THE UNIVERSITY OF MANITOBA

THE INFLUENCE OF EUGENOL, A SPICE-BASED PHENOLIC ANTIOXIDANT ON THE GROWTH OF MEAT SAUSAGE LACTIC ACID BACTERIA

by
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AKINTOKUNBO ADEMOLA ADEJUMO

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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DEDICATION

With due gratitude to the Almighty God,
this thesis is dedicated to,

my parents,
Chief and Mrs. Akin Adejumo,

my wife,
Toyin,

my son,
Akinlolu,

and to the rest of my family,
all of whose love and patience has sustained me,

and especially to,
the peoples of Nigeria and Canada.

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ABSTRACT.

The antibacterial activity of eugenol the major active principle of clove oil was investigated using two homofermentative Lactobacillus species, designated as lactose (LF) and non-lactose (NLF) fermenting. These organisms were isolated from an Iraqi fermented meat sausage, Pastirma.

Initial studies established that increasing concentrations of eugenol (0.0 -0.5 for the LF and 0.0-0.15%,v/v for the NLF) inhibited growth, pH and titratable acidity development in both the LF and the NLF cultures, and that the LF culture was more susceptible to the effects of eugenol. The minimum inhibitory concentration (MIC) of eugenol was 0.06 and 0.08%,v/v for the LF and the NLF organisms, respectively, using an inoculum of 10^5CFU/mL in APT broth. The length of cultivation also played a significant role in the susceptibility of these organisms to eugenol. Sublethal concentrations of eugenol (0.03% and 0.06% for the LF and NLF respectively) incorporated into APT broth, resulted in morphological changes to these organisms which were manifested by swelling. This cellular swelling was shown to increase biomass determinations during time-course studies. A decrease in cellular adenosine triphosphate (ATP) accumulation in both the LF and the NLF was observed as the eugenol concentration was increased, suggesting leakage of either ATP or phosphate, or less accumulation of ATP in the cell.

Filtrates obtained from these two organisms grown in the presence of increasing concentrations of eugenol showed increasing UV absorbancy at 260 and 280 nm, indicating leakage of intra-

cellular contents (nucleotides and proteinaceous materials, respectively). Increase in absorbance was detected before any appreciable lethality took place with both the LF and the NLF culture, suggesting that lethality is at least, partially a result of leakage, and not vice-versa.. Cell filtrates of LF and NLF treated with increasing concentrations of eugenol also showed increasing release or leakage of intracellular proteins.

Growth of both organisms in the presence of eugenol caused some significant changes in the cellular fatty acid type and composition of these organisms. In both cases, increasing eugenol concentrations significantly caused a decrease in the saturated:unsaturated fatty acid ratio. The higher ratio observed in the NLF may explain the higher degree of resistance of this organism to eugenol than the LF organism. Increasing concentrations of eugenol also appeared to cause increased synthesis of some fatty acids not normally found in the lactobacilli, such as branch chain (iso- and ante-iso-) and the saturated, but odd-numbered carbon (n-C15:0 and n-C17:0) fatty acids. Repeated transfer of eugenol-treated LF and NLF cultures into fresh media without eugenol, indicated that the organisms would attempt to recover from the effect of eugenol by synthesizing more of the saturated, and less of the unsaturated and unusual fatty acids.

Microscopic observations of the LF and NLF cells treated with eugenol indicated swelling and lysis of the cells. The treated cells were translucent, and appeared elongated compared to the normal cells (controls) for both organisms.

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1. INTRODUCTION.

Spices and herbs are common food materials, used mainly for their flavor and aroma properties. These properties largely reside with compounds such as alcohols, esters, terpenes, phenols, and organic acids (Pruthi, 1980; Shelef et al.,1980). Although spices contribute mainly to the development of flavor, they have also been shown to possess inhibitory/stimulatory microbial activity, thus influencing the growth of certain microorganisms (Karaioannoglou et al.,1977; Salzer et al.,1977; Zaika and Kissinger, 1979, 1981; Huhtanen, 1980; Skjelkvale and Nes, 1981). Many workers have demonstrated the antimicrobial activities of spices against a wide variety of microorganisms including bacteria (Hoffman and Evans, 1911; Beuchart, 1976; Shelef et al.,1980), molds and fungi (Kato et al.,1973; Sharma et al.,1979; Hitokoto et al.,1980; Azzouz and Bullerman, 1982) in a wide variety of food materials (Anand and Jahar, 1957; Bullerman, 1974; Salzer et al.,1977; Nkanga and Uraih, 1981) as well as in laboratory media (Shelef et al.,1980).

Although spices are still commonly used in their natural forms, extracts are becoming more increasingly popular. Such extracts include oleoresins and spice essential oils (Pruthi, 1980; Nes and Skjelkvale, 1982). Spice essential oils contain many volatile constituents, which are also known to possess antimicrobial properties. These antimicrobial components of essential oils include allicin in garlic, allyl isothiocyanate in mustard, cinnamic aldehyde and eugenol in cinnamon, and eugenol in cloves (Salzer, 1977; Shelef, 1983). However, although it has now

been recognized that both the essential oils and their components are even more potent in their antimicrobial activity than the natural spices, very limited research has been done in this area. Nevertheless, a few workers have recorded growth and end-product inhibition by these essential oils and their volatile components with fungi (Cavallito and Bailey,1944; Maruzzella and Liguori, 1958; Bullerman et al.,1977; Boonchird and Flegel, 1982; Jay and Rivers, 1984), and with Gram positive and Gram negative bacteria (Maruzzella and Henry, 1958; Katayama and Nagai, 1960; Miyao, 1975; Jay and Rivers, 1984).

Eugenol, the major active component of cloves and clove oil, carnation, laurel and cinnamon leaf oils, is a phenolic compound which is a derivative of o-methoxyphenol, possessing a hydroxyl group directly bonded to a benzene ring (Katayama and Nagai, 1960). Clove oil may contain as much as 95% eugenol (Pruthi,1980). This essential oil has already found uses as a flavoring agent, in cosmetics manufacture, and as an anaesthetic in dental surgery. It has been reportedly used in foods such as non-alcoholic beverages, ice cream, candies, baked goods, chewing gums and meats at various concentrations, mainly as an antioxidant. (Fenaroli, 1975). Though it is known to possess antimicrobial activity, (Hoffman and Evans, 1911; Katayama and Nagai 1960; Miyao 1975; Boonchird and Flegel 1982; Jay and Rivers, 1984) eugenol has not been widely investigated despite its approval for food use (FEMA, 1965; Council of Europe, 1974; Fenaroli, 1975).

Since eugenol is a phenolic compound, its antimicrobial activity has been linked to that of other phenolic antioxidants

and antimicrobial agents currently used in foods, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and mono-tertiary butyl hydroquinone (TBHQ)(Bullerman et al., 1977). It is now known that these phenolic antioxidants also have antimicrobial activity against bacteria, viruses, molds and protozoa (Chang and Branen, 1975; Snipes et al., 1975; Surak et al.,1976a, 1976b; Robach et al., et al., 1977; Kim et al., 1978; Davidson and Branen, 1980a, 1980b; Raccach, 1984) although very little research has been done on their mechanism of inhibition. (Branen et al., 1980). Raccach (1984), proposed that the antimicrobial activity of phenolic antioxidants appears to depend on the presence of a hydroxyl group on the molecule, the lipid solubility of the compound and the degree of steric hindrance. Several workers have reported that the mechanism of inhibition of phenolic compounds involves the function and composition of the cellular membrane, the synthesis of DNA, RNA, protein and lipid, and the function of the mitochondrion (Metcalfe, 1971; Surak et al., 1976a, 1976b; Hammerstedt et al., 1976; Wanda et al., 1976; Singer and Wan, 1977; Surak,1977; Davidson and Branen, 1980b; Degre' and Sylvestre,1983). Since eugenol is a a natural component of food, it would be considered more desirable and more acceptable to the discriminating consumer than the presently used synthetic phenolic antioxidants.

