 4.5). C14:0 was higher in these groups than in the controls, which had a significantly ($p<0.05$) lower percentage of C14:0 as they grew within 2h.

From 2h to 24h, C14:0 decreased significantly ($p<0.01$) in the adapted treatment group as cells grew (Table 4.1). The non-adapted treatment group showed a similar trend, but this was not significant ($p>0.05$). At 24h there were no differences among the inoculated groups and they had a significantly ($p<0.05$) lower C14:0 percentage than the sterile groups (Figure 4.6). Bacteria in the treatments grew and converted C14:0 to another fatty acid.

C15:0 fatty acid. At 2h C15:0 was not detected in any group (Figure 4.7). The control groups significantly ($p<0.01$) increased C15:0 from 2h to 24h (Table 4.1). They had a higher relative abundance of C15:0 than the other groups at 24h (Figure 4.8).

C16:1w7c fatty acid. At 2h C16:1w7c was at a higher percentage in the control groups than in the other groups (Figure 4.9). Compared to 2h, C16:1w7c decreased significantly ($p<0.005$) in the control groups at 24h (Table 4.1 and Figure 4.10). In contrast, the treatment groups significantly ($p<0.005$) produced

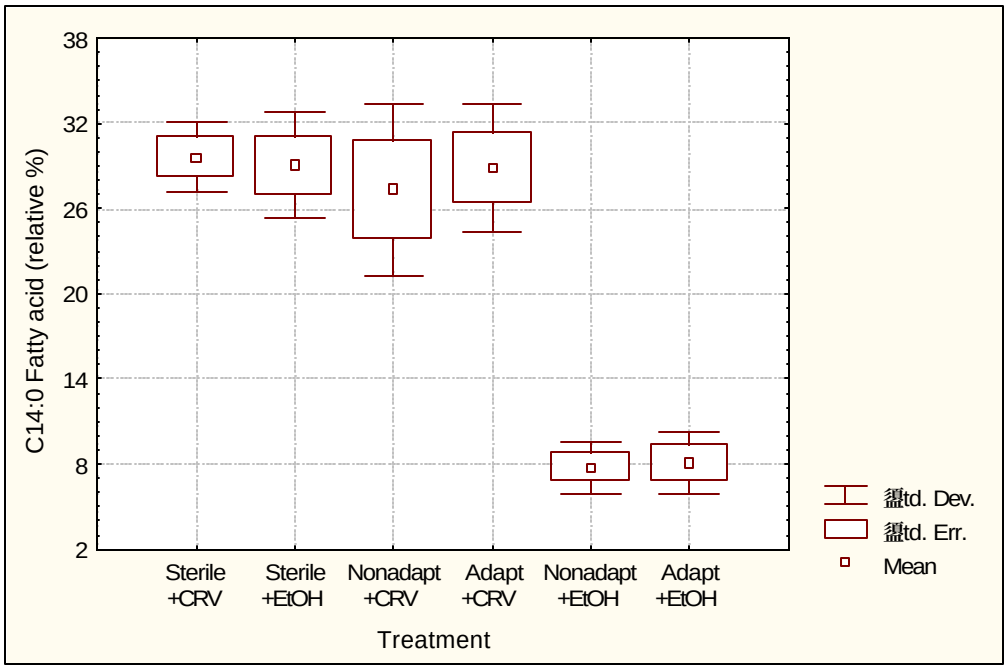


Figure 4.5. Comparison of C14:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

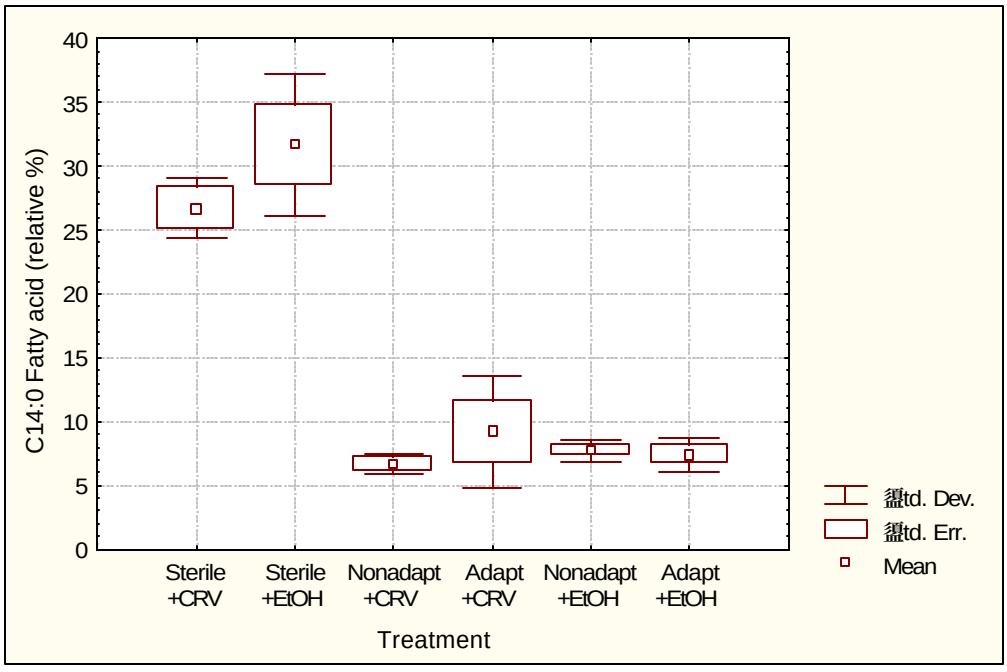


Figure 4.6. Comparison of C14:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

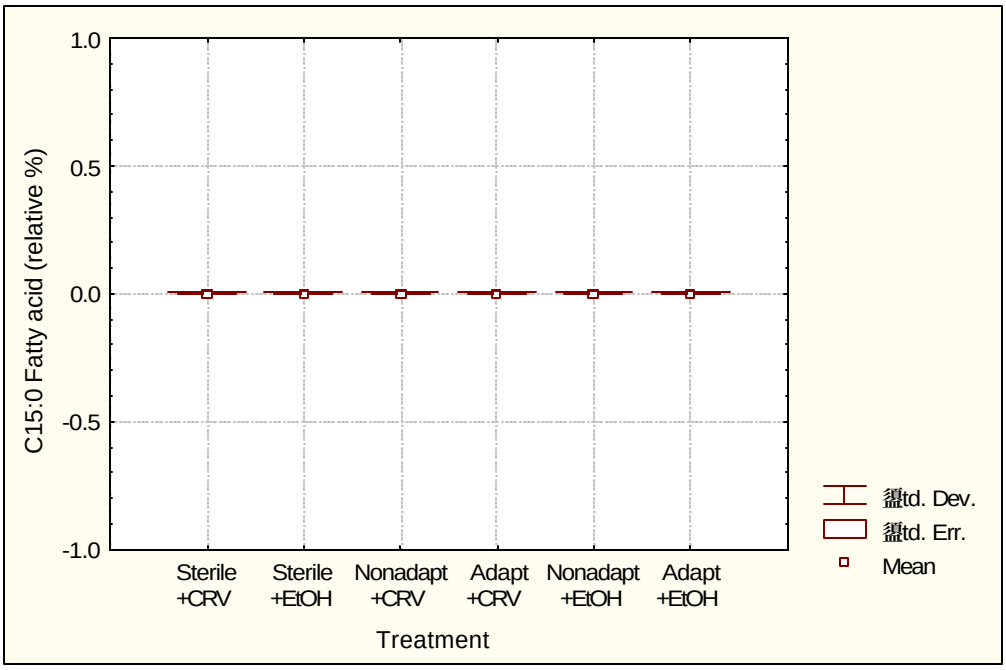


Figure 4.7. Comparison of C15:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

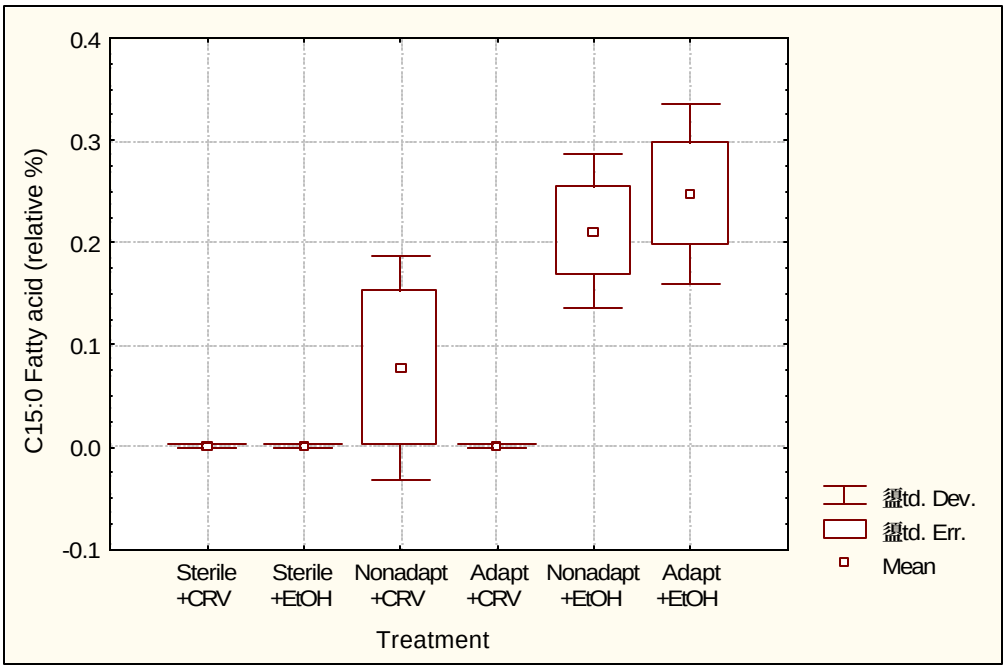


Figure 4.8. Comparison of C15:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

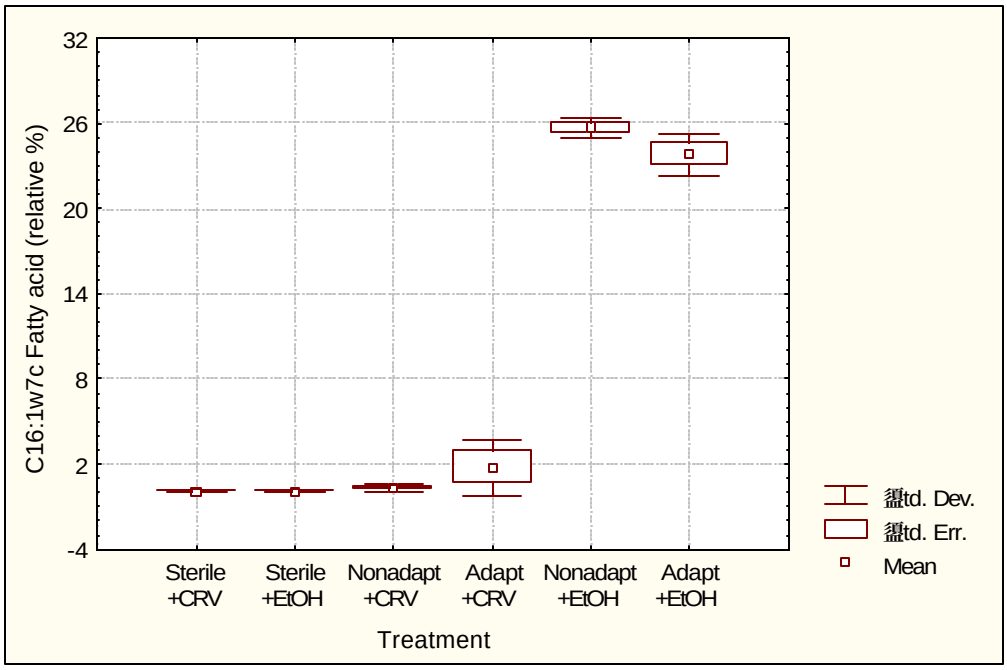


Figure 4.9. Comparison of C16:1w7c fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

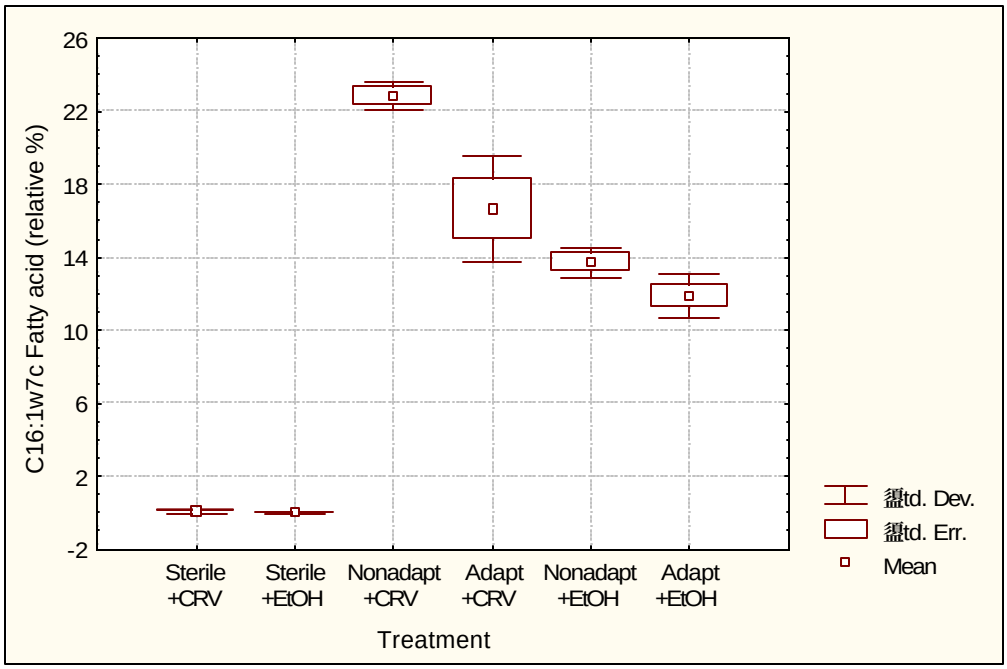


Figure 4.10. Comparison of C16:1w7c fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

more of this fatty acid with time. At 24h, the non-adapted treatment group produced the most C16:1w7c, followed by the adapted treatment group (Figure 4.10). The percentage was lower in the controls and least in the sterile groups.

C16:0 fatty acid. At 2h C16:0 was highest in the control groups as they grew (Figure 4.11). The percentage of fatty acid in the treatment groups was in between that of the control and sterile groups since they were still inhibited. From 2h to 24h, the non-adapted control and the adapted treatment group produced significantly ($p<0.02$ and $p<0.001$) more C16:0 (Table 4.1). In the non-adapted control there was a trend of increased C16:0 from over time, but this was not ($p>0.05$). At 24h C16:0 was more abundant in the inoculated groups than the sterile groups. The abundance of this fatty acid did not differ among inoculated groups (Figure 4.12).

Cy17:0 fatty acid. At 2h Cy17:0 was highest in the control groups (Figure 4.13). There were no differences among the sterile and treatment groups. Over time Cy17:0 significantly increased in the control groups and the non-adapted treatment group ($p<0.02$ and $p<0.001$) (Table 4.1). At 24h Cy17:0 was still the most relatively abundant in the control groups since they reached stationary phase earlier and second highest in the adapted treatment group (Figure 4.14). The abundance of Cy17:0 of the non-adapted treatment did not differ significantly ($p>0.05$) from its adapted counterpart.