This study was initiated to examine the antibacterial activity and possible inhibitory mechanism of eugenol against two lactic acid bacteria isolated from an Iraqi fermented meat sausage, Pastirma. The objectives of this study specifically include:

(I)the effect of eugenol on bacterial growth and by-product formation concomitant with intracellular materials leakage and lethality, and (II) the changes in the composition and type of cellular fatty acids in the bacteria, as affected by the presence of eugenol during growth.

2. REVIEW OF LITERATURE.

2.1. SPICES AND HERBS.

Spices and herbs are dried aromatic plant products used since antiquity to flavor foods and beverages. They are widely used to impart flavor and aroma to foods. Some spices are also used in perfumery and cosmetics, while others are used to soothe and heal through medicinal incorporation (Pruthi,1980). Included in herbs and spices are leaves (rosemary, sage), flowers and flower buds (clove), bulbs (garlic, onion), rhizomes (asafoetida), fruit (pepper, cardamom), and other parts of the plant. Frequently, blends of several spices are used. Spices are selected and classified on the basis of the flavor and color that they impart to foods, that is, hot (pepper), pungent (garlic), herbaceous (sage, rosemary), aromatic (clove, cinnamon), and coloring (turmeric; Clark, 1970).

2.1.1. Chemical components of spices and herbs.

The use of gas chromatography as an analytical method for the identification of spice and herb components has yielded important information regarding their flavor, aroma and color characteristics (Salzer, 1977). Many workers have reported that the major flavor components of spices and herbs consist of such compounds as alcohols, aldehydes, esters, terpenes and sesquiterpenes, phenols, organic acids and others, some of which have not yet been fully identified (Pruthi,1980; Shelef et al.,1980). Coloring pigments in spices and herbs include carotenoids and anthocyanin in red pepper, crocin in saffron, curcumin in tur-

meric, phenols in nutmeg, mace and cloves, and isothiocyanate in mustard seeds (Pruthi, 1980), while pungent principles such as zingerone and gingerol in ginger root, capsaicin in red pepper, eugenol in cloves, and isoeugenol in nutmeg, have been found to be derivatives of o-methoxyphenol (Kulka, 1967).

2.1.2. Antimicrobial properties of spices.

Many researchers have concluded that most spices possess antimicrobial activities against a wide variety of microorganisms. Hoffmann and Evans (1911) demonstrated the preservative actions of nutmeg, cinnamon, allspice, cloves, ginger, black pepper, Cayenne pepper and mustard in apple sauce and ketchup. Beuchart (1976) concluded that spices, and their essential oils as well as organic acids are particularly sensitive to Vibrio parahemolyticus. Shelef et al. (1980) tested the sensitivity of 22 Gram negative and 24 Gram positive bacteria to sage, rosemary and allspice at spice concentrations up to 2% in the growth media and concluded that Gram positive bacteria were more sensitive than Gram negative bacteria, with sage having the highest antimicrobial activity, followed by rosemary. Allspice was shown to be the least effective.

Huhtanen (1980) concluded that out of 33 alcoholic extracts of spices tested on Clostridium botulinum, mace and achiote were the most inhibitory. These spices gave a minimum inhibitory concentration (MIC) of 31 mg/L, followed by nutmeg, bay leaf,white and black pepper with an MIC of 125 mg/L; paprika, rosemary, oregano, turmeric and thyme each gave an MIC of 500 mg/L, while the

rest showed little or no inhibition at 2000 mg/L. Zaika and Kissinger (1981) reported that increasing concentrations of oregano from 0.5 to 8.0 g/L in a liquid medium resulted in either stimulation, delay, or inhibition of acid production and viability of both Lactobacillus plantarum and Pediococcus cerevisiae. Azzouz and Bullerman (1982), found that of 26 herbs, spices, plant components and commercial antifungal agents tested, cloves, mustard, cinnamon, garlic, allspice and oregano at the 2% level in potato dextrose agar, completely inhibited growth of seven mycotoxigenic molds for various times up to 21 days. Recent studies on in vitro antibacterial activities of some ground spices or their extracts are presented in Table 1.

2.1.3. Effects of spices on fermented meat products.

Nes and Skjelkvale (1982), investigated the effect of spices and oleoresins on the fermentation properties of 3 commercially available starter cultures of L. plantarum, and found that natural spices accelerated the fermentation process in the production of dry salami sausage, while oleoresins showed no effect. Karaioannoglou et al.(1977); Salzer et al.(1977); Zaika and Kissinger (1979, 1981) and Huhtanen (1980), in separate studies, reported that spices not only contribute to flavor development in dry sausage, but also possess inhibitory or stimulatory activity, thus influencing the growth of certain bacteria. Of the various bacteria studied, L. plantarum was found to be most frequently stimulated by the different spices tested, with respect to growth and acid production (Zaika and Kissinger,1979; Skjelkvale and

Table 1. Summary of recent studies of *in vitro* antimicrobial activities of some ground spices or their extracts (adapted from Shelef, 1983).

Spice	Inhibited Organisms	Source
Garlic	*S. typhimurium*, *E. coli*	Johnson and Vaughn, 1969.
	S. aureus, *E. coli*	Powers *et al.*, 1975.
	B. cereus	Karaioannoglou, 1977.
Onion	*A. flavus*, *A. parasiticus*	Sharma *et al.*, 1979.
Cloves	*P. cerevisiae*, *L. plantarum*	Zaika and Kissinger, 1979.
	Aspergillus (3 strains)	Hitokoto *et al.*, 1980.
	Bacteria, yeasts, molds	Kato *et al.*, 1973.
Cloves	mycotoxigenic *Aspergillus*	Azzouz and Bullerman, 1982.
Cinnamon	*Penicillium* (7 strains)	
Oregano	*Salmonella*	Julseth and Diebel, 1974.
	V. parahemolyticus	Beuchart, 1976.
	Toxigenic *Apergillus* (3 strains)	Llewellyn *et al.*, 1981.
Rosemary	*B. cereus*, *S. aureus*	Shelef *et al.*, 1980.
Sage	*V. parahemolyticus*	Shelef *et al.*, 1980.

Nes,1981). Zaika and Kissinger (1984) in another study, identified manganese as a factor in spices responsible for the enhancement of acid production by meat starter bacteria with cloves having the highest Mn^{++} content and exerting the greatest stimulatory effect. This effect was greatly dependent on the growth medium employed. These authors reported that in media such as APT or MRS broths, which give optimum growth of lactic acid bacteria, the content of Mn^{++} was too high ($7.1*10^{-4}$M Mn^{++} in APT and $2.2*10^{-4}$M in MRS) to allow the stimulatory effect of spices to be significant. Thus when a 0.1N HCl extract of cloves was added to APT medium ($4.3*10^{-6}$M added Mn^{++}), no significant enhancement of acid production was observed, since acid production by L. plantarum was already at a maximum in the medium (Zaika and Kissinger,1984).

Zaika et al.(1978) also reported that a mixture of 9 spices used in Lebanon bologna formulation enhanced the fermentation of these sausages. Nkanga and Uraih (1981) examined the effect of 1% of cloves, onion, ginger and black and white peppers on the growth of Staphylococcus aureus in meat homogenates (10% meat and 1% NaCl). The authors reported that growth in samples containing cloves and red pepper was inhibited; onion and black pepper were less inhibitory, and a level of 10% cloves or red pepper in meat homogenates was bactericidal against S. aureus. More recently, Al-Jalaly (1985) isolated two Lactobacillus spp. from an Iraqi fermented meat sausage, Pastirma, and investigated the effects of 10 spices and spice mixtures used in the sausage formulation on the isolates. It was found that increasing concentrations of the

spice mixture in APT broth progressively increased the time for maximum growth, titratable acidity and pH development in both cultures. Furthermore, investigation of the MIC of each individual spice present in the mixture showed that clove, black pepper and nutmeg were the most effective against the isolates, while cardamon, cinnamon, coriander and cumin were the least effective (Al-Jalaly,1985).