C17:0 fatty acid. At 2h none of the groups synthesized C17:0 (Figure 4.15). Compared to 2h, the controls produced more ($p<0.005$) C17:0 at 24h (Table 4.1). At 24h (Figure 4.16) the percentage of C17:0 was greatest in the

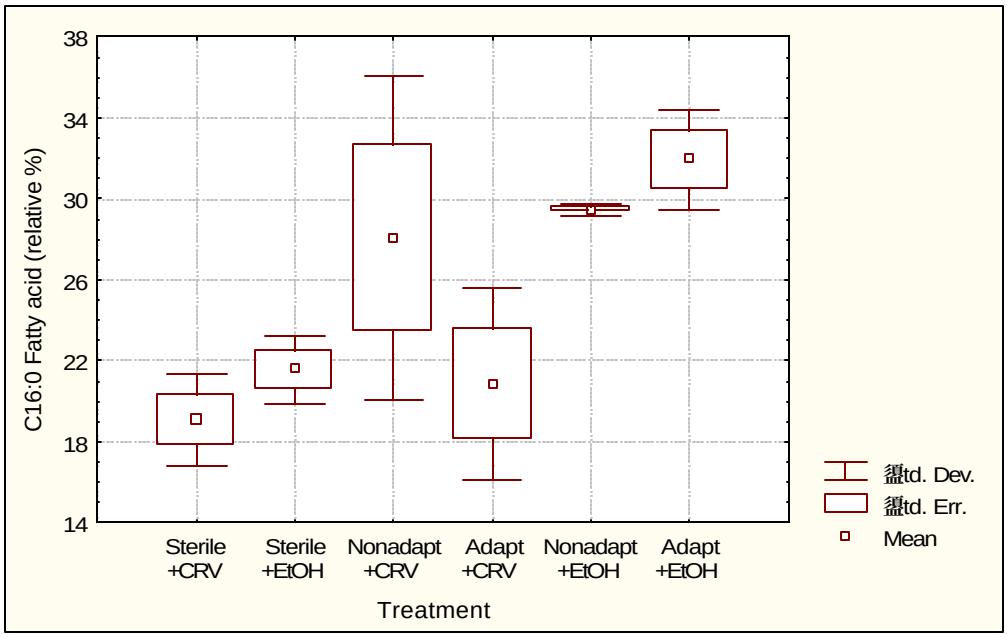


Figure 4.11. Comparison of C16:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

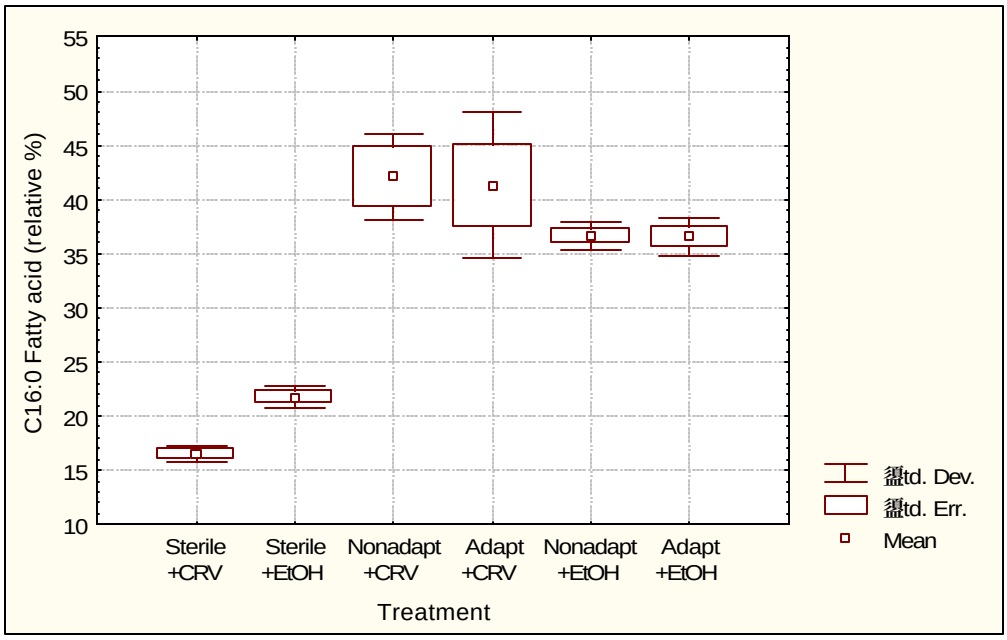


Figure 4.12. Comparison of C16:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

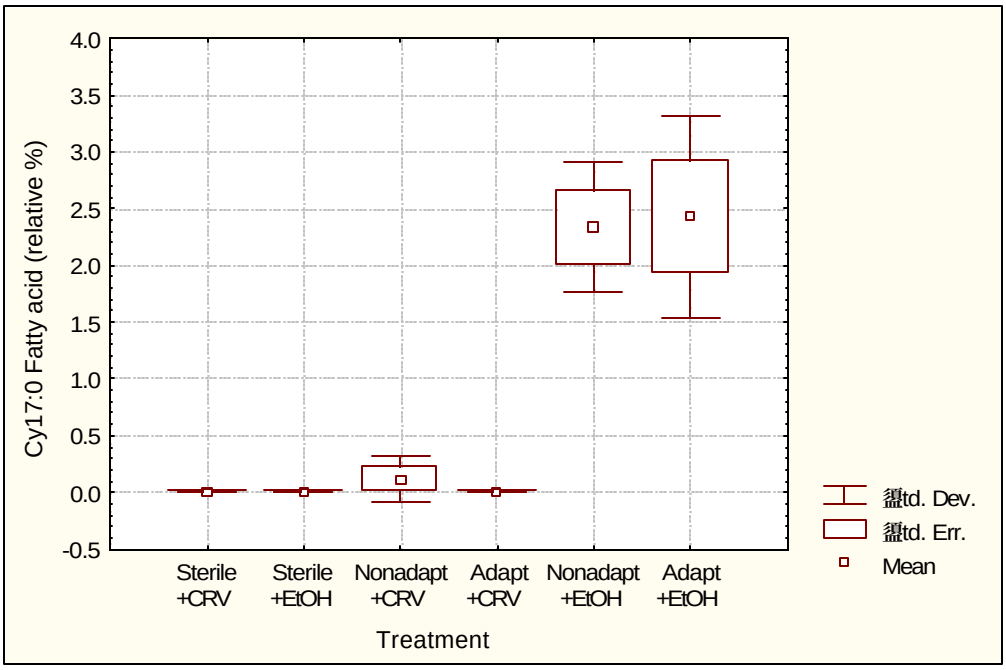


Figure 4.13. Comparison of Cy17:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

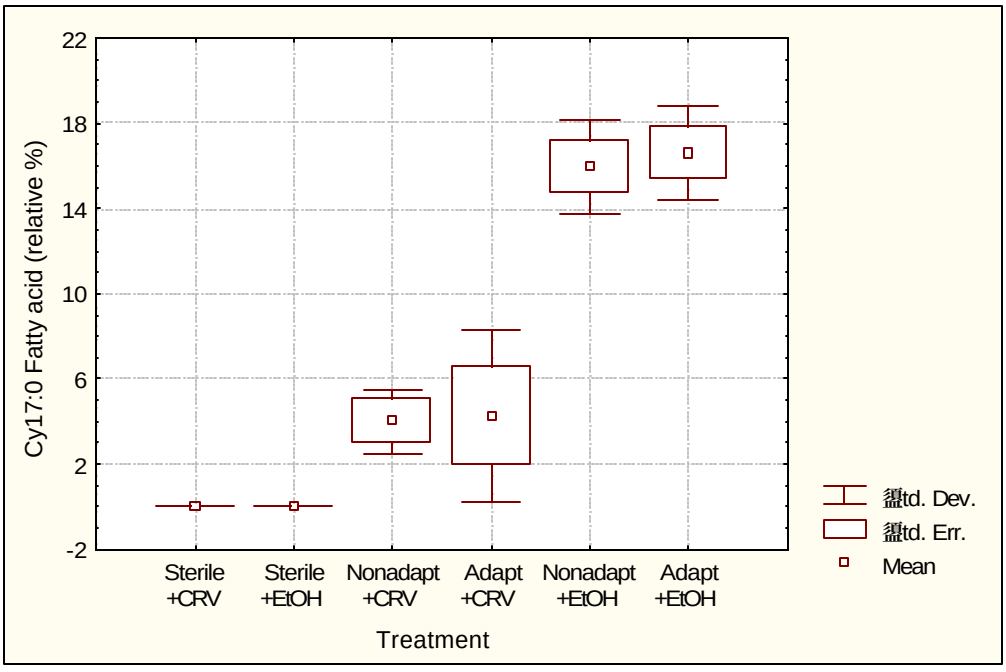


Figure 4.14. Comparison of Cy17:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

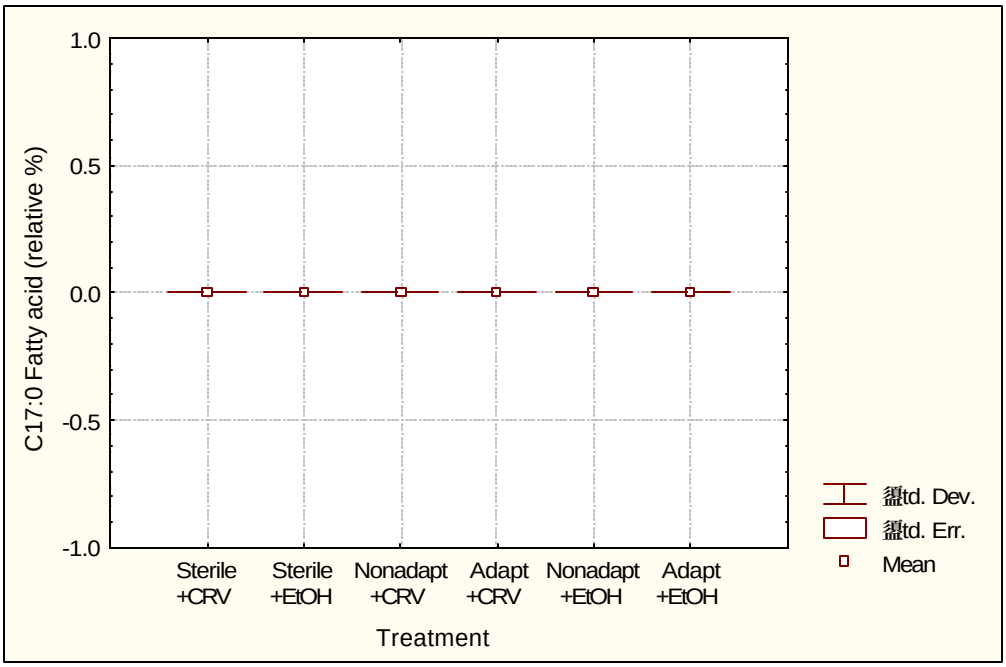


Figure 4.15. Comparison of C17:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

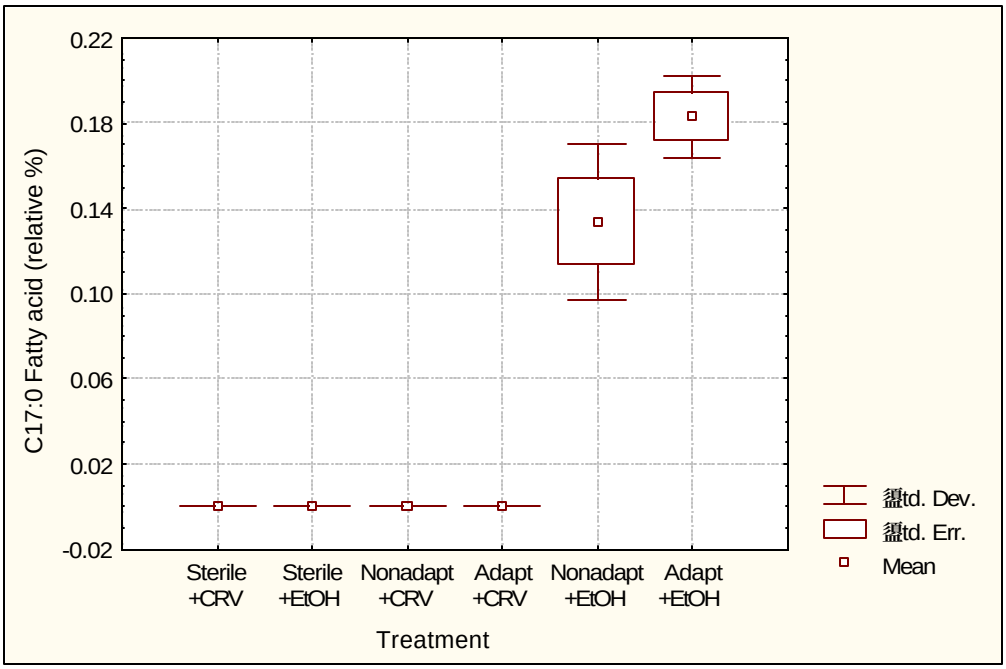


Figure 4.16. Comparison of C17:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

adapted control, followed by the non-adapted control. There was no significant ($p>0.05$) difference among the sterile and treatment groups.

C18:1w9c fatty acid. At 2h there was no definite pattern of differences among the groups due to the wide variability of the sterile and treatment groups, except that the controls appeared to have the lowest percentage of C18:1w9c and least variability (Figure 4.17). The abundance of C18:1w9c decreased in the non-adapted treatment and adapted control groups from 2h to 24h (Figure 4.18), but this was not significant ($p>0.02$). There were no significant changes in the other groups over time.

C18:1w7c fatty acid. At 2h the abundance of C18:1w7c was highest in the control groups (Figure 4.19). It was lowest in the sterile and treatment groups. From 2h to 24h, the non-adapted control significantly ($p<0.02$) decreased the proportion of C18:1w7c (Table 4.1). The adapted control exhibited a similar declining trend, but this was not significant ($p>0.02$). In contrast, the non-adapted and adapted treatment groups produced significantly ($p<0.005$) more of this fatty acid over time. At 24h, C18:1w7c was most abundant in the controls, followed by the treatment groups, and then the sterile groups (Figure 4.20).

C18:1w7t fatty acid. At 2h the percentages of C18:1w7t did not differ significantly ($p>0.05$) among the groups perhaps due do the variability within groups (Figure 4.21). It was detected in the sterile groups at 2 and 24h and in the treatment groups at 2h, but was depleted at 24h (Figure 4.22). It was present in the treatment groups because the cells were inhibited by CRV and had not yet metabolized it. There were no significant ($p>0.05$) changes across time within

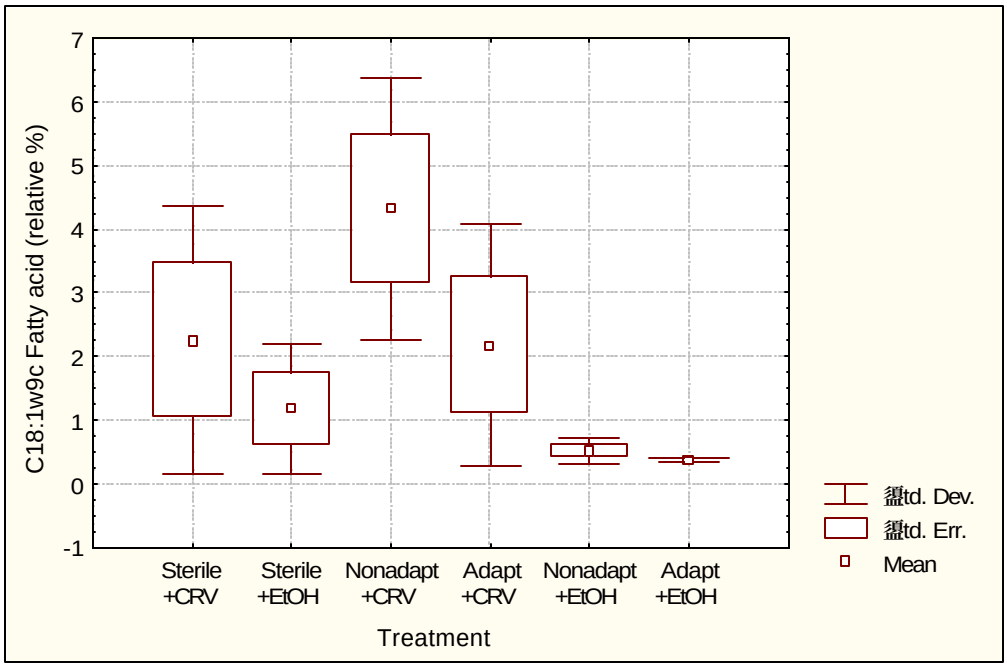


Figure 4.17. Comparison of C18:1w9c fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

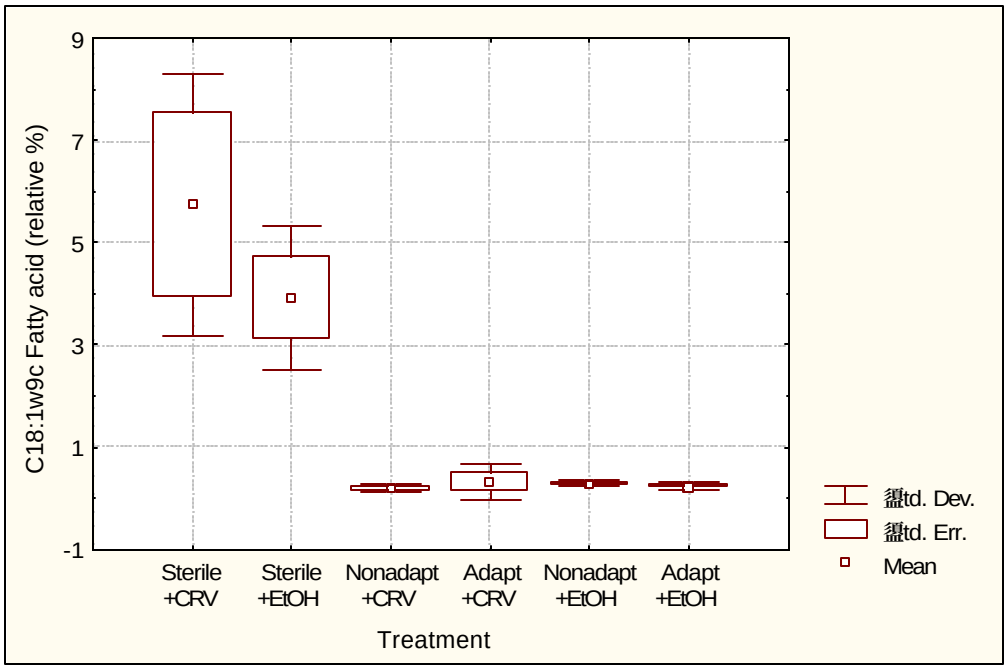


Figure 4.18. Comparison of C18:1w9c fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

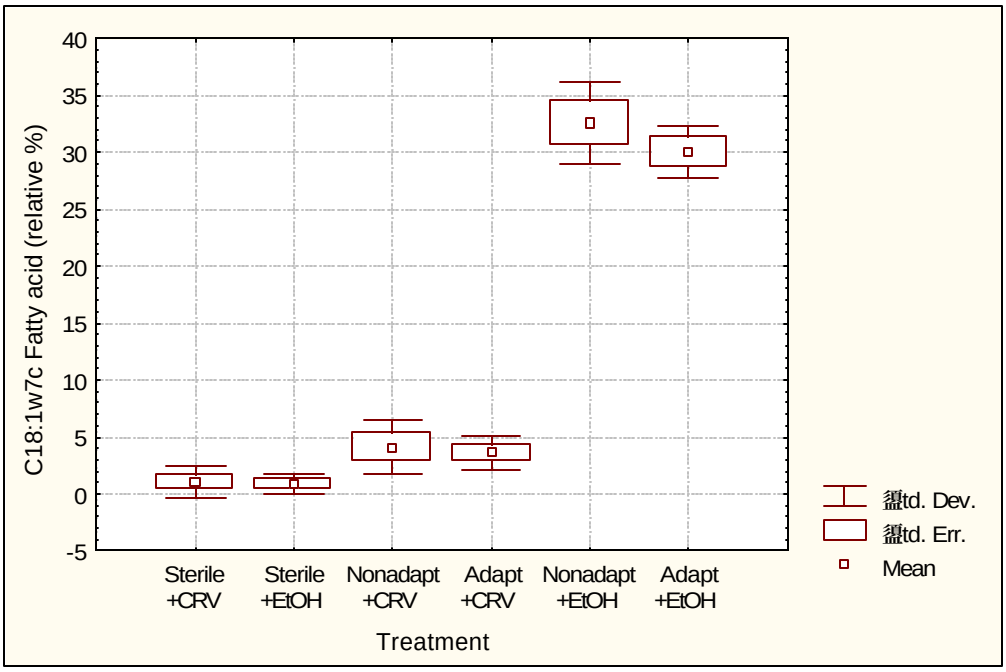


Figure 4.19. Comparison of C18:1w7c fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

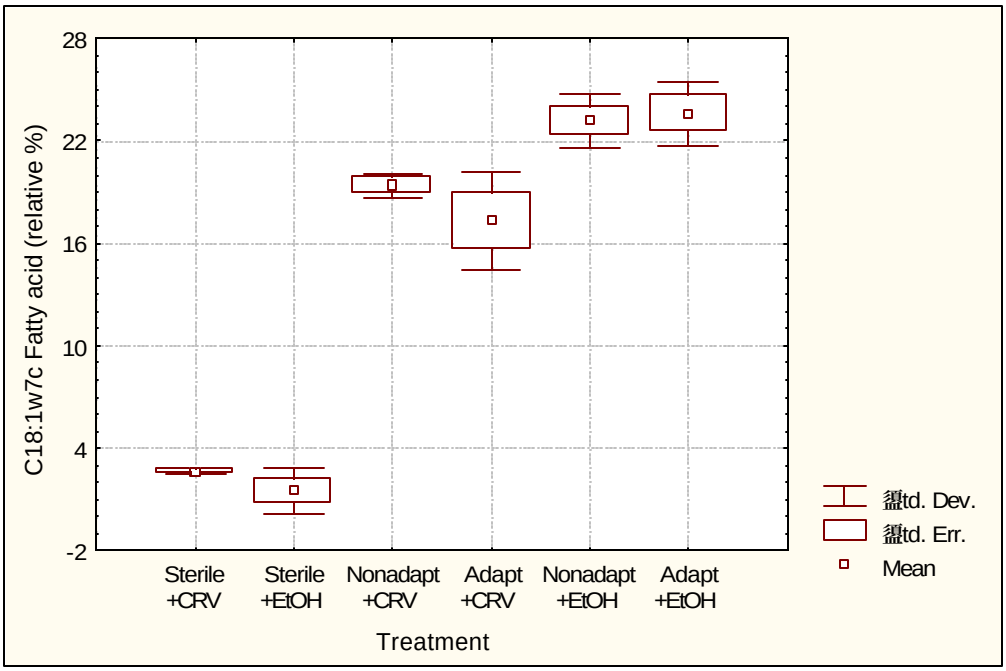


Figure 4.20. Comparison of C18:1w7c fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

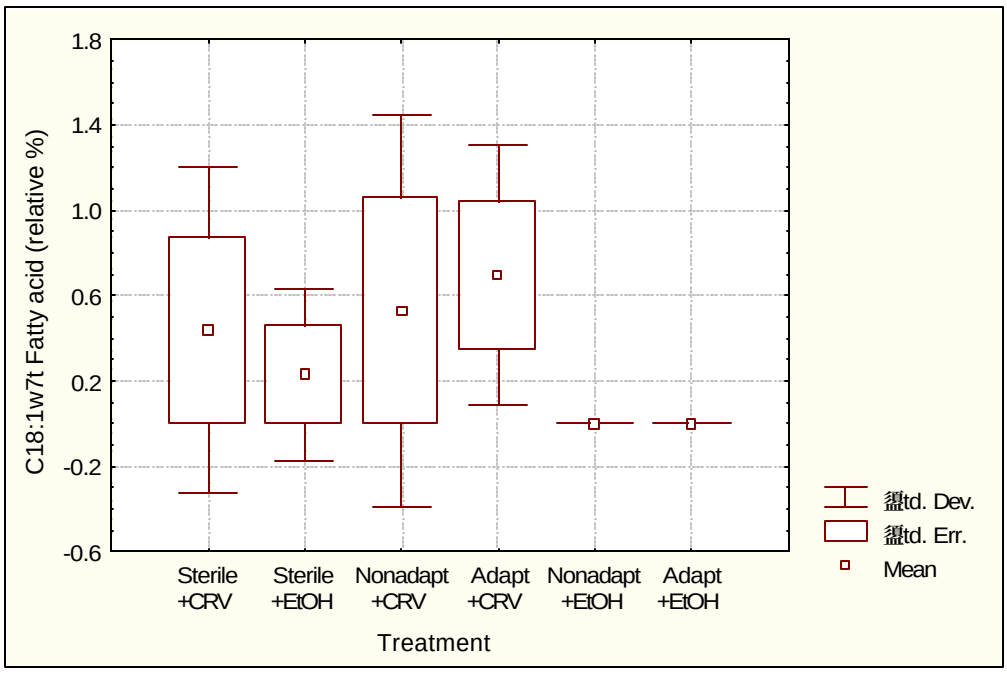


Figure 4.21. Comparison of C18:1w7t fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

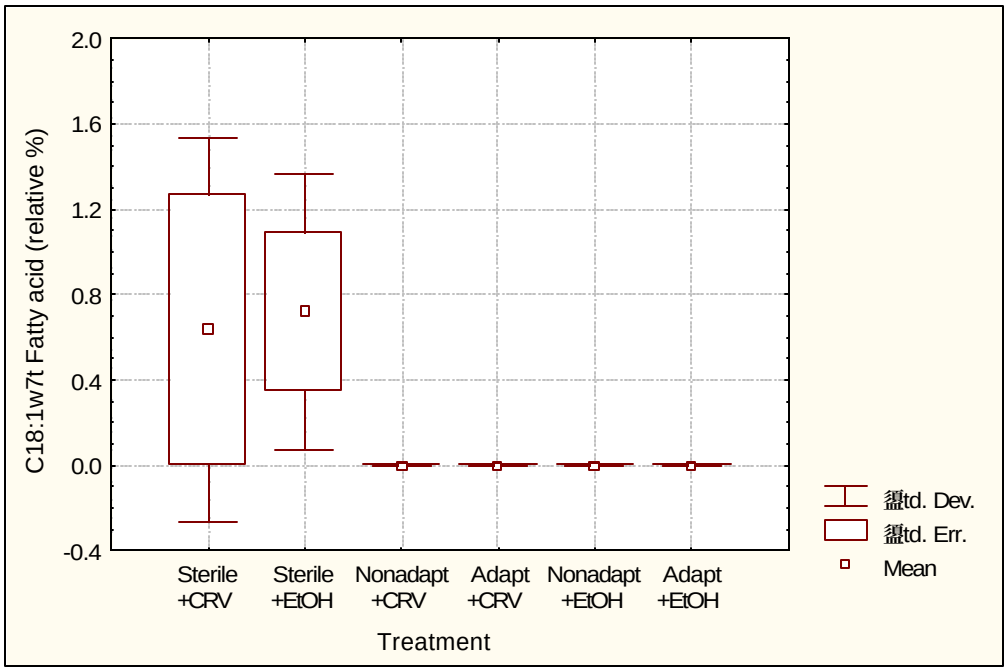


Figure 4.22. Comparison of C18:1w7t fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

each group. At 24h the sterile + EtOH group had significantly ($p<0.05$) more C18:1w7t than the control and adapted treatment groups. The wide variability in the sterile group may have been due to very small pellets that were obtained after centrifuging and washing (Figure 4.22).

C18:0 fatty acid. At 2h non-adapted control had decreased C18:0 to a proportion lower than the sterile groups and the non-adapted treatment group, which were similar (Figure 4.23). The percentage of C18:0 was higher in the non-adapted treatment than in the non-adapted control because cells were still inhibited by CRV. The percentage of C18:0 in the treatment groups was more similar to the sterile groups than the inoculated controls. The treatment groups had incorporated C18:0 but were unable to convert it as well as the inoculated controls.

Within each group C18:0 did not differ significantly ($p>0.05$) across time (possibly obscured by within-group variation), although there is a declining trend of C18:0 in the adapted treatment group. At 24h the treatment groups grew and converted C18:0 to another fatty acid. Therefore, there were no differences among the inoculated groups and C18:0 percentages were lower than in the sterile groups (Figure 4.24).

Cy19:0 fatty acid. At 2h Cy19:0 was not detected in any group (Figure 4.25). Compared to 2h, the controls formed more Cy19:0 at 24h as they entered stationary phase earlier ($p<0.01$) (Table 4.1). At 24h Cy19:0 was most abundant in the control groups and least in the sterile and treatment groups (Figure 4.26).

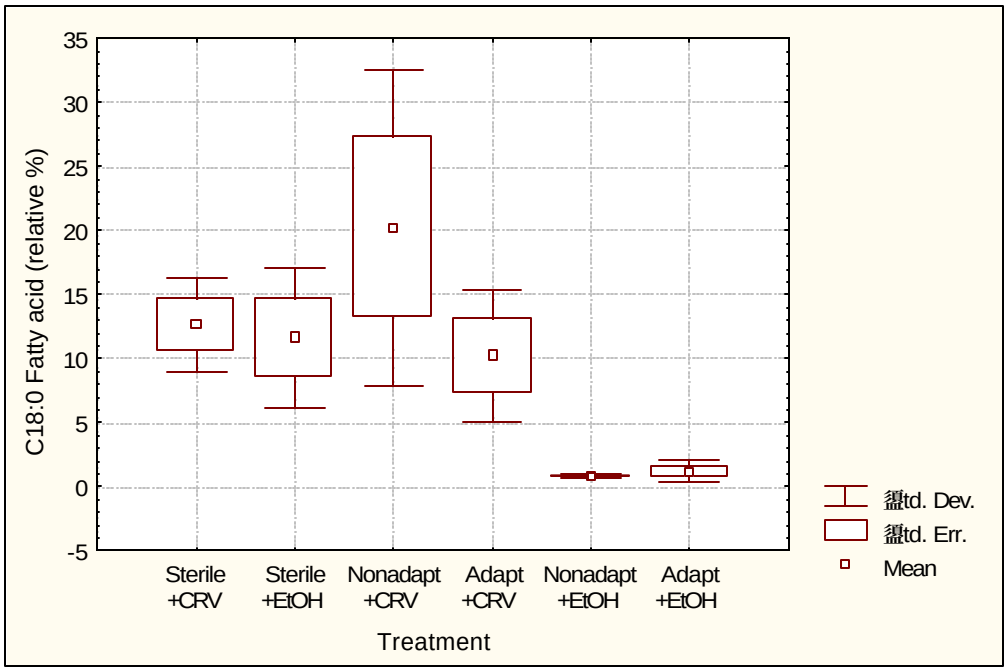


Figure 4.23. Comparison of C18:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

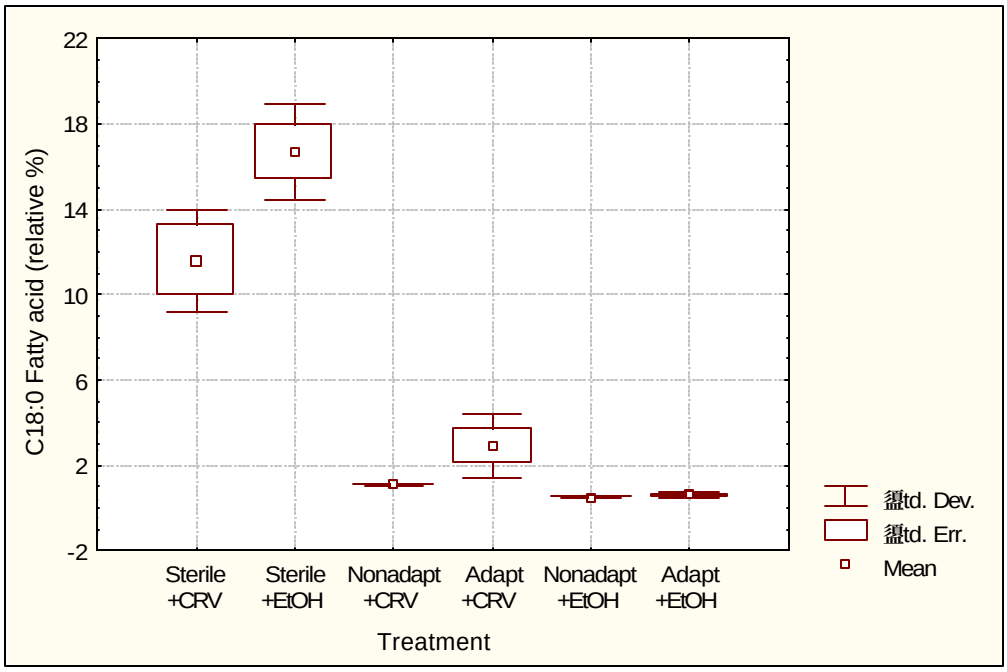


Figure 4.24. Comparison of C18:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

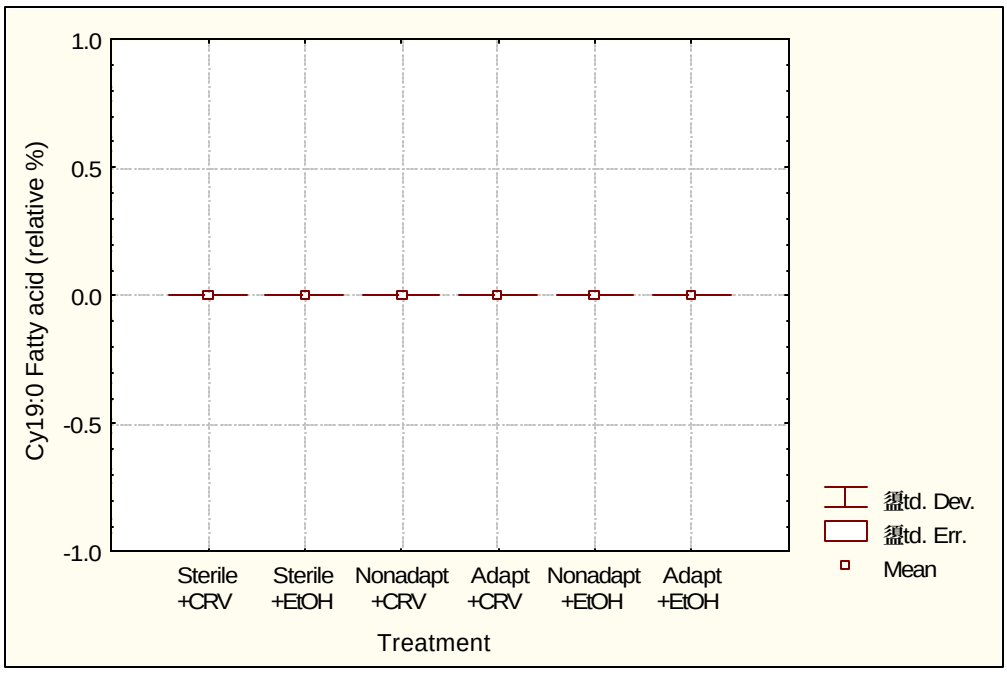


Figure 4.25. Comparison of Cy19:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

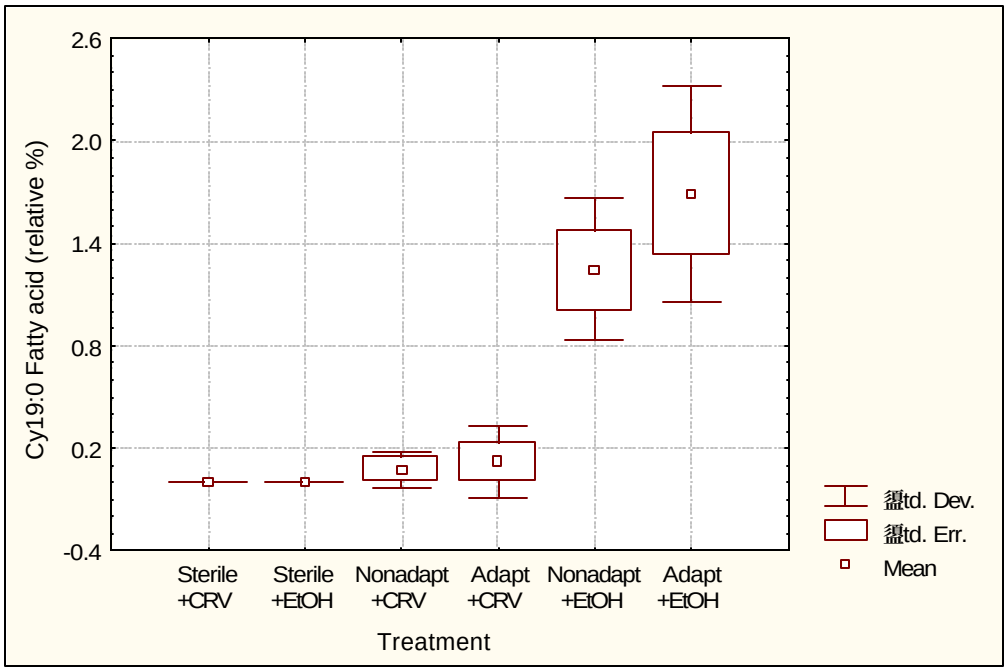


Figure 4.26. Comparison of Cy19:0 fatty acid of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

Saturated fatty acids. At 2h the percentages of saturated fatty acids were similar in the sterile and treatment groups (Figure 4.27). The fatty acids were much lower in the control groups since the cells were not inhibited. The percentage of SFA significantly ($p<0.005$) declined over time in the treatment groups as they grew, but it increased for the non-adapted control ($p<0.01$) (Table 4.1). At 24h, the abundance of SFA was highest in the sterile groups, followed by the adapted treatment and then the non-adapted treatment group (Figure 4.28). The controls contained the least SFA.