A summary of some recent studies on the microbial inhibition by spices in various foods is presented in Table 2.

2.1.4. Effect of spices on bacterial spores.

Very little is known regarding the influence of spices on bacterial spores. However, Karaioannoglou (1977) reported that garlic extract (1% in enriched BHI) inhibited the outgrowth of Bacillus cereus spores. Shelef et al.(1984), in studying the effect of sage on B. cereus spores, reported that the presence of sage allowed germination to occur, but outgrowth was inhibited, the degree of inhibition depending on the spore inoculum.

2.2. SPICE ESSENTIAL OILS.

Essential oils are the volatile oils obtained from plants or from parts thereof by (1) water distillation, (2) steam distillation, (3) enzymatic action followed by steam distillation or, (4) water and steam distillation (Pruthi, 1980). Extracts from spices include mainly essential oils, oleoresins, spice essences, emulsions, and spice decoctions.

Table 2. Microbial inhibition of some microorganisms by some spices in foods. (after Shelef, 1983)

Spice	Effective level, (%)	Food	Organism	Source
Cinnamon	3.0	mango pickle	A. niger	Anand and Jahar, 1957.
	1.0	raisin bread	A. parasiticus	Bullerman, 1974.
Cloves	0.6	mango pickle	A. niger	Anand and Jahar, 1957.
	1.0	meat homogenate (10%)	S. aureus	Nkanga and Uraih, 1981.
Red pepper	1.0	meat homogenate	S. aureus	
Pepper	.08 - .02	fresh sausage	E. coli	Salzer et al., 1977.
	0.3		lactobacilli	
			micrococci	

2.2.1. Volatile components of spice essential oils.

Most essential oils of spices consist of mixtures of hydrocarbons (terpenes, sesquiterpenes, etc.), oxygenated compounds (alcohols, esters, aldehydes, phenols, ketones, etc.) and a small percentage of non volatile residues such as waxes and paraffins (Salzer, 1977; Pruthi,1980). The advantages of spice essential oils include, hygiene, being free of all microorganisms, standard flavoring strength, their flavor quality is consistent with that of the raw material, they do not impart color to the product, they are free from enzymes and tannins. In addition, they are reasonably stable on storage and less bulky than the raw material (Pruthi, 1980).

The major active principles in spice essential oils are presented in Table 3.

2.2.2. In vitro antimicrobial activity of spice essential oils.

The association of the volatile essential oil fraction with the antimicrobial activity of spices has long been recognized. Janke (1923), reported that mustard oil, at a concentration of 0.006% suppressed the development of mycodermae in pickles, and a lower concentration was effective against acetic acid bacteria in pickled tomatoes packed in 2.5% acetic acid. Maruzzella and Liguori (1958) confirmed the *in vitro* antifungal activity of 92 volatile oils, with *Streptomyces venezuelae* being the most susceptible, and *Candida krusei* being the most resistant to the oils. Chamberland (1887) showed that cinnamon oil was lethal to spores of the anthrax bacillus, while Hoffmann and Evans (1911) and Cor-

Table 3. Spice essential oils and their major active principles.

Essential Oil	Major active principle	% Active principle	Source
Clove	eugenol	85 - 92	Pruthi, 1980.
		95	Merory, 1960.
		75	Salzer, 1977.
Cinnamon	cinnamic aldehyde	42 - 75	Pruthi, 1980.
		40 - 80	Salzer, 1977.
Ajowan	thymol	45 - 55	Pruthi, 1980.
Garlic	allicin	u[1]	Pruthi, 1980.
Turmeric	curcurmin	u	
Oregano	carvacol	u	
Thyme	thymol	u	Salzer, 1977.
Pimento (allspice)	eugenol methyl ether	u	
	eugenol	60 - 80	
Nutmeg	B-pipene, sabinene	65 - 80	
Pepper	piperine	98	

[1] - Undetermined

ran and Edgar (1933), demonstrated the germicidal properties of spices and essential oils in experiments aimed at food preservation. Prasad and Joshi (1929), recommended a method of preserving fruits using an infusion of ground cloves and salt. Frazier (1967), reported that cinnamon and clove oils were the most effective inhibitors of yeasts and bacteria; in high concentrations these compounds permitted mold growth but inhibited asexual spore formation.

Maruzzella and Henry (1958) also reported that when 35 volatile oils were tested against 5 bacteria, eucalyptus, cinnamon and oreganum oils exhibited the greatest antibacterial activity, with Gram positive bacteria showing more susceptibility to the volatile oils than Gram negative bacteria. Bullerman et al.(1977) reported that cinnamon and clove oils were inhibitory towards growth and aflatoxin production by Aspergillus parasiticus at 200 to 250 mg/L, respectively. Recent studies on in vitro antimicrobial activities of some spice essential oils are presented in Table 4.

2.2.3. Active antimicrobial compounds in spice essential oils.

Data on the essential oil content and some of the recognized antimicrobial components in selected spices are summarized in Table 5.

Limited studies have been reported concerning the antimicrobial effects of the primary constituents of spice essential oils. Bullerman et al. (1977) reported growth and aflatoxin inhibition by cinnamaldehyde and eugenol, at levels above 200 mg/L, and con-

Table 4. Recent reports on *in vitro* antimicrobial activities of spice essential oils (after Shelef, 1983)

Spice essential oils	Inhibited organisms	Source
Oregano, thyme	*V. parahemolyticus*	Beuchart, 1976.
Sage	*B. cereus*	Shelef *et al.*, 1984.
Rosemary	*S. aureus*	Farbood *et al.*, 1976.
Garlic (Allicin)	*Candida albicans*, other pathogenic fungi.	Yamada and Azuma, 1977.
Cinnamon, clove	*A. parasiticus*	Bullerman *et al.*, 1977.

Table 5. Essential oils of some spices with antimicrobial activities (after Shelef,1983)

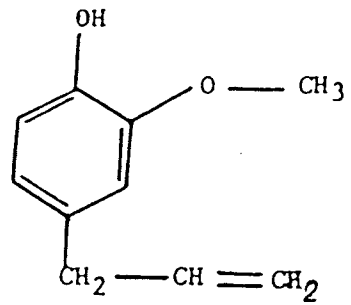
Spice	Proximate essential oil content, (%)	Antimicrobial components
Garlic	0.3-0.5	allicin
Mustard	0.5-1.0	allyl isothiocyanate
Cinnamon	0.5-2.0	cinnamic aldehyde
		eugenol
Clove	16.0-18.0	eugenol
Sage	0.7-2.0	thymol eugenol
Oregano	0.8-0.9	thymol carvacol

cluded that the levels of these two compounds commonly found in candies and baked goods could be effective as growth inhibitors of fungi. Other researchers have reported the inhibitory effects of various spice essential oil components against various microorganisms implicated in either food spoilage or toxin production (Hoffmann and Evans,1911; Cavallito and Bailey,1944; Maruzzella and Liguori, 1958; Katayama and Nagai,1960; Boochird and Flegel,1982).