Monounsaturated fatty acids. At 2h UFA were highest in the control group (Figure 4.29). They were lowest in the sterile and treatment groups. Compared to 2h, UFA significantly ($p<0.005$) increased at 24h for the treatment groups, but decreased for the non-adapted control ($p<0.01$) (Table 4.1). At 24h the abundance of UFA was highest in the control groups, followed by the non-adapted treatment and then the adapted treatment group (Figure 4.30). These fatty acids were lowest in the sterile groups.

Ratio of Cy17:0 to C16:1w7c. At 2h there were no differences in the ratios among the groups (Figure 4.31). There was a larger standard error for the non-adapted CRV due to variation in both Cy17:0 and its precursor, which reflected the range in sensitivity. Over time, this ratio significantly ($p<0.005$) increased in the control groups, indicating earlier entry into the stationary phase (Table 4.1). At 24h the ratio was highest in the control groups and lowest in the sterile and treatment groups (Figure 4.32).

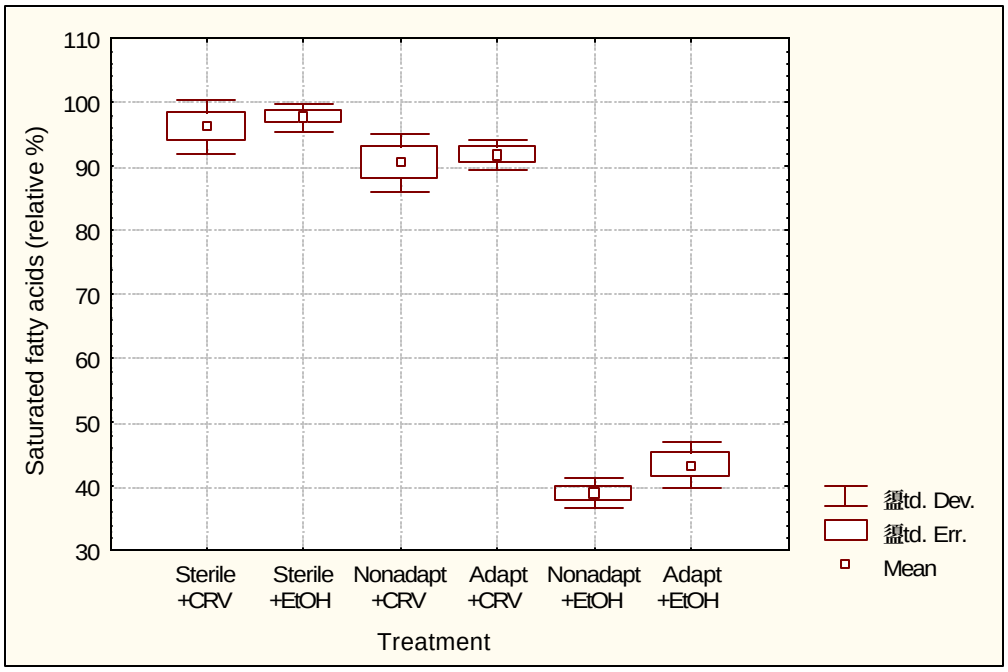


Figure 4.27. Comparison of saturated fatty acids of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

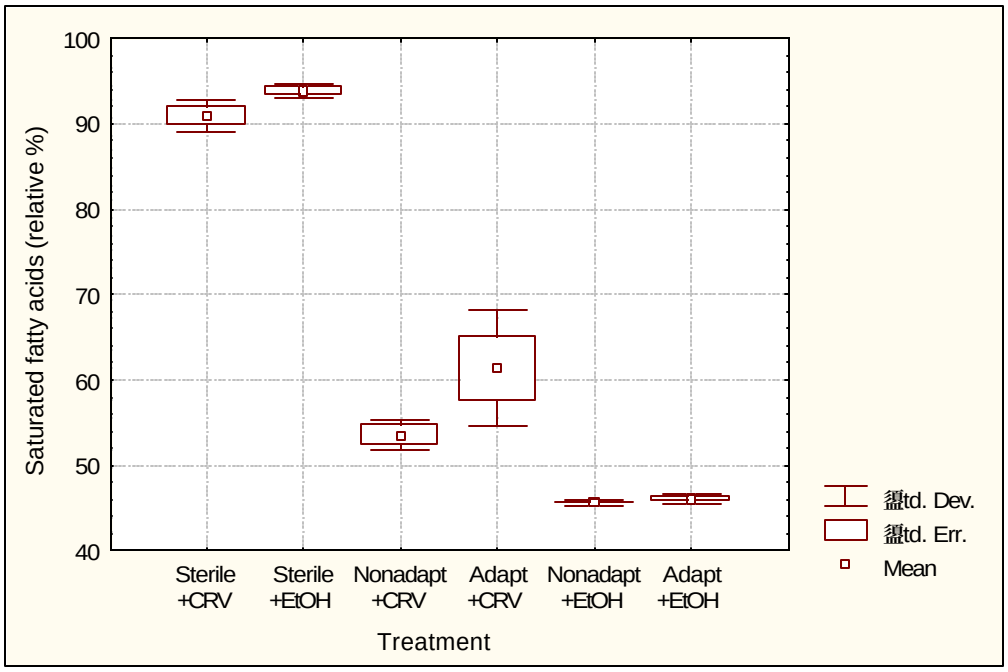


Figure 4.28. Comparison of saturated fatty acids of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

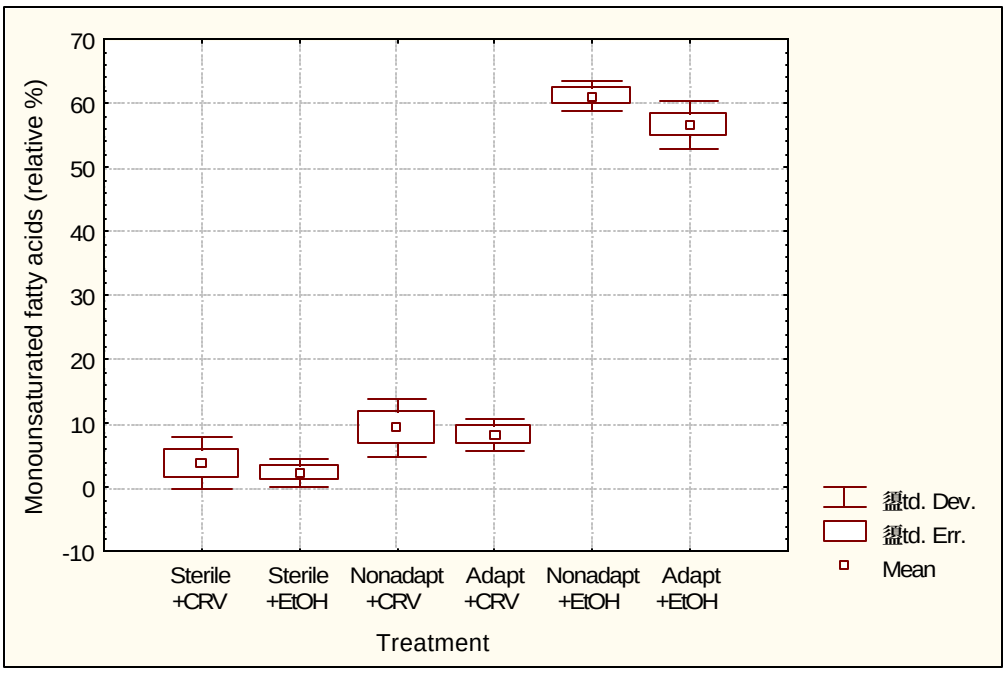


Figure 4.29. Comparison of monounsaturated fatty acids of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

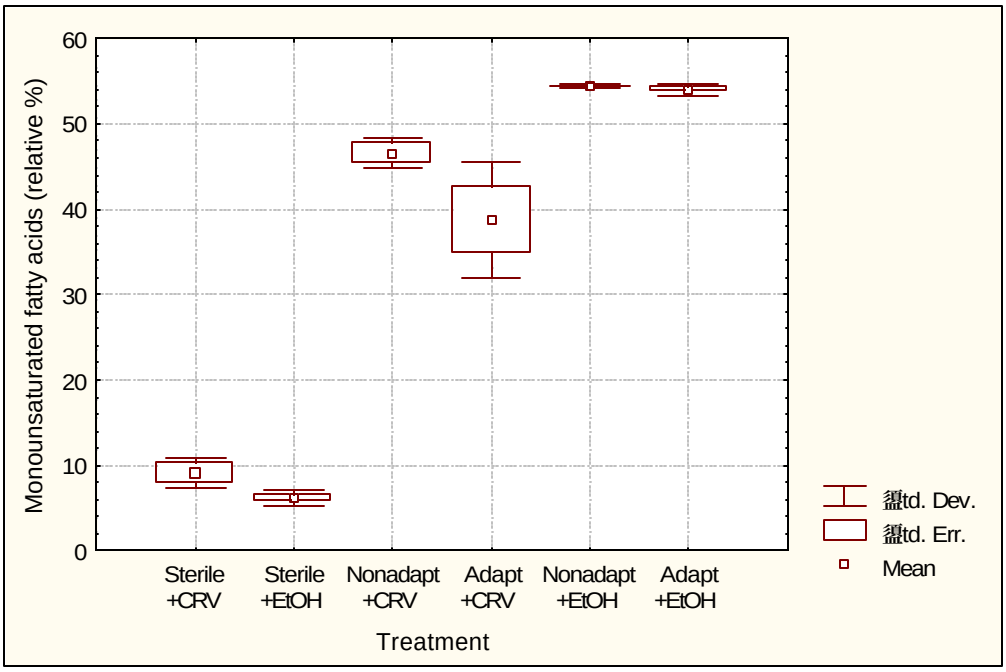


Figure 4.30. Comparison of monounsaturated fatty acids of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

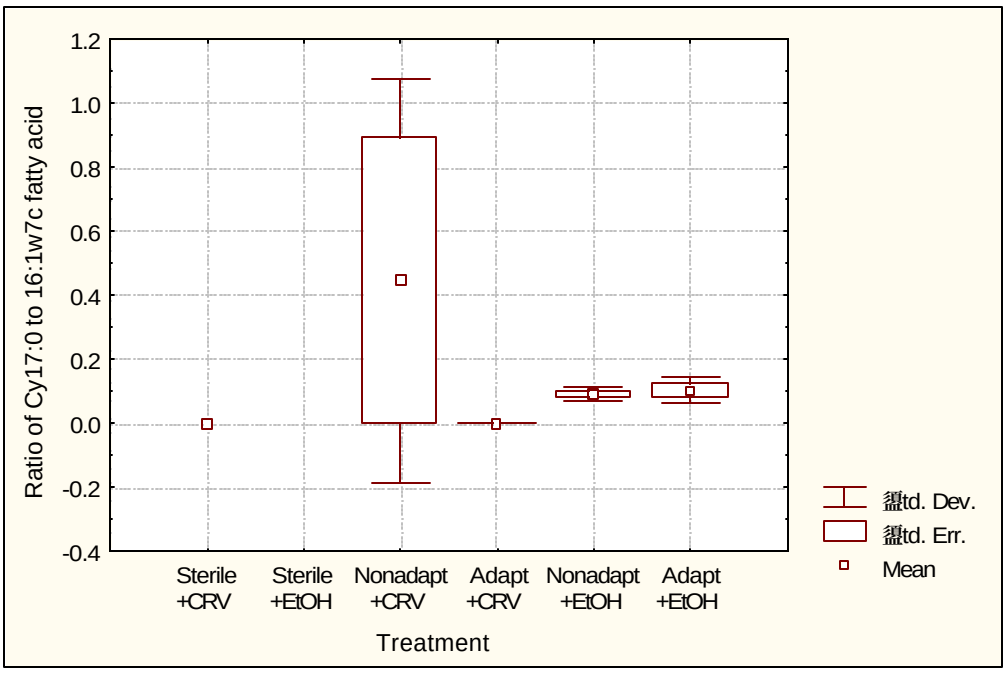


Figure 4.31. Comparison of ratio of Cy17:0 to 16:1w7c fatty acids of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

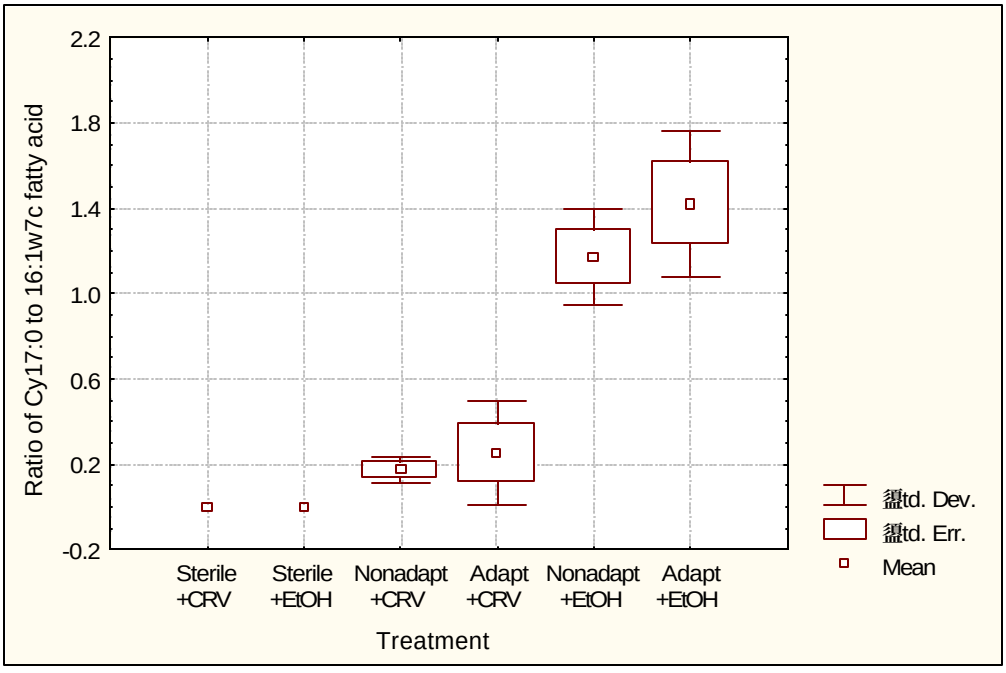


Figure 4.32. Comparison of Cy17:0 to 16:1w7c fatty acids of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

Ratio of Cy19:0 to C18:1w7c. The ratios were zero because Cy19:0 was undetected at 2h (Figure 4.33). From 2h to 24h, this ratio increased significantly ($p<0.02$) for the non-adapted control (Table 4.1). A similar trend occurred in the adapted control, but this was not significant ($p>0.05$). At 24h the ratio was highest in the control groups and lowest in the sterile and treatment groups (Figure 4.34).

Ratio of saturated to unsaturated fatty acids. At 2h, this ratio was lowest in the control groups, which were able to grow and decrease the proportion of SFA (Figure 4.35). The ratio was highest for the sterile groups, followed by the treatment groups, although there is some overlap between the sterile + CRV and treatment groups.

Compared to 2h, this ratio drastically ($p<0.02$) decreased at 24h in the adapted treatment group (and numerically in the non-adapted treatment group), but increased significantly ($p<0.01$) in the non-adapted control group (Table 4.1). At 24h, the ratio was lowest in all the inoculated groups (Figure 4.36). It was highest in the sterile + EtOH group followed by the sterile + CRV group.

Discussion

Anaerobes, facultative anaerobes, and also some aerobic bacteria synthesize lipids via the anaerobic pathway, which has been studied in *E. coli*. The starting reactants are acetyl-CoA and 3 malonyl-ACP. A trans double bond is produced during elongation. From that point, it can be reduced to form a

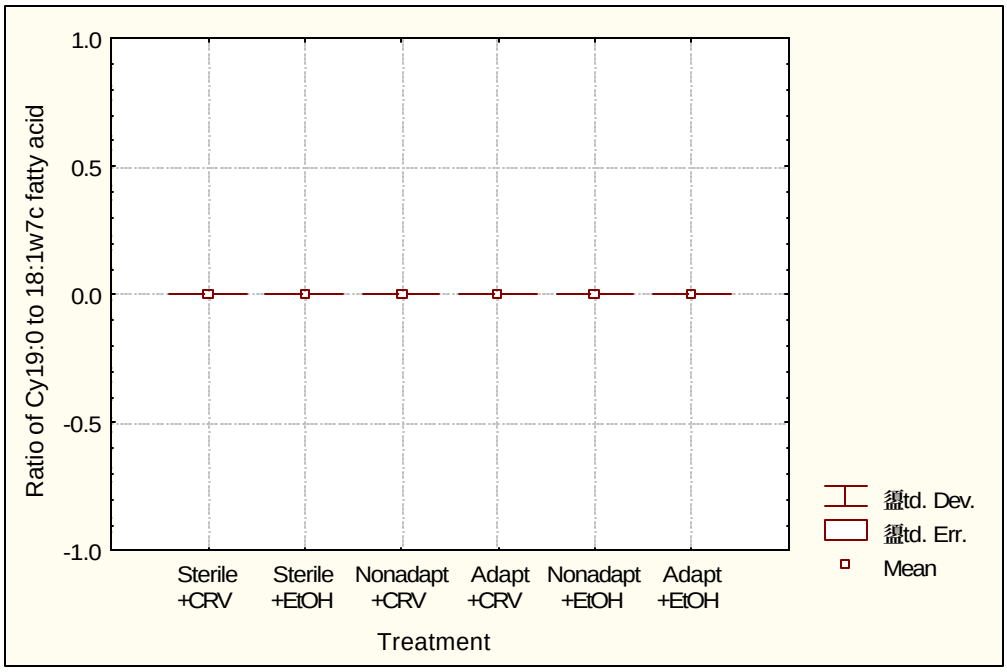


Figure 4.33. Comparison of ratio of Cy19:0 to 18:1w7c fatty acids of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

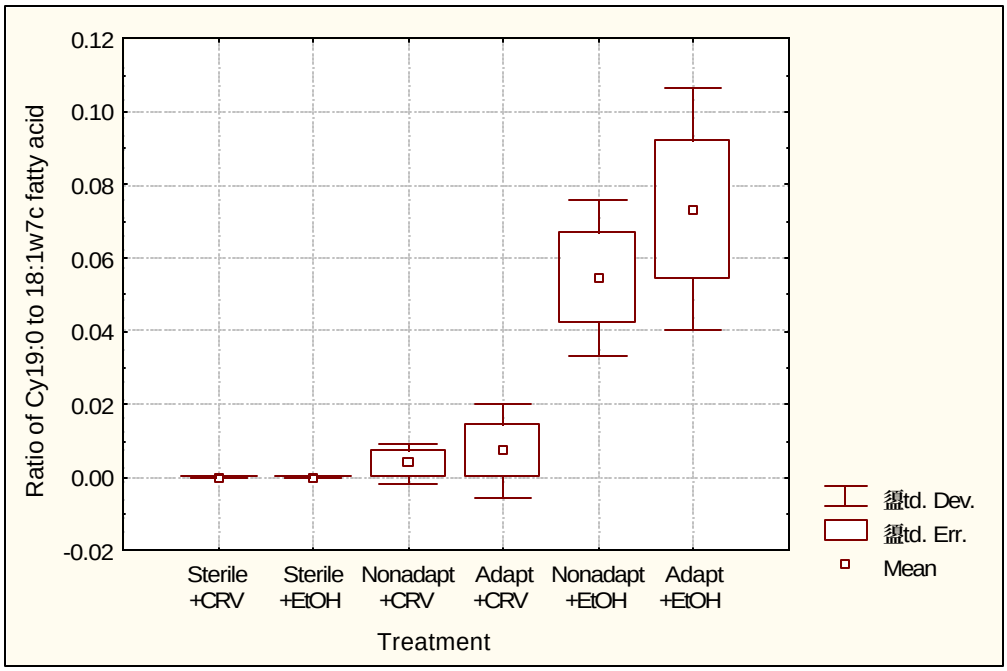


Figure 4.34. Comparison of Cy19:0 to 18:1w7c fatty acids of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

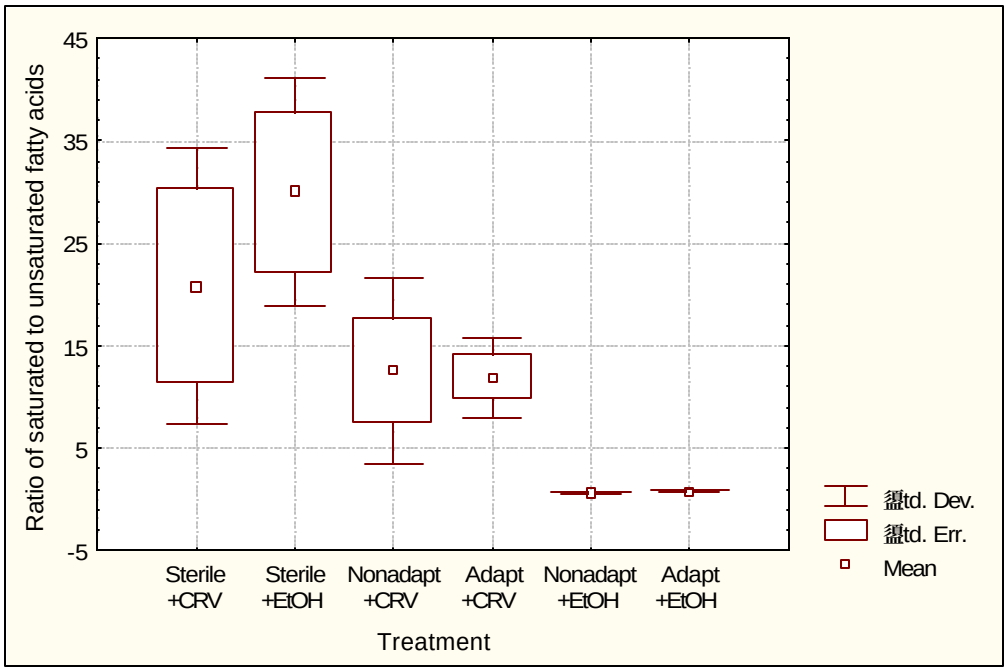


Figure 4.35. Comparison of ratio of saturated to unsaturated fatty acids of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 2-hr incubation

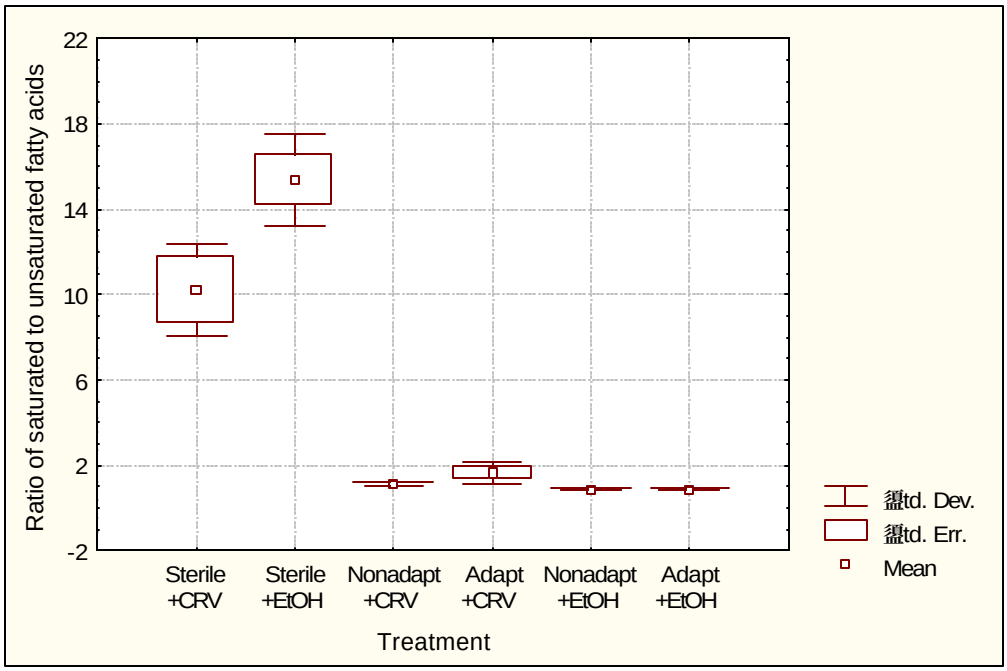


Figure 4.36. Comparison of ratio of saturated to unsaturated fatty acids of CRV-adapted or non-adapted cells with or without CRV to inoculated controls (+EtOH) and sterile controls after 24-hr incubation

saturated fatty acid or isomerized into a cis-monoenoic fatty acid. This cis fatty acid cannot be reduced and remain unchanged with further elongation. These saturated and cis-unsaturated chains are incorporated into phospholipids of the membrane. Since the degree of saturation cannot change after synthesis, the ratio of SFA to UFA cannot be changed in cells that cease growth. Phospholipid biosynthesis quickly decelerates if growth stops. However, post-synthesis modification of cis-monoenoic fatty acids to their cyclopropyl derivatives and trans-isomers can still occur (Diefenbach and others 1992; Keweloh and Heipieper 1996).

The uptake of fatty acids from the culture medium can occur by acyl-chain unspecific or active or passive transport. They are oxidized or incorporated in membrane lipids, which is regulated. Biosynthesis is regulated as well (Diefenbach and others 1992; Keweloh and Heipieper 1996).

Membrane fluidity has been correlated with changes in fatty acid composition (Mazzotta and Montville 1997). Certain types of lipid may have a greater influence on the fluidity than other. For example, converting a cis-monoenoic acid to its trans-isomer greatly reduces fluidity, but not as significantly as a change in cis to saturated fatty acids (Keweloh and Heipieper 1996).

The melting points of unsaturated fatty acid (UFA) and branched chain fatty acids are lower than that of saturated fatty acids (SFA). There are hydrophobic interactions among acyl chains of SFA. In membranes rich with SFA the membrane is packed and rigid. The chain can extend at its maximum if

the saturated chain rotates into a trans-conformation (Keweloh and Heipieper 1996).

In contrast, the double bond in cis-UFA creates a 30° angle in the acyl chain. This kink disrupts the ordered packing of the acyl chains in the membrane (Keweloh and Heipieper 1996). Therefore, an increase in cis UFA causes a decrease in the temperature for the transition from the gel to the liquid-crystal membrane phase and an increase in fluidity (Keweloh and Heipieper 1996; Casadei and others 2002).

A shortening in the acyl chain length will also lower the melting temperature and increase fluidity. A change in the degree of saturation is thought to be more effective than a change in the chain length (Keweloh and others 1991). Cyclopropyl fatty acids (CFA) are more physically similar to cis-monoenoic fatty acids, which are precursors of CFA. Trans-fatty acids are more stable and resemble SFA (Guckert and others 1986). Trans-UFA acyl chains are long and extended, packing more rigidly and occupying lower molar volume in the membrane. Trans UFA decrease the phase transition temperature, but not as drastically as cis UFA (Diefenbach and others 1992; Keweloh and Heipieper 1996).

The ratio of UFA to SFA indicates membrane fluidity. Casadei and colleagues (2002) developed a fluidity index that was calculated as the ratio of UFA plus cyclopropane fatty acids (CFA) in stationary phase to SFA. They extracted phospholipids from *E. coli* grown at different temperatures and measured the phase transition temperatures by DSC.

Casadei and colleagues (2002) confirmed the validity of the fluidity index as an indicator of membrane fluidity. They found that there was a direct linear relationship between the phase transition temperature and growth temperature and then an inverse linear relationship between the phase transition temperature and the fluidity index of phospholipids. The proportion of SFA increased while the proportion of UFA decreased with increasing growth temperature in stationary and exponential phase cells. The fluidity index was slightly greater in exponential than stationary phase cells. They reported that the abundance of CFA increased and C18:1 and C16:1 decreased in stationary phase cells. Since most of the treatment groups in this present data contained mostly SFA, a ratio was calculated of SFA to UFA plus CFA.

The measurement of the ester-linked phospholipid fatty acids (PLFA) is useful. The PLFA content quantitatively indicates the viable biomass since they have a high turnover rate and are present in consistent abundance in cell membranes. When cells die or lyse, hydrolysis of phospholipids quickly occur and the polar head group and diglyceride remain (White and Ringelberg 1997).

In addition to providing an estimate of the viable biomass, PLFAs can indicate nutritional and physiological status using the ratio of trans-monoenoic fatty acids to their cis homologues and the ratio of cyclopropyl derivatives to their monoenoic fatty acid precursors (White and Ringelberg 1997). During starvation of cultures of *Vibrio cholerae*, the trans/cis ratio increased. The cis-monoenoic fatty acids (such as 16:1w7c and 18:1w7c) decreased and the SFA, CFA, and

trans-monoenoic fatty acids increased. During starvation, membrane fluidity decreased overall (Guckert and others 1986).

When loss of phospholipid occurred during starvation, cis-monoenoic acids were preferentially utilized to possibly enhance survival. Cis fatty acids have faster turnover than saturated fatty acids and are more easily metabolized. During starvation, bacteria may synthesize trans-monoenoic acids, which bacteria do not readily metabolize, or transmethylate unstable cis- monoenoic fatty acids to form their cyclopropyl derivatives to survive (Guckert and others 1986).

It has been suggested that CFA are more stable to turnover and degradation (Guckert and others 1986). CFAs were formed (with a decline in their cis-fatty acid precursors) in *Vibrio cholerae* and *Pseudomonas putida* as cells aged and entered the stationary phase (Guckert and others 1986; Diefenbach and others 1992; Loffeld and Keweloh 1996). It has been hypothesized that CFA are formed under stress when growth stops (Guckert and others 1986).

The objectives in our study were to:

1) Determine the effect of exposure to CRV on the fatty acid composition of the bacterial membrane of *Salmonella*.

Compared to the non-adapted *Salmonella* at 2h, the non-adapted treatment had lower ($p<0.05$) C16:1w7c, Cy17:0, C18:1w7c, and unsaturated fatty acid (UFA) and higher ($p<0.05$) C14:0, C18:1w9c, C18:0, saturated fatty acid (SFA), and SFA/UFA ratio. The SFA to UFA ratio was drastically ($p<0.05$)

greater than the control. Therefore, CRV exposure caused a less fluid membrane. The total PLFA was numerically much lower, indicating that CRV inhibited growth.

At 24h, the non-adapted treatment had lower ($p<0.05$) total phospholipid fatty acid (PLFA), lower ($p<0.05$) C15:0, Cy17:0, C17:0, C18:1w7c, 19:0, and UFA than the non-adapted control and higher ($p<0.05$) C16:1w7c and SFA. The SFA to UFA ratio was only slightly higher, but not significant as it was at 2h. This suggested that CRV exposure for 24h decreased membrane fluidity. The total PLFA of the non-adapted treatment was significantly ($p<0.05$) lower because of growth inhibition by CRV.

2) Determine whether changes in the fatty acid composition of the bacterial membrane of *Salmonella* occur due to growth in the presence of CIN as a possible adaptive response to CRV.

At 2h there were no significant ($p>0.05$) differences in the total PLFA or fatty acids between the treatment groups as a function of incubation time, especially within the non-adapted group in which some cells may have exhibited a range of sensitivity to CRV. The SFA to UFA ratio was numerically higher in the non-adapted treatment group. The effect of CRV adaptation was unclear at 2h. Since the concentration used for adaptation and treatment corresponded to a 2.5% probability of lethality, the effect of CRV was not pronounced.

At 24h the only significant ($p<0.05$) difference was a lower abundance of C16:1w7c in the adapted treatment, which led to lower ($p<0.05$) unsaturated fatty acid and higher ($p<0.05$) saturated fatty acids. The SFA to UFA ratio was slightly

higher in the adapted treatment group. This suggested that adaptation to CRV decreased the fluidity of the membrane.

3) Determine whether reversal of these changes in fatty acid composition occurs when the stressor, CRV, is removed.

There were no differences at 2h. However, at 24h the adapted control produced more C17:0 than the non-adapted. Although this difference in fatty acid was not seen in the adapted treatment, it seems that adaptation of a less fluid membrane was maintained.

4) Determine whether any adaptive changes in the fatty acid composition are maintained after subsequent exposure to the CRV.

Compared to the adapted control at 2h, the adapted treatment group was found to have more C12:0, C14:0, and SFA and less C16:1w7c, C16:0, Cy17:0, C18:1w7c, and UFA. The SFA to UFA ratio was much greater in the treatment. Therefore, even after CRV exposure the adaptation of less fluid membrane is maintained.

At 24h it had higher SFA and lower total PLFA, C15:0, C16:1w7c, Cy17:0, C17:0, C18:1w7c, Cy19:0, and UFA. The SFA to UFA ratio was only slightly higher, but not significant as it was at 2h. This suggested that adaptation with subsequent CRV exposure for 24h decreased membrane fluidity, keeping the adaptive response. The total PLFA was significantly ($p<0.05$) lower, indicating that CRV inhibited growth.

5) Determine the effect of growth in the presence of CRV (adaptation) and subsequent CRV treatment on affect fatty acid composition.

The adapted treatment group had more C12:0, C14:0, C16:0, SFA, and and less C16:1w7c, Cy17:0, C18:1w7c, and UFA at 2h. The SFA to UFA ratio was significantly ($p<0.05$) and drastically greater than the non-adapted control.

At 24h, this group had a lower total PLFA, C16:1w7c, Cy17:0, C17:0, C18:1w7c, Cy19:0 and monounsaturated fatty acids and higher C12:0 and C15:0. The SFA to UFA ratio was only slightly higher, but not significant as it was at 2h. CRV adaptation and treatment decreased membrane fluidity and inhibited growth.

Overall, most of the changes were in the longer chain fatty acids than in the shorter chain fatty acids. It appears that both treatment groups became more fluid and the control groups became less fluid with time. At 2h the SFA to UFA ratios of the treatment groups were significantly ($p<0.05$) greater than those of the control groups. However at 24h, the magnitude of the difference decreased. Perhaps this response to 2h incubation is more similar to an adaptation.

Significant changes from 2h to 24h occurred mostly with the control groups and occasionally with the adapted treatment group. However, significant differences may not have been detected due to variability within these treatment groups. At 2h, the proportions of C12:0, C14:0 and C18:0 of the treatment groups were more similar to that sterile controls or in between that of the sterile controls and inoculated controls. It is hypothesized that the bacteria of the treatment groups had incorporated these fatty acids from the medium but were not able to convert them to other fatty acids because growth was inhibited by CRV treatment. With time, the non-adapted treatment overcame the inhibition

and was able to grow and synthesize phospholipids (as evidenced by the numerical increase in total PLFA and decrease in these fatty acids) for a more favorable proportion of SFA to UFA ratio that is closer to homeostasis such as in the non-adapted + EtOH group.