2.3. EUGENOL.

Cloves contain essential oil ranging from 15 to 20%, of which 85 to 95% is eugenol (Merory,1960; Pruthi,1980). Eugenol, a phenolic compound, is the main constituent of several important essential oils such as oils of clove, clove stem and leaf, pimento, cinnamon leaf, laurel leaf and carnation (Food Chemicals Codex,1972; Fenaroli, 1975; Opdyke,1975; Salzer,1977; Pruthi,1980). It is a colorless to pale yellow liquid, with a strongly aromatic odor of clove, and a spicy pungent taste. Eugenol is also known as eugenic acid; 4-allyl- guaiacol; 1-hydroxy-2-methoxy-4-allylbenzene; 4-allyl-2-methoxyphenol or 2-methoxy-4-prop-2-enylphenol. It has a molecular weight of 164.21, and the empirical formula, $C_{10}H_{12}O_2$. Eugenol is a derivative of o-methoxyphenol, possessing a hydroxyl group directly bonded to a benzene ring (Figure 1) and darkens and thickens upon exposure to air. It is slightly soluble in water, and is miscible with ether, alcohol, chloroform and with fixed oils (Fenaroli,1975).

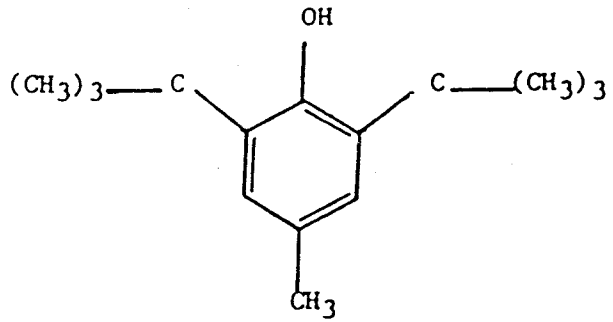
Figure 1 The chemical structures of eugenol, BHA and BHT.



Eugenol

(4-allyl-2-methoxyphenol)

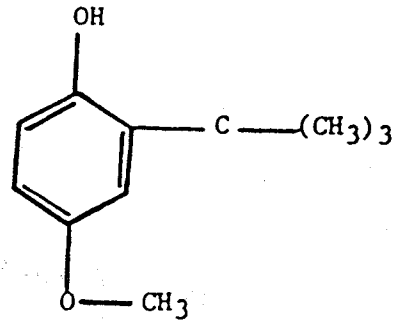
$C_{10}H_{12}O_2$ Mol. Wt. 164.21

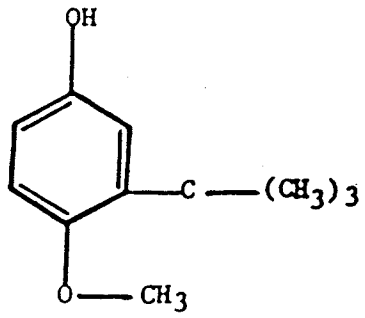


Butylated hydroxytoluene(BHT)

(2,6-di-tert-butyl-o-cresol;
4-methyl-2-6-di-tert-butylphenol)

$C_{15}H_{24}O$ Mol. Wt. 220.34





Butylated hydroxyanisole(BHA)

3-BHA(3-tert-butyl-4-hydroxyanisole;
2-tert-butyl-4-methoxyphenol)

2-BHA(2-tert-butyl-4-hydroxyanisole;
3-tert-butyl-4-methoxyphenol)

$C_{11}H_{16}O_2$ Mol. Wt. 180.24

2.3.1. Current status and reported uses of eugenol.

Eugenol has already found uses as a flavoring agent in foods, in cosmetics and perfume manufacture and as an anesthetic in dental surgery (Food Chemicals Codex,1972; Fenaroli,1975; Opdyke,1975). Eugenol was granted generally recognized as safe (GRAS) status by the Flavoring Extract Manufacturer's Association (FEMA,1965), and is approved by the U.S. Federal Department of Agriculture as GRAS for food use. The Council of Europe (1974), included eugenol in the list of artificial flavoring substances that may be added to foodstuffs without hazard to public health. Eugenol has also been reportedly used in a wide variety of foods at various approved concentrations (Table 6.). The incorporation of eugenol in foods is to be expected, since it is obtained as a natural product from naturally edible plant materials.

2.3.2. The antimicrobial activity of eugenol.

Although the literature does not abound on the antimicrobial activity of eugenol, a few workers have nonetheless confirmed this property. Early reports by Hoffmann and Evans (1911) confirmed the preservative quality of eugenol in ketchup and apple-sauce, as well as in growth media. Katayama and Nagai (1960) in an attempt to study the chemical structure and antibacterial activity of various terpenes, including eugenol, showed that at dilutions of 2000 or more, eugenol was inhibitory towards the growth of Bacillus subtilis, Salmonella enteritidis, Staphylococcus aureus, Pseudomonas aeruginosa, Proteus morganii and Escherichia coli (Table 7.). Miyao (1975) reported that eugenol was

Table 6. Reported uses of eugenol (Fenaroli, 1975)

Food item	Concentration (mg/L)
Non alcoholic beverages	1.4
Ice cream, ice, etc.	3.1
Candy	32.0
Baked goods	33.0
Gelatins and puddings	0.6
Chewing gum	500.0
Condiments	9.6-100.0
Meats	40.0-2000.0

Table 7. The antibacterial activity of various terpenes, volatile alcohols and aldehydes. (after Katayama and Nagai,1960)

Compound	Bacteria tested			
	B. subtilis	E. coli	S. aureus	P. aeruginosa
	Dilutions[1]			
Eugenol	<2000	<2000	<2000	<2000
Benzyl alcohol	20	20	20	200
n-Hexyl alcohol	100	100	20	100
Vanillin	2000	2000	2000	200
Cinnamic aldehyde	200	20	200	20
Acetaldehyde	20	20	20	20
Salycylaldehyde	2000	<2000	<2000	1000
Benzaldehyde	20	100	20	100
Isoborneol	2000	-	1000	1000
Thymol	1000	<2000	1000	<2000
Carvone	100	20	20	20
α-Pipene	10	10	10	10
ß-Pipene	100	20	20	100

[1]Maximum dilution at which bacterial growth was inhibited.

effective against 6 microbial strains isolated from Vienna sausage, and that dipping the sausage in a 50% ethanol solution containing 2% eugenol for 10 seconds prolonged their shelf life at 10°C by 12 days. Bullerman et al.(1977) in their studies with eugenol and cinnamic aldehyde reported that at 125 mg/L, eugenol exhibited inhibitory action against growth and aflatoxin production by Aspergillus parasiticus in a yeast-extract sucrose medium. Similarly, eugenol has also been confirmed to possess in vitro antifungal activity against the pathogenic yeasts Candida albicans and Cryptococcus neoformans by Boonchird and Flegel (1982).

The antibacterial activity of eugenol, in comparison to some other terpenes, volatile alcohols and aldehydes of plant origin whose antibacterial activities have also been established, is presented in Table 7. Eugenol, vanillin and salycylaldehyde all of which possess a hydroxyl group directly bonded to a cyclohexane or benzene ring, showed antibacterial activity in dilutions of over or about 2000. It is thus presumed that addition of condiments containing such terpenes to fish, ham and sausage will have an antiseptic and antifungal effect (Pruthi,1980).

More recently, Jay and Rivers (1984) tested 21 flavoring compounds against a variety of organisms and reported that eugenol, among others, was one of the most effective. Activity of eugenol was highest at pH 6.0, decreasing at pH 8.0. The authors also reported that eugenol was generally more effective against fungi than against bacteria, with the lactic acid bacteria being the most resistant. Their findings suggested that compounds used

in acid foods, ostensibly as flavoring agents, may exert antimicrobial effects when considered in context with all parameters of growth. Since eugenol is a phenolic compound, its antimicrobial activity has been linked to that of synthetic phenolic antioxidants presently used in foods such as butylated hydroxyanisole (BHA); butylated hydroxytoluene (BHT) and monotertiary butylhydroquinone (TBHQ) (Bullerman et al.,1977).