The controls were expected to increase in this ratio because C16:1w7c and C18:1w7c decline and CFA increase in stationary phase (Casadei and others 2002). These changes occurred in the controls from 2 to 24h. Over time, SFA increased in the controls, which was a sign of starvation, along with the decrease in C16:1w7c and C18:1w7c and significant increase in CFA (Guckert and others 1986). The treatments did not seem to have entered stationary phase over time because there were significant increases in C16:1w7c and C18:1w7c. However, there were a significant increase Cy17:0 in the non-adapted treatment and a numerical increase in Cy17:0 in the adapted treatment as well as numeric increases in Cy19:0.

Essential oil components, such as CRV, act on the membrane, as because they are lipophilic (Sikkema and others 1995; Ultee and others 1999). Lipophilic compounds accumulate in the cell membrane, which was demonstrated in liposomes synthesized from *E. coli* lipids (Sikkema and others 1995). As the CRV interacts with phospholipid bilayer, it may increase membrane permeability and cause cellular constituents to leak (Ultee and others 1998).

It has been suggested that CRV occupies more area than the typical space between the fatty acid chains of two adjoining phospholipid molecules.

This interferes with the van der Waals interactions between the chains and therefore affect fluidity (Ultee and others 2000b; Ultee and others 2002). If the membranes become too fluid, additional CRV can continue to permeate the membrane, accumulate, and cause more damage (Ultee and others 1998).

It was found that CRV expanded the liposomal membrane using fluorescent probes (Ultee and others 2002). It is believed that this swelling leads to a destabilized membrane and therefore a leakage of ions. During exposure to CRV, an influx of protons and efflux of potassium ions in *Bacillus cereus* was reported (Ultee and others 1999). This leakage was also evident in the decrease of the pH gradient across the membrane (Ultee and others 2002). Griffin and others (1999) categorized CRV in a group of oxygenated terpenoids with high antimicrobial activity associated with higher hydrogen binding capacity, this hypothesis is reasonable. The leakage of ions leads to a drop in the membrane potential and depletion of the intracellular ATP pool (Ultee and others 2002). It was found that 300 mg/L of CRV reduced viable counts of *Bacillus cereus* cells in 30 min and depleted intracellular ATP within 7 min without a proportional increase in extracellular ATP (Ultee and others 1999).

Similarly, Helander and others (1998), reported that CRV at 300 mg/L (MIC = 451 mg/L) increased the uptake of hydrophobic fluorescent probe NPN in *E. coli* O157:H7 and S. Typhimurium and decreased intracellular ATP in *E. coli* O157:H7, but with a smaller increase in extracellular ATP. These effects indicated that CRV disintegrated the outer membrane (Helander and others 1998). It was suggested that CRV impaired the cell membrane and increased

permeability because of the increased fluorescence of nuclear stain EB after *Staphyloccus aureus* and *Pseudomonas aeruginosa* were exposed to 0.1% CRV (Lambert and others 2001).

Ultee and others (2000) investigated the adaptation of *Bacillus cereus* to CRV. Overnight cultures were diluted 100-fold in BHI+0.5% glucose and incubated with or without CRV (0 - 60 mg/L) at 30°C for 4h. These exponentially growing cells were centrifuged and washed with 25 mM phosphate buffer (pH 7.0) or buffer with added 60 mg/L CRV (sublethal). The pellet was measured by Fourier-transformed infrared spectra, which determined the degree of interaction between acyl chains. This interaction indicated the membrane ordering and fluidity.

They reported that after CRV was added to non-adapted cells there was a decrease in the melting temperature (T_m) of the membrane (20.5°C to 12.6°C). Compared to non-adapted cells, cells adapted to 60 mg/L exhibited a less fluid membrane because the T_m increased from 20.5°C to 28.3°C. When adapted cells were exposed again to CRV, the T_m decreased again to 19.5°C, which was the same value as non-adapted cells with no exposure to CRV (Ultee and others 2000b).

In the same study, *iso*-C13:0, C14:0 and *iso*-C15:0 fatty acids (which are higher melting) increased in the membrane lipid composition of *B. cereus* cells incubated with 60 mg/L CRV. *Cis*-C16:1 fatty acid decreased. These changes correlated with the decrease in membrane fluidity of cells adapted to CRV. However, the decrease in long chain fatty acids (C18:0) did not correlate with the

decrease in fluidity. Cells adapted to CRV had additional phospholipids compared to non-adapted cells. There were no noticeable differences in proportions of phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), and phosphatidylglycerol (PG). No glycolipids were detected. They reported that *B. cereus* did not metabolize CRV since 98% of it remained after 74h of incubation. Cells adapted to CRV were less sensitive when subsequently exposed to CRV as indicated by longer times to kill 50% of cells (Ultee and others 2000b).

In this present study, the microorganism, adaptation process, comparison of various treatment groups was different from the study of Ultee and others (2000). The concentration of CRV (871 mg/L) was considerably higher. Their finding that adaptation reduced the fluidity of the membrane agreed with the data in this study. In contrast, they reported an increase in fluidity with exposure to CRV, which contradicted with this study. This study found that the SFA to UFA ratio was higher in the non-adapted treated cells. When adapted cells were subsequently exposed to CRV in this study, the SFA to UFA ratio was greater than that of the non-adapted control. This disagreed with the finding of Ultee and others (2000), which reported that the fluidity of the adapted cells subsequently exposed to CRV was similar to the non-adapted control.

In a related study of late-log phase *Pseudomonas putida* cells, which were adapted to 0.5g phenol in agar and subsequently exposed to 0.25 – 1.25g phenol/l for 3h, it was found that C16:0 and C16:1 trans was greater and C16:1cis and C18:1cis was less than the adapted control (Heipieper and others

1992). A comparison of results in this study reveals both agreements and differences. The adapted treatment group had a lower relative percentage of C16:0, C16:1w7c, and C18:1w7c at 2h .

Keweloh and others (1991) found that exponentially growing *E. coli* K-12 cells exposed for 4h with 0.5 or 1g/l phenol in the growth medium had higher amounts of C16:0 than in the control, which differed slightly from this present study. The treatment groups had lower C16:0 percentages than the controls at 2h, but they were higher than the controls at 24h. They also reported that the proportions of C16:1 and C18:1 were lower in the treated cells with an increase in cyclopropane fatty acids, which agreed with this study. Overall, they found that there was a higher degree of saturation, which was similar to this study. It was suggested that greater saturation results in a more rigid membrane that compensates for increase in fluidity and permeability caused by phenol. Saturated fatty acids have acyl chains that are packed more closely, which could limit the entry of phenol and therefore, its toxic effect (Keweloh and others 1991). In the same way, the increase of saturation upon exposure and adaptation to CRV could (a phenolic compound) reduce its accessibility into or its impact on the cell.

The effect of Tween 20 was not investigated in this study. It is possible that use of 1% Tween 20 affected the fatty acid composition. *L. monocytogenes* grown in BHI broth with 0.1% Tween at 30°C and harvested in mid-log phase was found to have lower C15:0, C17:0, anteiso-, and iso-fatty acids and higher straight-chain, even-numbered fatty acids with no significant change in anionic

phospholipid. Despite these modifications that were expected to decrease fluidity, no significant change in fluidity was detected. Cells adapted to Tween were more sensitive because of increased binding to nisin (Li and others 2002).

Significant findings

Compared to the non-adapted *Salmonella* at 2h, the non-adapted treatment had lower ($p<0.05$) C16:1w7c, Cy17:0, C18:1w7c, and unsaturated fatty acid (UFA) and higher ($p<0.05$) C14:0, C18:1w9c, C18:0, saturated fatty acid (SFA), and SFA/UFA ratio. At 24h, the non-adapted treatment had lower ($p<0.05$) total phospholipid fatty acid (PLFA), lower ($p<0.05$) C15:0, Cy17:0, C17:0, C18:1w7c, Cy19:0, and UFA than the non-adapted control and higher ($p<0.05$) C16:1w7c and SFA. At 2h there were no significant differences between the fatty acid compositions of adapted *Salmonella* treated with CRV and non-adapted cells treated with CRV. At 24h, the adapted cells treated with CRV had lower ($p<0.05$) C16:1w7c and UFA and higher ($p<0.05$) SFA than non-adapted cells treated with CRV. The SFA/UFA ratio was numerically higher in the adapted *Salmonella* than the non-adapted cells at 24h. Since the fatty acid composition and total PLFA of the non-adapted control and adapted controls were similar, the adaptive response had been reversed since the stressor, CRV, was not used to treat the adapted controls.

Most of the changes from 2 to 24h occurred in the membrane of the non-adapted and adapted control *Salmonella*. Over time there was a decrease in

C16:1w7c ($p<0.01$), C18:1w7c, and UFA ($p<0.01$) and an increase in total PLFA ($p<0.01$), C16:0, Cy17:0 ($p<0.01$), SFA, Cy17:0/ C16:1w7c ratio ($p<0.01$), and SFA/UFA ratio in the control groups. In the adapted and non-adapted treated *Salmonella* there was a decrease in C14:0, SFA ($p<0.01$), and SFA/UFA ratio and an increase in C16:0, Cy17:0, C18:1w7c ($p<0.01$), and UFA from 2 to 24h.

Since fatty acid composition is one of the factors that affect membrane fluidity it appears that short- and long-term treatment with CRV at sub-lethal concentrations decreased the fluidity of the *Salmonella* membrane by significantly increasing the proportion of saturated fatty acids. This decreased fluidity may prevent additional CRV from permeating. The effect of bacterial growth in the presence of CRV on the fatty acid composition was much smaller. Determination of any changes in the phospholipid head groups would be useful because of their affect on fluidity. Measurement of the membrane transition temperature from gel-to-liquid-crystalline phase by Fourier-transformed infrared spectrometry would indicate the interaction between their acyl chains and therefore fluidity. A nuclear magnetic resonance spectrometer or a Langmuir-Blodgett trough can also measure fluidity. Investigation about the target site that CRV acts upon and the specific interactions of CRV with membrane lipids and proteins using differential scanning calorimetry would contribute to the understanding of its mechanism of action and the bacterial response.

REFERENCES

Brul S, Coote P. 1999. Preservative agents in foods: Mode of action and microbial resistance mechanisms. International Journal of Food Microbiology 50:1-17.

Casadei MA, Manas P, Niven G, Needs E, Mackey BM. 2002. Role of membrane fluidity in pressure resistance of *Escherichia coli* NCTC 8164. Applied and Environmental Microbiology 68:5965-5972.

Denich TJ, Beaudette LA, Lee H, Trevors JT. 2003. Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. Journal of Microbiological Methods 52:149-182.

Diefenbach R, Heipieper HJ, Keweloh H. 1992. The conversion of cis into trans unsaturated fatty acids in *Pseudomonas putida P8*: Evidence for a role in the regulation of membrane fluidity. Applied Microbiology and Biotechnology 38:382-387.

Friedman M, Henika PR, Mandrell RE. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes,* and *Salmonella enterica*. Journal of Food Protection 65:1545-1560.

Guckert JB, White DC *Proceedings of the Fourth International Symposium of Microbial Ecology*, p pp 455-459.

Guckert JB, Hood MA, White DC. 1986. Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio Cholerae*: increases in the trans/cis ratio and proportions of cyclopropyl fatty acids. Applied and Environmental microbiology 52:794-801.

Guckert JB, Antworth CP, Nichols PD, D.C. W. 1985. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. FEMS Microbiology Ecology 31:147-158.

Hammer K, Carson C, Riley T. 1999. Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology 86:985-990.

Heipieper H, Diefenbach R, Keweloh H. 1992. Conversion of cis unsaturated fatty acids to trans, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. Applied and Environmental Microbiology 58:1847-1852.

Helander I, Alakomi H, Latva KK, Mattila ST, Pol I, Smid E. 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. Journal of Agricultural and Food Chemistry 46:3590-3595.

Juneja VK, Davidson PM. 1993. Influence of altered fatty acid composition on resistance of *Listeria monocytogenes* to antimicrobials. Journal of Food Protection 56:302-305.

Keweloh H, Heipieper HJ. 1996. Trans unsaturated fatty acids in bacteria. Lipids 31:129-137.

Keweloh H, Diefenbach R, Rehm HJ. 1991. Increase of phenol tolerance of *Escherichia coli* by alterations of the fatty acid composition of the membrane lipids. Archives of Microbiology 157:49-53.

Kim J, Marshall MR, Wei CI. 1995a. Antibacterial activity of some essential oil components against five foodborne pathogens. Journal of Agricultural and Food Chemistry 43:2839-2845.

Kim JM, Marshall MR, Cornell JA, Preston JF, Wei CI. 1995b. Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella typhimurium* in culture medium and on fish cubes. Journal of Food Science 60:1364-1368.

Lambert R, Skandamis P, Coote P, Nychas G. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of Applied Microbiology 91:453-462.

Li J, Chikindas ML, Ludescher RD, Montville TJ. 2002. Temperature and surfactant induced membrane modifications that alter *Listeria monocytogenes* nisin sensitivity by different mechanisms. Applied and Environmental Microbiology 68:5904-5910.

Lis-Balchin M, Deans SG. 1997. Bioactivity of selected plant essential oils against *Listeria monocytogenes*. Journal of Applied Microbiology 82:759-762.

Loffeld B, Keweloh H. 1996. Cis/trans isomerization of unsaturated fatty acids as possible control mechanism of membrane fluidity in *Pseudomonas putida P8*. Lipids 31:811-815.

Mazzotta AS, Montville TJ. 1997. Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10 degree C and 30 degree C. Journal of Applied Microbiology 82:32-38.

Russell NJ, Evans RI, ter Steeg PF, Hellemons J, Verheul A, Abee T. 1995. Membranes as a target for stress adaptation. International Journal of Food Microbiology 28:255-261.

Seltmann G, Holst O. 2002. The bacterial cell wall. Berlin: Springer-Verlag.

Sikkema J, de Bont JAM, Poolman B. 1994. Interactions of cyclic hydrocarbons with biological membranes. Journal of Biological Chemistry 269:8022-8028.

Sikkema J, de Bont JAM, Poolman B. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews 59:201-222.

Ultee A, Gorris LG, Smid EJ. 1998. Bactericidal action of carvacrol towards the food-borne pathogen *Bacillus cereus*. Journal of Applied Bacteriology 85:211-218.

Ultee A, Kets EPW, Smid EJ. 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 65:4606-4610.

Ultee A, Bennik MHJ, Moezelaar R. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 68:1561-1568.

Ultee A, Slump RA, Steging G, Smid EJ. 2000a. Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice. Journal of Food Protection 63:620-624.

Ultee A, Kets EPW, Alberda M, Hoekstra FA, Smid EJ. 2000b. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. Archives of Microbiology 174:233-238.

Valero M, Salmeró n MC. 2003. Antibacterial activity of 11 essential oils against *Bacillus cereus* in tyndallized carrot broth. Journal of Food Microbiology 85:73-81.

White DC, Ringelberg DB. 1997. Utility of the signature lipid biomarker analysis in determining the in situ viable biomass, community structure, and nutritional/physiologic status of deep subsurface microbiota. In: P. S. Amy and D. L. Haldeman. The microbiology of the terrestrial deep subsurfarce. Boca Raton: CRC Lewis Publishers.

Yousef AE, Courtney PD. 2003. Basics of stress adaptation and implications in new-generation foods. In: A. E. Yousef and V. K. Juneja. Microbial stress adaptation and food safety. Boca Raton: CRC Press. 31-54.