2.4. PHENOLIC ANTIOXIDANTS.

Phenolic antioxidants such as BHA, BHT and TBHQ, are commonly used to prevent those changes in flavor quality and nutritive value that result from oxidation of unsaturated fats and fat-containing products. In addition, they are known to possess antimicrobial activity (Branen et al., 1980; Raccach, 1984).

2.4.1. The chemical nature of phenolic antioxidants.

The most frequently used phenolic antioxidants in foods in the United States are BHA, BHT, TBHQ and propylgallate (PG). Less frequently used is 2,4,5-trihydroxybutyrophenone (THBP) (Sims and Fioriti,1980). These compounds are low molecular weight (166-220) hindered phenols containing 1 to 3 hydroxylgroups (Figure 1). The steric hindrance brought about by the substituted alkyl group(s) makes these materials relatively non-polar (Raccach, 1984). Phenolic antioxidants are practically insoluble in water, but soluble in organic solvents such as ethanol and propylene glycol. BHA and BHT, each having one hydroxyl group, are soluble (up to 50%) in fats and oils. PG, THBP and TBHQ with more than one hydroxyl group have a low solubility (<1-10%) in lipids (Raccach, 1984).

2.4.2. The antimicrobial properties of phenolic antioxidants.

Phenolic compounds have been known for many years to possess antimicrobial activity, but Ward and Ward (1967) were the first to report the antimicrobial activity of phenolic antioxidants. It is now known that these antioxidants have antimicrobial activity against growth and by-product formation in bacteria (Gram positive and negative, spore and non-spore formers, spoilage and pathogenic), molds, yeasts, viruses and protozoa.

Several Gram positive and Gram negative bacteria are inhibited by phenolic antioxidants, however, Gram positive bacteria are generally more sensitive (Branen et al.,1980). Ward and Ward (1967) reported the antimicrobial effectiveness of BHT on Salmonella senftenberg. Davidson et al.(1975) and Chang and Branen (1975) have also reported inhibition of growth by BHA on some strains of Salmonella typhimurium and growth and enterotoxin production inhibition by Staphylococcus aureus. Chang and Branen (1975) also reported total inhibition of enteropathogenic E. coli growth by 400 mg/L BHA in nutrient broth at 37°C, while Davidson and Branen (1980a) reported delayed growth of Pseudomonas fragi at 22°C, and total inhibition of growth at 7°C by 100 mg/L BHA. Robach et al.(1977) found that 50 mg/L BHA totally inhibited growth of Vibrio parahemolyticus in trypticase soy broth, while in a crab meat homogenate, 400 mg/L BHA was required to totally inhibit V. parahemolyticus. Clostridium perfringens (3 strains) were found to be completely inhibited by 100 mg/L BHA at 30°C (Klindworth et al.,1979). Vardaman et al. (1978) reported that 10 mg/L BHT was totally inhibitory to Mycoplasma synoviae iso-

lates in Mycoplasma media, while in in vivo studies with chickens, higher concentrations of up to 400 mg/L BHT did not have any significant effect on the isolates.

Chang and Branen (1975) reported that in a glucose-salts medium, 1000 mg/L BHA inhibited growth and production of aflatoxins B_1, B_2, G_1 and G_2 by Aspergillus parasiticus spores, while 250 mg/L BHA totally inhibited growth and aflatoxin production by A. parasiticus mycelia. Inhibition of species of Geotrichium, Penicillium and Aspergillus by BHA have also been reported (Ahmad, 1979). Beggs et al. (1978), reported that the pathogenic yeasts, Candida albicans and C. parasilopsis were inhibited by sub-inhibitory concentrations of antioxidants, exhibiting fungistatic or fungicidal properties.

Viruses are not excluded from the inhibitory properties of phenolic antioxidants. Snipes et al. (1975) found that lipid-containing viruses such as ϕ 6 (a bacteriophage) and herpes simplex were 50% inactivated by 10^{-4} to 10^{-5}M BHT, while non lipid-containing viruses such as poliovirus were not inactivated. Wanda et al. (1976) also found that 30 to 100 uM BHT totally inactivated ϕ 6 phage, and that the action of BHT was enhanced by the presence of calcium, barium and strontium. These workers then concluded that in the presence of BHT, the phage lost its ability to attach to the host cell. Bacteriophage PMZ was reported to be inhibited by BHT in the first 5 minutes of exposure, and in the presence of 0.2 mM BHA, the virus was completely disrupted, causing it to release DNA (Cupp et al.,1975). Kim et al.(1978), reported BHT inhibition of the lipid-containing human and murine

cytomegaloviruses (CMV) and Semliki Forest Virus (SFV), although Vaccinia virus, which also contains lipid was not inhibited.

Surak et al. (1976a) reported the BHA-induced growth inhibition of the protozoan Tetrahymenas pyriformis, by 50% and also 50% inhibition of DNA, RNA and protein syntheses within 10 minutes after addition of 20 mg/L BHA.

2.4.3. Inhibitory mechanisms of phenolic antioxidants.

Limited studies have been reported concerning the mechanism of microbial inhibition by phenolic antioxidants, nevertheless, several workers have made attempts to elucidate the mechanism. Some principles associated with the mechanisms are presented in Table 8.

(a) Intracellular leakage:

Some studies have shown that phenolic antioxidants react with the cellular membrane, impairing both its function and integrity. Vas (1953) proposed that phenols attacked the cytoplasmic membrane, releasing intracellular constituents. Judis (1963) also found that in the presence of phenolic disinfectants, there was a loss of 50% of the Na-glutamate-3,4-^{14}C and 12% of the $NaH_2{}^{32}PO_4$ from E. coli. Davidson (1979) reported that BHA caused leakage of intracellular proteins from Pseudomonas fluorescens, as a result of membrane damage. Degre' and Sylvestre (1983) reported that Staphylococcus aureus treated with BHA (>100 ug/mL), lost UV (260 nm) absorbing material, suggesting leakage of nucleotides. The same phenomenon was observed by Davidson and Branen (1980b) with Pseudomonas fluorescens and

Table 8. Inhibitory mechanism of phenolic antioxidants (after Raccach, 1984)

Antioxidant	Microorganism/System	Effect	Reference
BHA	S. aureus	1. Leakage of nucleotides. 2. Lysis of protoplasts.	Degre' and Sylvestre, 1983.
BHA	Pseudomonas	1. Intracellular leakage. 2. Changes in cellular lipids.	Davidson and Branen, 1980b.
BHT	T. pyriformis	Intracellular leakage.	Surak et al., 1976b.
BHT	Bacteriophage Q 6	Disruption of envelope.	Wanda et al., 1976.
BHA/TBHQ	T. pyriformis	Inhibition of DNA, RNA and protein synthesis.	Surak et al., 1976a; Surak, 1977.
TBHQ	T. pyriformis	Inhibition of acetate metabolism.	Surak, 1977.
BHT	Liposomes	1. Increased membrance fluidity. 2. Decreased ^{22}Na permeability.	Singer and Wan, 1977.
BHT	Liposomes	Disruption	Eletr et al., 1974.
BHT	Bovine sperm	Depression of mitochondrial function.	Hammerstedt et al., 1976.
BHT	Rhesus monkey epithelial cells	Inhibition of RNA synthesis.	Metcalfe, 1971.

P. fragi exposed to 100 to 200 mg/L BHA. These organisms lost UV (280 nm) absorbing material (suggesting intracellular leakage of proteinaceous material) and previously incorporated ^{14}C-labelled compounds, with BHA-treated P. fluorescens experiencing a higher amount of intracellular leakage than BHA-treated P. fragi. According to these workers, intracellular leakage was observed before death occurred. Surak et al. (1976b) reported that BHT caused disruption of the cell membrane, resulting in intracellular leakage from the cilliated protozoa, Tetrahymenas pyriformis, while Singer and Wan (1977) reported that BHT reduced the loss of initially trapped ^{22}Na by liposomes.

(b) Effects on cellular membrane integrity:

Judis (1963) concluded that the loss of the labelled materials from E. coli was due to a weakening or destruction of the permeability barrier of the cell membrane. Bernheim (1972) proposed that phenol reacts primarily with the phospholipid component of the cell membrane of Pseudomonas aeruginosa, subsequently causing an increase in the permeability of the cell membrane. Phenol was later shown to cause rapid swelling of P. aeruginosa cells, which further substantiated this hypothesis (Bernheim, 1974). Toluene, another phenolic compound, was shown to cause considerable cytoplasmic membrane damage in E. coli (Woldringh, 1973; De Smet et al., 1978). De Smet et al. (1978) also found that the outer membrane of E. coli remained relatively intact.

Phenolic antioxidants may depress the phase transition of biological membranes. Singer and Wan (1977) reported that BHT lowered the melting temperature (by 20°C) of the fatty acyl

chains of vesicles made of saturated phospholipids. Hammerstedt et al. (1976) made a similar observation with bovine sperm membranes and BHT. Depression of the phase transition temperature expands the "fluid" state of the membranes over a wider range of temperature. Singer and Wan (1977) suggested that it would be necessary for a compound which was to interact with the membrane to contain a hydroxyl group, and to be lipid soluble. BHA and BHT have these two prerequisites. BHT for example, by virtue of its hydroxyl group, would be concentrated close to the membrane-water interface and not "buried" deep within the membrane lipid bilayer. In this position, BHT could easily disrupt the phospholipid packing, resulting in alteration of the phase transition temperature (Singer and Wan, 1977). In reality, BHA in most cases is more effective than BHT (Kabara, 1980), due to differences in its chemical structure. BHA, with only one tertiary butyl group, can better orient itself at the water interphase as compared to BHT, which contains two tertiary groups (Kabara, 1980).

(c) Interaction with cellular lipids:

Phenolic antioxidants may induce changes in the cellular lipids of the organism. Davidson and Branen (1980b) reported that 100 mg/L BHA increased the amount of phosphatidyl glycerol and lowered the amount of phosphatidyl ethanolamine in P. fragi but not in P. fluorescens, suggesting alteration in the amount of synthesis of these phospholipids, leading to a change of charge distribution of the membrane, and thereby affecting protein-lipid interactions or membrane alterations. The same authors also observed that 50 mg/L BHA caused a decline in the ratio of unsa-

turated to saturated fatty acids in P. fluorescens and an increase of that ratio in P. fragi. These changes in fatty acids probably occurred in order to maintain a proper "fluid" state of the cellular membrane (Davidson and Branen, 1980b).

(d) Effects on synthesis and metabolism:

Surak (1977) reported that TBHQ, but not BHA inhibited the metabolism of ^{14}C-acetate in the ciliated protozoa, T. pyriformis. Phenolic antioxidants were also found to inhibit the synthesis of nucleic acids. BHA and TBHQ inhibited the syntheses of DNA and RNA in T. pyriformis (Surak et al.,1976a; Surak, 1977), but did not find any effect of 20 mg/L BHA on the cellular membrane of the protozoa even though both DNA and RNA syntheses were inhibited. Metcalf (1971) reported that BHT inhibited RNA synthesis in Rhesus monkey kidney epithelial cells, and found a correlation between lipid solubility of a range of BHT analogues and the capacity to inhibit RNA synthesis. Cellular metabolism was probably depressed by structural changes of the cellular membrane, resulting in decreased permeability to essential metabolites. Oleuropein, a phenolic glycoside, was found to cause a decrease in the ATP content of resting cells of Lactobacillus plantarum, but did not have any effect on the rate of glycolysis (Juven et al.,1971).

Phenolic antioxidants have also been implicated in the inhibition of protein and lipid synthesis. TBHQ, for instance, decreased the incorporation of ^{14}C-acetate into lipids and proteins of T. pyriformis and also inhibited the incorporation of ^{14}C-amino acids into the proteins of the same organism (Surak,

1977). Raccach and Henningsen (1982) reported that 50 ug/mL TBHQ slightly decreased (10-15%) the synthesis of staphylococcal DNase, and that 7.5 ug/mL TBHQ inhibited the synthesis of D-lactic acid more than L-lactic acid. These workers suggested that this was probably the result of a greater inhibition of either the synthesis or activity of D-Lactic acid dehydrogenase as compared to L-Lactic acid dehydrogenase.

The inhibition of DNA, RNA and protein synthesis strongly suggests that phenolic antioxidants interact with the cellular genetic material or with the enzymes responsible for the synthesis of these compounds (Branen et al.,1980; Raccach, 1984). The function of mitochondria was also impaired by phenolic antioxidants. BHT in bovine sperm reduced the oxidative decarboxylation of pyruvate before entering the tricarboxylic acid cycle and thus reduced total oxygen consumption (Hammerstedt et al.,1976).

(e) Other effects:

Woldringh (1973) reported that treatment of E. coli cells with 0.25% toluene caused partial dissolution of the plasma membrane concomitant with displacement of nuclear material towards the cell periphery. Sgaragli et al.(1977) reported that monocyclic compounds with an aliphatic chain containing at least 2 carbon atoms,interact with biomembranes producing solubilization of proteins. Among the benzene derivatives tested were BHA and BHT.

2.5. RELATIONSHIP BETWEEN EUGENOL AND PHENOLIC ANTIOXIDANTS.

The relationship between eugenol and other phenolic antioxi-

dants as to its antimicrobial activity, has not been established. The only link so far has been the fact that eugenol is a phenolic compound, and seems to exert antimicrobial action through the possession of a free hydroxyl group directly bonded to a benzene ring (Katayama and Nagai,1960; Figure 1). This is also true of BHA, BHT, TBHQ, PG and THBP, all of which have at least one hydroxyl group bonded to the ring. (Raccach,1984; Figure 1.) However, eugenol does not possess any butyl group [$C(CH_3)_3$],as do the other phenolic compounds. Another similarity is that eugenol is an o-methoxyphenol derivative (2-methoxyphenol) as is BHA (4-methoxyphenol). Yet another similarity between eugenol, BHA and BHT lies in their possession of one hydroxyl group, which makes them up to 50% soluble in fats and oils (Food Chemicals Codex, 1972; Raccach, 1984). From the explanations regarding the role of hydroxyl groups and their interactions with cell membrane (Singer and Wan, 1977), it can be suggested that eugenol, by virtue of its one hydroxyl group, and lipid solubility, would act in a similar fashion as BHA and BHT, thereby altering the surface dipole potential of the membrane or the organization of interfacial water itself.

Since eugenol, as a food grade phenolic compound has been confirmed to possess antimicrobial activity against a wide variety of microorganisms commonly found in foods, its antimicrobial activity and mechanism of inhibition can therefore be linked to those of BHA, BHT and other phenolic antioxidants.

2.6. ENVIRONMENTAL FACTORS AFFECTING THE INHIBITORY EFFECTIVENESS OF PHENOLIC ANTIOXIDANTS.

While the antimicrobial activity of phenolic antioxidants has been confirmed, this property is modified by a number of factors, most of which are environmental (Branen et al.,1980; Raccach, 1984). These factors include:

Effect of microbial species/strain: Phenolic antioxidants seem to be more effective against Gram positive than against Gram negative bacteria (Branen et al.,1980). Thus, the latter organisms invariably require higher amounts of phenolic antioxidants for inhibition to be effective. These authors also cautioned that because of the highly selective inhibitory effect of these compounds, their application in food products could lead to selection for certain microorganisms.