APPENDIX

SAS program to compare the effectiveness of essential oil components tested against *L. monocytogenes* using microdilution method

```
/* This SAS program generates SAS Probit models (probability of lethality) to
compare the effectiveness of essential oil components tested against L.
monocytogenes using microdilution method (microtiter plates)*/

data multi;
     input stock $ chemical $ Dose Response N;
     dose=log10(dose); label dose=Log10(Dose) ppm;
          datalines;
Nov17 CRV 3200 2 2
Nov17 CRV 3000 2 2
Nov17 CRV 2800 2 2
Nov17 CRV 2600 1 2
Nov17 CRV 2400 2 2
Nov17 CIN 1200 2 2
Nov17 CIN 1000 2 2
Nov17 CIN 800 2 2
Nov17 CIN 600 0 2
Nov17 CIN 400 0 2
Nov17 CIN 200 0 2
*rest of data not included in appendix;
;
  proc sort; by chemical dose;
  symbol v=dot color=blue;

  * For each chemical, test for a stock effect;
  goptions ftext='Arial/bold' htext=12pt;
  proc probit order=internal data=multi; by chemical;
     class stock;
     Stock: model Response/N=  Dose stock / inversecl;
          predpplot var=dose level=('None') cfit=red haxis=2.5 to 3.7 by .1
cframe=ligr inborder;
          ippplot var=dose level=('None') cfit=red vaxis=2.5 to 3.7 by .1 cframe=ligr
inborder;

         * Summarize data across stocks;

  proc summary nway data=multi; class chemical dose; var Response N;
     output out=multisum sum=;
  proc print data=multisum; var chemical dose Response N;

   * For each chemical, analyze summarized data;
```

```
  proc probit order=internal data=multisum; by chemical;
     Overall: model Response/N=  Dose / lackfit aggregate inversecl;
     predpplot var=dose level=('None') cfit=red haxis=2.5 to 3.7 by .1 cframe=ligr
inborder
        vref(intersect)=(0.50,0.99) vreflabels=('Probability=0.50' 'Probability=0.99');
          lpredplot var=dose level=('None') cfit=red haxis=2.5 to 3.7 by .1
cframe=ligr inborder
        vaxis = -15 to 5 by 5;
          ippplot var=dose level=('None') cfit=red vaxis=2.5 to 3.7 by .1 cframe=ligr
inborder
        href(intersect)=0.50 hreflabels=('Probability=0.50');
          ippplot var=dose level=('None') cfit=red vaxis=2.5 to 3.7 by .1 cframe=ligr
inborder
        href(intersect)=0.99 hreflabels=('Probability=0.99');

  proc probit order=internal data=multisum;
     class chemical;
     Chemical: model Response/N = chemical chemical*Dose / lackfit aggregate
noint inversecl;
  run;
  data fill; input chemical $;
     do dose = 2.4 to 3.8 by 0.025;
              output;
     end;
  datalines;
CIN
CRV
EUG
RHO
  data new; set multisum fill;
  proc sort data=new; by chemical dose;
  proc genmod order=internal data=new;
     class chemical;
          output out=stats l=lower p=prob u=upper;
          model Response/N = chemical chemical*Dose / noint dist=binomial
link=probit type3 wald;
  run;

 data forplot; length chemical $ 8; set stats;
    p = Response/N;
    if p = 0 then prob = 0;
         else if p = 1 then prob = 1;
         else if 0 < p < 1 then prob = p;
         if p ne . then do; output; return; end;
    chemical=substr(chemical,1,3)||' prd'; output;
```

```
          prob=lower; chemical=substr(chemical,1,3)||' lci'; output;
          prob=upper; chemical=substr(chemical,1,3)||' uci'; output;
 proc sort data=forplot; by chemical dose;
 proc gplot data=forplot;
    plot prob*dose=chemical / haxis = 2.2 to 3.6 by 0.1
              vaxis = 0 to 1 by 0.1 grid nolegend;
         /* Chemicals in the footnote statement should be in alphabetical order,
and the order of colors should match that given in the symbol statements which
follow*/
          /*footnote c=black 'Chemical:  ' c=blue '  CIN  ' c=red now black'  CRV  '
c=big now vpar '  EUG  ' c=orange '  RHO  ';*/
    label  prob=Probability;
          symbol1 v=circle color=blue i=none;
          symbol2 v=none color=blue i=join line=2;
          symbol3 v=none color=blue i=join line=1;
          symbol4 v=none color=blue i=join line=2;
    symbol5 v=x color=black i=none;
          symbol6 v=none color=black i=join line=2;
          symbol7 v=none color=black i=join line=1;
          symbol8 v=none color=black i=join line=2;
          symbol9 v=triangle color=vpar i=none;
          symbol10 v=none color=vpar i=join line=2;
          symbol11 v=none color=vpar i=join line=1;
          symbol12 v=none color=vpar i=join line=2;
          symbol13 v=square color=orange i=none;
          symbol14 v=none color=orange i=join line=2;
          symbol15 v=none color=orange i=join line=1;
          symbol16 v=none color=orange i=join line=2;

 run; quit; run; footnote;
```

SAS program to compare the effectiveness of essential oil components tested against *L. monocytogenes* using macrodilution method

```
/* This SAS program generates SAS Probit models (probability of lethality) to
compare the effectiveness of essential oil components tested against L.
monocytogenes using macrodilution method (glass tubes)*/

data multi;
     input stock $ chemical $ Dose Response N;
     dose=log10(dose); label dose=Log10(Dose) ppm;
          datalines;
Jun20 CRV 3200 4 4
Jun20 CRV 3000 4 4
```

```
Jun20 CRV 2800 4 4
Jun20 CRV 2600 4 4
Jun20 CIN 1200 4 4
Jun20 CIN 1000 4 4
Jun20 CIN 800 4 4
Jun20 CIN 600 4 4
*rest of data not included in appendix;
;
  proc sort; by chemical dose;
  symbol v=dot color=blue;

  * For each chemical, test for a stock effect;
  goptions ftext='Arial/bold' htext=12pt;
  proc probit order=internal data=multi; by chemical;
     class stock;
     Stock: model Response/N=  Dose stock / inversecl;
          predpplot var=dose level=('None') cfit=red haxis=2.4 to 3.8 by .1
cframe=ligr inborder;
          ippplot var=dose level=('None') cfit=red vaxis=2.4 to 3.8 by .1 cframe=ligr
inborder;

         * Summarize data across stocks;

  proc summary nway data=multi; class chemical dose; var Response N;
     output out=multisum sum=;
  proc print data=multisum; var chemical dose Response N;

   * For each chemical, analyze summarized data;

  proc probit order=internal data=multisum; by chemical;
     Overall: model Response/N=  Dose / lackfit aggregate inversecl;
     predpplot var=dose level=('None') cfit=red haxis=2.5 to 3.7 by .1 cframe=ligr
inborder
        vref(intersect)=(0.25,0.95) vreflabels=('Probability=0.25' 'Probability=0.95');
          lpredplot var=dose level=('None') cfit=red haxis=2.5 to 3.7 by .1
cframe=ligr inborder
        vaxis = -15 to 5 by 5;
          ippplot var=dose level=('None') cfit=red vaxis=2.5 to 3.7 by .1 cframe=ligr
inborder
        href(intersect)=0.95 hreflabels=('Probability=0.95');
          ippplot var=dose level=('None') cfit=red vaxis=2.5 to 3.7 by .1 cframe=ligr
inborder
        href(intersect)=0.25 hreflabels=('Probability=0.25');

  proc probit order=internal data=multisum;
```

```
      class chemical;
      Chemical: model Response/N = chemical chemical*Dose / lackfit aggregate
noint inversecl;
   run;
   data fill; input chemical $;
       do dose = 2.4 to 3.8 by 0.025;
                output;
       end;
   datalines;
CIN
CRV
EUG
LNO
RHO
   data new; set multisum fill;
   proc sort data=new; by chemical dose;
   proc genmod order=internal data=new;
      class chemical;
            output out=stats l=lower p=prob u=upper;
            model Response/N = chemical chemical*Dose / noint dist=binomial
link=probit type3 wald;
   run;

 data forplot; length chemical $ 8; set stats;
     p = Response/N;
     if p = 0 then prob = 0;
           else if p = 1 then prob = 1;
           else if 0 < p < 1 then prob = p;
           if p ne . then do; output; return; end;
           chemical=substr(chemical,1,3)||' prd'; output;
           prob=lower; chemical=substr(chemical,1,3)||' lci'; output;
           prob=upper; chemical=substr(chemical,1,3)||' uci'; output;
 proc sort data=forplot; by chemical dose;
 proc gplot data=forplot; legend1;
     plot prob*dose=chemical / haxis = 2.2 to 3.6 by 0.1
                vaxis = 0 to 1 by 0.1 grid nolegend;
          /* Chemicals in the footnote statement should be in alphabetical order,
and the order of colors should match that given in the symbol statements which
follow*/
           /*footnote c=black 'Chemical:  ' c=blue '  CIN  ' c=red now black'  CRV  '
c=big now vpar '  EUG  ' c=orange '  RHO  ';*/
     label prob=Probability;
           symbol1 v=circle color=blue i=none;
           symbol2 v=none color=blue i=join line=2;
           symbol3 v=none color=blue i=join line=1;
```

```
        symbol4 v=none color=blue i=join line=2;
    symbol5 v=x color=black ci=black cv=black i=none;
        symbol6 v=none color=black i=join line=2;
        symbol7 v=none color=black i=join line=1;
        symbol8 v=none color=black i=join line=2;
        symbol9 v=triangle color=vpar cv=vpar i=none;
        symbol10 v=none color=vpar i=join line=2;
        symbol11 v=none color=vpar i=join line=1;
        symbol12 v=none color=vpar i=join line=2;
        symbol13 v=dot color=lime cv=lime i=none;
        symbol14 v=none color=lime i=join line=2;
        symbol15 v=none color=lime i=join line=1;
        symbol16 v=none color=lime i=join line=2;
        symbol17 v=square color=orange cv=orange i=none;
        symbol18 v=none color=orange i=join line=2;
        symbol19 v=none color=orange i=join line=1;
        symbol20 v=none color=orange i=join line=2;

 run; quit; run; footnote;
```

SAS program to compare the macrodilution and microdilution methods within a component against *L. monocytogenes*

```
/* This SAS program compares the macrodilution and microdilution methods
within a component, against L. monocytogenes */

data lmtubes;
     input stock $ chemical $ Dose Response N;
     method='tubes';
     dose=log10(dose); label dose=log10(dose);
          org='lm';
          datalines;
Jun20 CRV 3200 4 4
Jun20 CRV 3000 4 4
Jun20 CRV 2800 4 4
Jun20 CRV 2600 4 4
Jun20 CIN 1200 4 4
Jun20 CIN 1000 4 4
Jun20 CIN 800 4 4
Jun20 CIN 600 4 4
*rest of data not included in appendix;
;
goptions ftext='Arial/bold' htext=12pt;
data lmmicro;
```

```
      input stock $ chemical $ Dose Response N;
      method='micro';
      dose=log10(dose); label dose=log10(dose);
           datalines;
Oct23 LNO 2800 0 4
Oct23 LNO 2400 0 4
Oct23 LNO 2000 0 4
Oct23 LNO 1600 0 4
Oct23 LNO 1200 0 4
Oct24 LNO 2800 0 4
Oct24 LNO 2400 0 4
Oct24 LNO 2000 0 4
Oct24 LNO 1600 0 4
Oct24 LNO 1200 0 4
*rest of data not included in appendix;
;
data multi; set lmtubes lmmicro;

          * Summarize data across stocks;

   proc summary nway data=multi; class chemical method dose; var Response N;
       output out=multisum sum=;
   proc print data=multisum; var chemical method dose Response N;

    * For each chemical, analyze summarized data;

   proc probit order=internal data=multisum; by chemical;
      class method;
      Overall: model Response/N=  Dose method / lackfit aggregate inversecl;
           predpplot var=dose level=('None') cfit=red haxis=2.4 to 3.8 by .1
cframe=ligr inborder
         vref(intersect)=0.95 vreflabels=('Probability=0.95');
           predpplot var=dose level=('None') cfit=red haxis=2.4 to 3.8 by .1
cframe=ligr inborder
         vref(intersect)=0.25 vreflabels=('Probability=0.25');
           lpredplot var=dose level=('None') cfit=red haxis=2.4 to 3.8 by .1
cframe=ligr inborder
         vaxis = -15 to 5 by 5;
           ippplot var=dose level=('None') cfit=red vaxis=2.4 to 3.8 by .1 cframe=ligr
inborder
         href(intersect)=0.95 hreflabels=('Probability=0.95');
           ippplot var=dose level=('None') cfit=red vaxis=2.4 to 3.8 by .1 cframe=ligr
inborder
         href(intersect)=0.25 hreflabels=('Probability=0.25');
```

```
  proc probit order=internal data=multisum;
     class chemical method;
     Chemical: model Response/N = chemical method chemical*method
chemical*Dose
     method*Dose / lackfit aggregate inversecl;
          where chemical eq 'CIN' or chemical eq 'CRV' or chemical eq 'EUG' or
chemical eq 'RHO';
  run;
```

SAS program to compare the effectiveness of essential oil components tested against *S.* Typhimurium using microdilution method

```
/* This SAS program generates SAS Probit models (probability of lethality) to
compare the effectiveness of essential oil components tested against S.
Typhimurium using microdilution method (microtiter plates)*/

data multi;
     input stock $ chemical $ Dose Response N;
     dose=log10(dose); label dose=Log10(Dose) ppm;
          datalines;
Nov17 CRV 1600 2 2
Nov17 CRV 1400 2 2
Nov17 CRV 1200 0 2
Nov17 CRV 1000 0 2
Nov17 CRV 800 0 4
Nov17 CIN 800 2 2
Nov17 CIN 600 2 2
Nov17 CIN 400 2 2
Nov17 CIN 200 0 4
*rest of data not included in appendix;
;
goptions ftext='Arial/bold' htext=12pt;
  proc sort; by chemical dose;
  symbol v=dot color=blue;

  * For each chemical, test for a stock effect;

  proc probit order=internal data=multi; by chemical;
     class stock;
     Stock: model Response/N=  Dose stock / inversecl;
          predpplot var=dose level=('None') cfit=red haxis=2.2 to 3.7 by .1
cframe=ligr inborder;
          ippplot var=dose level=('None') cfit=red vaxis=2.2 to 3.7 by .1 cframe=ligr
inborder;
```

```
        * Summarize data across stocks;

  proc summary nway data=multi; class chemical dose; var Response N;
     output out=multisum sum=;
  proc print data=multisum; var chemical dose Response N;

   * For each chemical, analyze summarized data;

  proc probit order=internal data=multisum; by chemical;
     Overall: model Response/N=  Dose / lackfit aggregate inversecl;
          predpplot var=dose level=('None') cfit=red haxis=2.2 to 3.7 by .1
cframe=ligr inborder
        vref(intersect)=(0.25,0.95) vreflabels=('Probability=0.25' 'Probability=0.95');
          lpredplot var=dose level=('None') cfit=red haxis=2.2 to 3.7 by .1
cframe=ligr inborder
        vaxis = -15 to 5 by 5;
          ippplot var=dose level=('None') cfit=red vaxis=2.2 to 3.7 by .1 cframe=ligr
inborder
        href(intersect)=0.95 hreflabels=('Probability=0.95');
          ippplot var=dose level=('None') cfit=red vaxis=2.2 to 3.7 by .1 cframe=ligr
inborder
        href(intersect)=0.25 hreflabels=('Probability=0.25');

  proc probit order=internal data=multisum;
     class chemical;
     Chemical: model Response/N = chemical chemical*Dose / lackfit aggregate
noint inversecl;
  run;
  data fill; input chemical $;
     do dose = 2.2 to 3.7 by 0.025;
              output;
      end;
  datalines;
CIN
CRV
EUG
LNO
RHO
  data new; set multisum fill;
  proc sort data=new; by chemical dose;
  proc genmod order=internal data=new;
     class chemical;
          output out=stats l=lower p=prob u=upper;
```

```
          model Response/N = chemical chemical*Dose / noint dist=binomial
link=probit type3 wald;
  run;

 data forplot; length chemical $ 8; set stats;
    p = Response/N;
    if p = 0 then prob = 0;
          else if p = 1 then prob = 1;
          else if 0 < p < 1 then prob = p;
          if p ne . then do; output; return; end;
    chemical=substr(chemical,1,3)||' prd'; output;
          prob=lower; chemical=substr(chemical,1,3)||' lci'; output;
          prob=upper; chemical=substr(chemical,1,3)||' uci'; output;
 proc sort data=forplot; by chemical dose;
  proc gplot data=forplot;
    plot prob*dose=chemical / haxis = 2.2 to 3.6 by 0.1
              vaxis = 0 to 1 by 0.1 grid nolegend;
         /* Chemicals in the footnote statement should be in alphabetical order,
and the order of colors should match that given in the symbol statements which
follow*/
          /*footnote c=black 'Chemical:  ' c=blue '  CIN  ' c=red now black'  CRV  '
c=big now vpar '  EUG  ' c=orange '  RHO  ';*/
    label  prob=Probability;
          symbol1 v=circle color=blue i=none;
          symbol2 v=none color=blue  i=join line=2;
          symbol3 v=none color=blue i=join line=1;
          symbol4 v=none color=blue i=join line=2;
    symbol5 v=x color=black i=none;
          symbol6 v=none color=black i=join line=2;
          symbol7 v=none color=black i=join line=1;
          symbol8 v=none color=black i=join line=2;
          symbol9 v=triangle color=vpar i=none;
          symbol10 v=none color=vpar i=join line=2;
          symbol11 v=none color=vpar i=join line=1;
          symbol12 v=none color=vpar i=join line=2;
          symbol13 v=dot color=lime i=none;
          symbol14 v=none color=lime i=join line=2;
          symbol15 v=none color=lime i=join line=1;
          symbol16 v=none color=lime i=join line=2;
          symbol17 v=square color=orange i=none;
          symbol18 v=none color=orange i=join line=2;
          symbol19 v=none color=orange i=join line=1;
          symbol20 v=none color=orange i=join line=2;

 run; quit; run; footnote;
```