Effect of stressed microorganisms: Ray (1979) reported that stressed cells have damage to their permeability barrier (cell wall and cytoplasmic membrane) that renders them more susceptible to many compounds. The author also reported that the combined effect of stress and TBHQ on lactic acid bacteria and fungi was synergistic.When TBHQ (10 ug/mL) was added to non-stressed organisms, a 41% reduction in lactic acid production was observed. Stressed organisms in the absence and presence of TBHQ (10 ug/mL) gave 20.8 and 76.5% reduction in lactic acid production respectively, this value being higher than the sum (61.8%) of TBHQ and stress alone.

Effect of type and concentration of phenolic antioxidants: In general, the antimicrobial activity of a phenolic antioxidant

decreases with the increase in the degree of hindrance (Raccach, 1984). Increasing the concentration of an antioxidant will increase its antimicrobial activity. This is especially necessary in food products, to overcome the antagonistic effect of certain food components such as fats and proteins. Eubanks and Beuchart (1982) reported that the growth of Saccharomyces cerevisiae in ice cream mix containing 2% peanut oil, was retarded by BHA (200 mg/L) and TBHQ (500 mg/L). TBHQ was more effective than BHA, while PG (up to 2000 mg/L) was ineffective. No explanation, however, was given for the superiority of TBHQ over BHA, but it was suggested that this could be a result of a concentration effect or that TBHQ was less affected by food components (Eubanks and Beuchart, 1982). In another study (Gailani, 1981), BHA, BHT, PG and TBHQ, employed separately in concentrations up to 400 mg/L, failed to prolong the shelf life of refrigerated (4°C) ground pork. It was suggested that higher concentrations of the phenolic antioxidant were required to achieve an extended shelf life.

Effect of concentration of microbial challenge: It appears that the antimicrobial activity of phenolic antioxidants decreases with increased microbial challenge, (Raccach, 1984), thus the use of the term, minimal inhibitory concentration (MIC) becomes valid only when the concentration of the tested microorganism is given. Pierson and Reddy (1982), reported that TBHQ or THBP were most efficient in delaying toxic swells produced by Clostridium botulinum, types A and B, with an inoculum of 8000 spore/g of comminuted pork at 27°C. THBP or TBHQ were most efficient in delaying these toxic swells with an inoculum of 1000 and 100

spore/g, respectively, while BHA, BHT and Nordihydroguiaretic acid (NDGA) were not as efficient as TBHQ and THBP.

Effect of pH : Klindworth et al.(1979) tested the effect of BHA (100 mg/L) on C. perfringens at pH values ranging from 5.5 to 8.5. Although BHA at all pH values tested showed partial inhibition, the maximal inhibition occurred at the extremes. It was concluded that the increased susceptibility was due to pH sensitivity rather than to the BHA. Ahmad (1979), found that inhibition of A. flavus by 50, 100 and 150 mg/L BHA generally decreased when the pH varied from 4.0 to 9.0. At 200 mg/L BHA however, 90-100% inhibition was observed at all pH values and it was concluded that 200 mg/L BHA would be useful over a wider range of pH values. More recently, Herald and Davidson (1983) in studies with E. coli and S. aureus, reported that there was an increase in the apparent inhibitory activity by hydroxycinnamic acids as the pH decreased from 6.0 to 5.0. Since the pKa of these compounds has been reported to be near pH 4.5 (Ong and Nagel, 1978), there would be a higher percentage of the undissociated acid present at lower pH levels. Frazier and Westhoff (1978) have already shown, with other pH-dependent antimicrobial compounds, that effectiveness increases as the percentage of undissociated acid increases.

Effect of food components: Food components such as protein and fat are known to bind and/or solubilize phenolic antioxidants. (Cornell et al.,1971; Cornell 1979). Robach et al.(1977) showed that BHA inhibition of Vibrio parahemolyticus decreased in the presence of lipids in a crab-meat homogenate. The BHA concentra-

tion needed for total inhibition in crab meat was 8 times higher than that needed for total inhibition in a nutrient broth. Corn oil was also found to depress the antimicrobial activity of phenolic antioxidants (Ahmad, 1979; Klindworth et al.,1979; Rico-Munoz and Davidson, 1983a). Two principal theories have been advanced to explain why the presence of lipids decreases the antimicrobial activity of BHA (Branen et al.,1980). First, since BHA has a non-polar character, it might migrate and solubilize in any lipid present in the medium, making it unavailable to act on microorganisms. Second, the antimicrobial property of BHA may be related to its antioxidant properties; therefore if BHA is used up in preventing autoxidation, the antimicrobial activity may also be lost. Prindle and Wright (1977), proposed that when phenolic antioxidants act as free radical traps, they lose a proton or an electron, and the free hydroxyl group becomes unavailable for antimicrobial activity.

The presence of proteins has also been found to have an effect on the inhibitory effectiveness of phenolic antioxidants. Rico-Munoz and Davidson (1983a) found that concentrations of 3 and 6% casein were required to obtain the same reduction in the antimicrobial activity of TBHQ (50 mg/L) and BHA (300 mg/L), respectively, in the growth of S. aureus.

Effect of carriers of phenolic antioxidants: In order to assure the dispersion of phenolic antioxidants in food products, they are usually incorporated using carriers. Nickerson and Starr (1960) and Ayres (1960), reported that an antioxidant system made up of BHA and BHT (100 mg/L each), and citric acid (500 mg/L)

delayed growth of the microbial flora of pork patties, attaining a spoilage level of $2.1*10^7$/g on the 11th day of storage (4.4-6.1°C). When carriers such as propylene glycol (100 mg/L) or sorbitan trioleate (1000 mg/L) were introduced into the product with the antioxidant system, the shelf life (microbial aspect) of the patties was decreased by 7 days. The carriers, by facilitating the dispersion of the antioxidant system in the fat portion of the meat, left only a small residue available for antimicrobial activity in the aqueous phase.

Effect of temperature: Wanda et al.(1976) noted the effect of temperature on BHT inactivation of bacteriophage 6. No inactivation was found when the virus was exposed to 0.03mM BHT at 0°C, however, at 25°C, the same concentration inactivated 99.99% of the phage in 30 minutes. A large drop in the degree of inactivation occurred when the exposure temperature was decreased from 20°C to 15°C; the percent survival increased from 2 to 90%. These workers postulated that the increased order of the lipid alkyl chains at lower temperatures decreased the partitioning of BHT into the viral membrane. Klindworth et al. (1979) reported that all cultures of C. perfringens tested, were inhibited by 150 mg/L BHA at 37°C. At 45°C, however, this concentration was not always inhibitory. While the reason for this was not clear, the authors theorized that the reduced inhibition was related to other external factors such as culture age or pH of the medium.

Effect of combination of phenolic antioxidants with temperature and food additives (other than antimicrobials): The antimicrobial activity of phenolic antioxidants in foods is affected by the

environmental temperature and by food additives such as carbohydrates, metal ions, etc.(Raccach, 1984). Raccach (1984) in a study with the fermentative activity of lactic acid bacteria, reported that the reduction of the fermentation temperature from 35 to 27°C enhanced the antimicrobial activity of an antioxidant system (30 ug/g meat each, of BHA, BHT and citric acid) against Pediococcus pentosaceus in Genoa sausage. Davidson and Branen (1980a) found that prior exposure of Pseudomonas fluorescens to BHA increased its resistance to the lethality of the phenolic antioxidant. Rico-Munoz and Davidson (1983b) found that Ca^{++} (4100 mg/L) increased the antimicrobial activity of BHA against S. aureus. The authors explained that Ca^{++} neutralize the negative charges on the cellular membrane, facilitating the penetration of BHA. It appears that the effect of calcium on phenolic antioxidants may be concentration dependent (Raccach, 1984).

3. MATERIALS AND METHODS.

3.1. ORGANISMS AND MAINTENANCE.

Two homofermentative, mesophilic, Lactobacillus species, designated as lactose and non-lactose fermenting, (LF and NLF, respectively) were supplied by Al-Jalaly (1985), after biochemical tests indicated an apparent absence of the enzyme lactase in the NLF, as a result of the organism's inability to ferment lactose in the growth media. The organisms were originally isolated from an Iraqi fermented meat sausage - Pastirma.