SAS program to compare the effectiveness of essential oil components tested against *S.* Typhimurium using macrodilution method

```
/* This SAS program generates SAS Probit models (probability of lethality) to
compare the effectiveness of essential oil components tested against S.
Typhimurium using macrodilution method (glass tubes) and includes contrasts
that were from the results of the dissertation*/

data multi;
     input stock $ chemical $ Dose Response N;
     dose=log10(dose); label dose=Log10(Dose) ppm;
          datalines;
Mar16 CRV 1600 4 4
Mar16 CRV 1400 3 4
Mar16 CRV 1200 1 4
Mar16 CRV 1000 0 4
Mar16 CRV  800 0 4
Mar16 CIN  400 4 4
Mar16 CIN  200 0 4
*rest of data not included in appendix;
;
 goptions ftext='Arial/bold' htext=12pt;
  proc sort; by chemical dose;
  symbol v=dot color=blue;

  * For each chemical, test for a stock effect;

  proc probit order=internal data=multi; by chemical;
     class stock;
     Stock: model Response/N=  Dose stock / inversecl;
          predpplot var=dose level=('None') cfit=red haxis=2 to 3.4 by .1
cframe=ligr inborder;
          ippplot var=dose level=('None') cfit=red vaxis=2 to 3.4 by .1 cframe=ligr
inborder; run;

         * Summarize data across stocks;

  proc summary nway data=multi; class chemical dose; var Response N;
      output out=multisum sum=;
  proc print data=multisum; var chemical dose Response N;

   * For each chemical, analyze summarized data;
 goptions ftext='Arial/bold' htext=12pt;
```

```
  proc probit order=internal data=multisum; by chemical;
    Overall: model Response/N=  Dose / lackfit aggregate inversecl;
          predpplot var=dose level=('None') cfit=red haxis=2 to 3.4 by .1
cframe=ligr inborder
        vref(intersect)=(0.25,0.95) vreflabels=('Probability=0.25' 'Probability=0.95');
          lpredplot var=dose level=('None') cfit=red haxis=2 to 3.4 by .1 cframe=ligr
inborder
        vaxis = -15 to 5 by 5;
          ippplot var=dose level=('None') cfit=red vaxis=2 to 3.4 by .1 cframe=ligr
inborder
        href(intersect)=0.95 hreflabels=('Probability=0.95');
          ippplot var=dose level=('None') cfit=red vaxis=2 to 3.4 by .1 cframe=ligr
inborder
        href(intersect)=0.25 hreflabels=('Probability=0.25'); run;

  proc probit order=internal data=multisum;
    class chemical;
    Chemical: model Response/N = chemical chemical*Dose / lackfit aggregate
noint inversecl;
  run;
  data fill; input chemical $;
     do dose = 2 to 3.4 by 0.025;
              output;
     end;
  datalines;
CIN
CRV
EUG
LNO
  data new; set multisum fill;
  proc sort data=new; by chemical dose;
  proc genmod order=internal data=new;
    class chemical;
          output out=stats l=lower p=prob u=upper;
          model Response/N = chemical chemical*Dose / noint dist=binomial
link=probit type3 wald;
          contrast 'CIN vs. CRV int' chemical 1 -1 0 0 / wald;
          contrast 'CRV vs. EUG int' chemical 0 1 -1 0 / wald;
          contrast 'EUG vs. LNO int' chemical 0 0 1 -1 / wald;
          contrast 'CIN vs. LNO int' chemical 1 0 0 -1 / wald;
          contrast 'CRV vs. LNO int' chemical 0 1 0 -1 / wald;
          contrast 'CIN vs. avg CRV-LNO int' chemical 1 -0.5 0 -0.5  / wald;
          contrast 'CIN vs. avg CRV-LNO slope' chemical*Dose 1 -0.5 0 -0.5  /
wald;
          contrast 'CIN vs. CRV slope' chemical*Dose 1 -1 0 0 / wald;
```

```
          contrast 'CRV vs. EUG slope' chemical*Dose 0 1 -1 0 / wald;
          contrast 'EUG vs. LNO slope' chemical*Dose 0 0 1 -1  / wald;
          contrast 'CIN vs. LNO slope' chemical*Dose 1 0 0 -1 / wald;
          contrast 'CRV vs. LNO slope' chemical*Dose 0 1 0 -1 / wald;
          estimate 'CIN vs. CRV int' chemical 1 -1 0 0;
          estimate 'CRV vs. EUG int' chemical 0 1 -1 0;
          estimate 'EUG vs. LNO int' chemical 0 0 1 -1;
          estimate 'CIN vs. CRV slope' chemical*Dose 1 -1 0 0;
          estimate 'CRV vs. EUG slope' chemical*Dose 0 1 -1 0;
          estimate 'EUG vs. LNO slope' chemical*Dose 0 0 1 -1;
          estimate 'CRV vs. LNO int' chemical 0 1 0 -1;
          estimate 'CRV vs. LNO slope' chemical*dose 0 1 0 -1;
          contrast 'CIN vs. CRV int/slope' chemical 1 -1 0 0, chemical*dose 1 -1 0
0 / wald;
     contrast 'CIN vs. LNO int/slope' chemical 1 0 0 -1, chemical*dose 1 0 0 -1 /
wald;
          contrast 'CRV vs. LNO int/slope' chemical 0 1 0 -1, chemical*dose 0 1 0 -
1 / wald;
          contrast 'CIN vs. avg CRV-LNO int/slope' chemical 1 -0.5 0 -0.5,
chemical*Dose 1 -0.5 0 -0.5  / wald;
  run;

 data forplot; length chemical $ 8; set stats;
    p = Response/N;
    if p = 0 then prob = 0;
          else if p = 1 then prob = 1;
          else if 0 < p < 1 then prob = p;
          if p ne . then do; output; return; end;
    chemical=substr(chemical,1,3)||' prd'; output;
          prob=lower; chemical=substr(chemical,1,3)||' lci'; output;
          prob=upper; chemical=substr(chemical,1,3)||' uci'; output;
 proc sort data=forplot; by chemical dose;
  proc gplot data=forplot;
    plot prob*dose=chemical / haxis = 2.2 to 3.6 by 0.1
              vaxis = 0 to 1 by 0.1 grid nolegend;
         /* Chemicals in the footnote statement should be in alphabetical order,
and the order of colors should match that given in the symbol statements which
follow*/
          /*footnote c=black 'Chemical:  ' c=blue '  CIN  ' c=red now black'  CRV  '
c=big now vpar '  EUG  ' c=orange '  RHO  ';*/
          label  prob=Probability;
          symbol1 v=circle color=blue i=none;
          symbol2 v=none color=blue i=join line=2;
          symbol3 v=none color=blue i=join line=1;
          symbol4 v=none color=blue i=join line=2;
```

```
    symbol5 v=x color=black i=none;
        symbol6 v=none color=black i=join line=2;
        symbol7 v=none color=black i=join line=1;
        symbol8 v=none color=black i=join line=2;
        symbol9 v=triangle color=vpar i=none;
        symbol10 v=none color=vpar i=join line=2;
        symbol11 v=none color=vpar i=join line=1;
        symbol12 v=none color=vpar i=join line=2;
        symbol13 v=dot color=lime i=none;
        symbol14 v=none color=lime i=join line=2;
        symbol15 v=none color=lime i=join line=1;
        symbol16 v=none color=lime i=join line=2;

 run; quit; run; footnote;
```

SAS program to compare the macrodilution and microdilution methods within a component against *S.* Typhimurium

```
/* This SAS program compares the macrodilution and microdilution methods
within a component, against S. Typhimurium */

data sttubes;
     input stock $ chemical $ Dose Response N;
     method='tubes';
     dose=log10(dose); label dose=log10(dose);
          org='st';
          datalines;
Dec9 RHO 1800 0 5
Dec9 RHO 1600 0 5
Dec9 RHO 1400 0 5
Dec9 RHO 1200 0 5
Jan22 RHO 1400 0 3
Jan22 RHO 1000 0 3
Feb8 RHO 1800 0 3
Feb8 RHO 1600 0 3
Feb13 RHO 2800 0 3
Feb13 RHO 2000 0 3
*rest of data not included in appendix;
;
 goptions ftext='Arial/bold' htext=12pt;
data stmicro;
     input stock $ chemical $ Dose Response N;
     method='micro';
     dose=log10(dose); label dose=log10(dose);
```

```
            datalines;
Nov17 CRV 1600 2 2
Nov17 CRV 1400 2 2
Nov17 CRV 1200 0 2
Nov17 CRV 1000 0 2
Nov17 CRV 800 0 4
Nov17 CIN 800 2 2
Nov17 CIN 600 2 2
Nov17 CIN 400 2 2
Nov17 CIN 200 0 4
*rest of data not included in appendix;
;
data multi; set sttubes stmicro;

          * Summarize data across stocks;

   proc summary nway data=multi; class chemical method dose; var Response N;
       output out=multisum sum=;
   proc print data=multisum; var chemical method dose Response N;

    * For each chemical, analyze summarized data;

   proc probit order=internal data=multisum; by chemical;
      class method;
      Overall: model Response/N=  Dose method / lackfit aggregate inversecl;
            predpplot var=dose level=('None') cfit=red haxis=2.4 to 3.8 by .1
cframe=ligr inborder
          vref(intersect)=0.95 vreflabels=('Probability=0.95');
            predpplot var=dose level=('None') cfit=red haxis=2.4 to 3.8 by .1
cframe=ligr inborder
          vref(intersect)=0.25 vreflabels=('Probability=0.25');
            lpredplot var=dose level=('None') cfit=red haxis=2.4 to 3.8 by .1
cframe=ligr inborder
          vaxis = -15 to 5 by 5;
            ippplot var=dose level=('None') cfit=red vaxis=2.4 to 3.8 by .1 cframe=ligr
inborder
          href(intersect)=0.95 hreflabels=('Probability=0.95');
            ippplot var=dose level=('None') cfit=red vaxis=2.4 to 3.8 by .1 cframe=ligr
inborder
          href(intersect)=0.25 hreflabels=('Probability=0.25');

   proc probit order=internal data=multisum;
      class chemical method;
      Chemical: model Response/N = chemical method chemical*method
chemical*Dose
```

```
      method*Dose / lackfit aggregate inversecl;
            where chemical eq 'CIN' or chemical eq 'CRV' or chemical eq 'EUG' or
chemical eq 'LNO';
   run;
```

SAS program to compare the concentrations of components needed to be lethal against S. Typhimurium and *L. monocytogenes*

```
/* This SAS program compares the concentrations of components needed to be
lethal against S. Typhimurium and L. monocytogenes within the macrodilution
method */

data lmtubes;
      input stock $ chemical $ Dose Response N;
      method='tubes';
      dose=log10(dose); label dose=log10(dose);
            org='LM';
            datalines;
Jun20 CRV 3200 4 4
Jun20 CRV 3000 4 4
Jun20 CRV 2800 4 4
Jun20 CRV 2600 4 4
Jun20 CIN 1200 4 4
Jun20 CIN 1000 4 4
Jun20 CIN 800 4 4
Jun20 CIN 600 4 4
*rest of data not included in appendix;
;
goptions ftext='Arial/bold' htext=12pt;
data sttubes;
      input stock $ chemical $ Dose Response N;
      method='tubes';
      dose=log10(dose); label dose=log10(dose);
            org='ST';
            datalines;
Mar16 CRV 1600 4 4
Mar16 CRV 1400 3 4
Mar16 CRV 1200 1 4
Mar16 CRV 1000 0 4
Mar16 CRV  800 0 4
Mar16 CIN  400 4 4
Mar16 CIN  200 0 4
*rest of data not included in appendix;
```

```
;
data multi; set lmtubes sttubes;

          * Summarize data across stocks;

   proc summary nway data=multi; class chemical org dose; var Response N;
      output out=multisum sum=;
   proc print data=multisum; var chemical org dose Response N;

    * For each chemical, analyze summarized data;

   proc probit order=internal data=multisum; by chemical;
      class org;
      Overall: model Response/N=  Dose org / lackfit aggregate inversecl;
            predpplot var=dose level=('None') cfit=red haxis=2.4 to 3.8 by .1
cframe=ligr inborder
         vref(intersect)=0.95 vreflabels=('Probability=0.95');
            predpplot var=dose level=('None') cfit=red haxis=2.4 to 3.8 by .1
cframe=ligr inborder
         vref(intersect)=0.25 vreflabels=('Probability=0.25');
            lpredplot var=dose level=('None') cfit=red haxis=2.4 to 3.8 by .1
cframe=ligr inborder
         vaxis = -15 to 5 by 5;
            ippplot var=dose level=('None') cfit=red vaxis=2.4 to 3.8 by .1 cframe=ligr
inborder
         href(intersect)=0.95 hreflabels=('Probability=0.95');
            ippplot var=dose level=('None') cfit=red vaxis=2.4 to 3.8 by .1 cframe=ligr
inborder
         href(intersect)=0.25 hreflabels=('Probability=0.25');

   proc probit order=internal data=multisum;
      class chemical org;
      Chemical: model Response/N = chemical org chemical*org  chemical*Dose
      org*Dose / lackfit aggregate inversecl;
            where chemical eq 'CIN' or chemical eq 'CRV' or chemical eq 'EUG' or
chemical eq 'RHO';
   run;
```

SAS program to compare the differences in biomass and fatty acids among CIN-treatment and control groups within 2h or 24h for *S*. Typhimurium

```
/* This SAS program compares the differences in biomass and fatty acids among
CIN-treatment and control groups within 2h or 24h for S. Typhimurium.  Arnold
Saxton's mmaov macro analyzed the imported 2h or 24h CIN data set using
```

Randomized Block Design with blocking on each replicate. The first %mmaov compares biomass among groups, averaging 2h and 24h together. Also includes comparing % fatty acids within a group and within 2h or 24h. Raw data not included in appendix. */

```
PROC IMPORT OUT= WORK.CIN
        DATAFILE= "C:\Documents and Settings\Valerie Ling\My
Documents\Val's Research\Results\CINLipids\CINData.xls"
        DBMS=EXCEL2000 REPLACE;
    SHEET="Sheet1$";
    GETNAMES=YES;
RUN;
%include 'c:\mmaov.sas';
%mmaov(cin,bio,class=block treat hour,fixed=treat hour(treat), random=block);
run;
data cin2; set cin; if hour eq 2;
data cin24; set cin; if hour eq 24;
%include 'c:\mmaov.sas';
%mmaov(cin2,bio,class=block treat,fixed=treat,random=block); run;
%mmaov(cin24,bio,class=block treat,fixed=treat,random=block); run;
%mmaov(cin2,c12  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin24,c12  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin2,c14  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin24,c14  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin2,c15  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin24,c15  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin2,c16  1w7c,class=block treat,fixed=treat,random=block); run;
%mmaov(cin24,c16  1w7c,class=block treat,fixed=treat,random=block); run;
%mmaov(cin2,c16  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin24,c16  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin2,cy17  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin24,cy17  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin2,c17  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin24,c17  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin2,c18  1w9c,class=block treat,fixed=treat,random=block); run;
%mmaov(cin24,c18  1w9c,class=block treat,fixed=treat,random=block); run;
%mmaov(cin2,c18  1w7c,class=block treat,fixed=treat,random=block); run;
%mmaov(cin24,c18  1w7c,class=block treat,fixed=treat,random=block); run;
%mmaov(cin2,c18  1w7t,class=block treat,fixed=treat,random=block); run;
%mmaov(cin24,c18  1w7t,class=block treat,fixed=treat,random=block); run;
%mmaov(cin2,c18  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin24,c18  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin2,cy19  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin24,cy19  0,class=block treat,fixed=treat,random=block); run;
%mmaov(cin2,nsats,class=block treat,fixed=treat,random=block); run;
```

%*mmaov*(cin24,nsats,class=block treat,fixed=treat,random=block); run;
%*mmaov*(cin2,monos,class=block treat,fixed=treat,random=block); run;
%*mmaov*(cin24,monos,class=block treat,fixed=treat,random=block); run;
%*mmaov*(cin2,cy17to16,class=block treat,fixed=treat,random=block); run;
%*mmaov*(cin24,cy17to16,class=block treat,fixed=treat,random=block); run;
%*mmaov*(cin2,cy19to18,class=block treat,fixed=treat,random=block); run;
%*mmaov*(cin24,cy19to18,class=block treat,fixed=treat,random=block); run;
%*mmaov*(cin2,satunsat,class=block treat,fixed=treat,random=block); run;
%*mmaov*(cin24,satunsat,class=block treat,fixed=treat,random=block); run;

proc sort data=cin; by treat hour block;
proc transpose data=cin out=cinout; by treat hour block; var c12_0--cy19_0;
proc sort data=cinout; by treat hour block descending col1; run;
proc anova; by treat hour; class _name_; model col1=_name_;
means _name_ / tukey lines; run; quit; run;

SAS program to compare the differences in biomass and fatty acids between the 2 or 24h data within a CIN-treatment or control group for *S.* Typhimurium

/* This SAS program compares the differences in biomass and fatty acids between the 2 or 24h data within a CIN-treatment or control group for S. Typhimurium. */

```
PROC IMPORT OUT= WORK.CIN
        DATAFILE= "C:\Documents and Settings\Valerie Ling\My Documen
ts\Val's Research\Results\CINLipids\CINData.xls"
        DBMS=EXCEL2000 REPLACE;
    SHEET="Sheet1$";
    GETNAMES=YES;
RUN;
```

*test for interaction term, where is the interaction? Use Bonferonni correction. This is alpha .10 divided by 6 follow-up tests used to test interaction between hour and treatment i.e. each pair - treat 1 at 2h vs 24h. .1/6 = 0.017 new alpha with B. correction to minimize Type I error, which increases with # of tests. We can be sure that the differences we find are true and not due to chance.';

```
 proc sort data=cin; by treat;run;
 proc glm data=cin;
 by treat;
 class hour;
 model bio=hour;
 means hour; lsmeans hour;
 run; quit;
```

```
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model c12  0=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model c14_0=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model c15_0=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model c16  1w7c=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model c16  0=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model cy17  0=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
```

```
 class hour;
 model c17_0=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model c18_1w9c=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model c18  1w7c=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model c18  1w7t=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model c18  0=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model cy19_0=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model nsats=hour;
 means hour; lsmeans hour;
```

```
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model monos=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model cy17to16=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model cy19to18=hour;
 means hour; lsmeans hour;
 run; quit;
proc sort data=cin; by treat;run;
proc glm data=cin;
 by treat;
 class hour;
 model satunsat=hour;
 means hour; lsmeans hour;
 run; quit;


/* To run the same SAS programs for CRV, import the CRV data file and use
“Find and Replace” under the “Edit” menu to find “CIN” and replace with “CRV”  */
```

VITA

Valerie W. Ling was born in New York City, NY in 1975. She attended public schools in Queens County and Stuyvesant High School of Science in Manhattan. In May 1997, she graduated from Cornell University, College of Agriculture and Life Sciences with a Bachelor of Science degree in Nutrition, Food, and Agriculture. She received a Master of Science degree in Recreation, Tourism, and Hospitality Management with a concentration in Foodservice and Lodging Administration from the University of Tennessee, Knoxville in August 1999. During her Master of Science program she completed the Dietetic Internship program and is currently a Registered Dietitian. As part of the Dietetic Internship she volunteered with the Food Safety and Education Staff at Food Safety and Inspection Service, U.S. Department of Agriculture, in Washington, D.C. She began pursuing her doctorate in Food Science and Technology at the University of Tennessee, Knoxville in August 1999 and will continue to work in the department.