The organisms were maintained on All Purpose Tween (APT) agar slants (APT, Difco) at 7°C after incubation at 30°C for 24 h. Growth on the slants was transferred every two weeks.

3.2. EUGENOL AND CLOVE POWDER.

Eugenol was obtained from Eastman Kodak Co., Rochester, N.Y., and was used without further purification. Eugenol was added directly into the sterile growth media, without sterilization.

Ground clove powder was obtained from a local retail outlet. In all cases, both the growth medium and the cloves were sterilized together at 15 psi for 15 min.

3.3. INITIAL INOCULUM.

Initial inocula were prepared from their respective slant growth in the following manner: a loopful of culture from a fresh slant was inoculated into 100 mL of sterile APT broth (APT,Difco)

contained in a 500 mL Erlenmeyer flask. The flask was then incubated for 24 h at 30°C. Colony forming units (CFU) were determined from the resultant growth by serial dilution using 99 mL phosphate buffer blanks (APHA,1978) and APT agar. All plates were incubated for 24 h at 30°C. Portions of the resultant growth (initial inoculum) were added to the treatment flasks such that the final concentration was 10^5CFU/mL.

3.4. GROWTH STUDIES.

3.4.1. Effect of eugenol on the rate of pH change.

Two milliliters of a 24-h LF culture were added to a series of 500 mL flasks, each containing 200 mL of APT broth and eugenol. (0, 0.02,0.03,0.04 and 0.05%,v/v). The flasks were incubated without agitation for 10 h at 30°C during which time 10 mL portions were removed from each flask on an hourly basis and centrifuged (6,000 * g for 20 min at 5°C). The pH of the resultant supernatant were then recorded. The NLF culture was similarly investigated using 0.0,0.03,0.06,0.09,0.12 and 0.15%,v/v eugenol. All treatments were performed in duplicate using a digital pH meter.

3.4.2. Effect of eugenol on growth, pH and titratable acidity development with extended incubation

One milliliter portions of a 24-h LF culture were added to a series of 250-mL flasks, each containing 100 mL APT broth and eugenol (0.0,0.03, and 0.05%,v/v.) Flasks were then incubated at 30°C for 96 h. At every 24-h period, 10 mL portions from each

flask were removed and pH, CFU/mL, and titratable acidity (TA) was determined. The TA was determined by taking 10 mL of the centrifuged broth (6,000*g for 20 min at 5°C),adding 50 mL of distilled water and titrating with 0.1N NaOH to a pH endpoint of 8.1 (AOAC,1975). The same procedure was followed for the NLF culture using 0.0,0,04,0.08 and 0.12%,v/v eugenol. All treatments were performed in duplicate.

3.4.3. Culture development in eugenol measured by biomass and absorbance

One milliliter portions of a 24-h LF culture were added to a series of 250 mL flasks containing 100 mL APT broth and eugenol (0.0,0.03 and 0.05%,v/v.) Flasks were then incubated at 30°C for 72 h. After each 24-h period, 5.0 mL portions were removed from each flask and vacuum filtered (0.45 µm; Millipore Corp., Bedford, MA) using pre-weighed filter discs. The filter discs were dried at 80°C for 2 h in a dessicator-oven, and then reweighed. The difference in weight before and after filtration was used to calculate biomass, expressed as mg/mL. Growth was also monitored by measuring the increase in absorbance at 600 nm (Davidson and Branen,1980a) of the cultures every 24 h. Blanks were prepared containing sterile APT broth and the respective concentration of eugenol.

The same procedures were used for the NLF culture using 0.0, 0.03,0.06,0.09 and 0.12%,v/v eugenol. All biomass and absorbance determinations are expressed as an average of two trials per treatment.

3.4.4. Growth, pH and titratable acidity development.

Clove Powder:

Clove powder, 0.0 to 0.6%,w/v was added to a series of 250-mL flasks each containing 100 mL APT broth. After sterilization, each flask was inoculated with 1.0 mL of a 24-h LF culture and incubated at 30°C for 24 h. The pH of the culture broths was measured using a digital pH meter at 0 and 24 h. Titratable acidity (TA) was determined at these time periods as previously described. Culture growth was monitored at 0 and 24 h by serial dilution in sterile phosphate buffer blanks and plating, using APT agar. All plates were incubated for 24 h at 30°C and then enumerated. The same procedure was performed with the NLF culture using 0.0 to 1.4%,w/v, clove powder.

Eugenol:

The effect of eugenol on pH, growth and TA development in the LF and NLF cultures was performed as described for clove powder. The concentrations of eugenol used were; 0.0,0.02,0.03, 0.04,0.05,0.06 and 0.07%,v/v and 0.0,0.02,0.04,0.06,0.08,0.10, 0.12 and 0.14%,v/v, for the LF and NLF cultures, respectively.

Minimum inhibitory concentration.

The minimum inhibitory concentration (MIC) of clove powder and eugenol was determined by arbitrarily selecting the minimum concentration of clove and eugenol which only allowed a pH drop of 0.5 units in 24 h.

Percentage inhibition of eugenol.

The results from the measurement of growth by absorbance at 600 nm (3.4.3.) were used to determine the percentage inhibition

(PI) of eugenol. Percentage Inhibition was calculated using the following formula (Davidson and Branen, 1980a):

$$P.I. = \frac{(\text{Control O.D.} - \text{Control Blank O.D.}) - (\text{Eugenol Treatment O.D.} - \text{Eugenol Blank O.D.})}{(\text{Control O.D.} - \text{Control Blank O.D.})} * 100$$

Control blanks were the absorbance readings at time 0 of the incubation period. Eugenol blanks were uninoculated flasks of APT broth with the appropriate concentration of eugenol (%,v/v). All absorbance measurements were performed in duplicate and the results expressed as average values.

3.4.5. Effect of eugenol and culture transfer on growth, pH and titratable acidity development.

One milliliter portions of a 24-h LF culture were added to each of 3 * 500 mL flasks, containing 100 mL APT broth and 0.0,0.03 and 0.05%,v/v, eugenol, respectively. The inoculated broths were incubated for 24 h at 30°C. One mL portions of the resultant growth from each flask was then re-inoculated into fresh APT broth containing the same concentration of eugenol. The freshly inoculated broths were incubated at 30°C for 24 h. Transferring of previous growth and incubation was repeated two more times. Growth (as CFU/mL), TA (as mL 0.1N NaOH used), and pH, were monitored at 0, 24 (1st transfer), 48 (2nd transfer), 72 (3rd transfer), and 96 h (4th transfer).

The same procedure was performed for the NLF culture using 0.0, 0.04,0.08 and 0.12%,v/v, eugenol. All transfers were performed in duplicate and results are expressed as average values.

3.4.6. Adenosine triphosphate content of cells grown in the presence of eugenol.

The adenosine triphosphate (ATP) content of the LF and NLF cultures grown in the presence of eugenol was determined using a modification of the firefly luciferase enzyme method described by Cheer et al. (1974) and DeLuca et al. (1979). One mL portions of 24-h LF cultures grown in APT broth at 30°C, were dispensed into a series of 250 mL flasks containing 100 mL APT broth and eugenol (0.0,0.02,0.04 and 0.06%,v/v). The final cell concentration in each flask was 10^5 CFU/mL; all flasks were incubated at 30°C. At time intervals of 0,12,24,36 and 48 h, one mL portions of the resultant growth was removed and pipetted directly into 10 mL boiling Tris buffer [Tris(hydroxymethyl)aminomethane]hydrochloride, 0.02M, pH 7.7 (TRIZMA)(Sigma Chemical Co.,St.Louis,MO). Following heating (2-4 min), the mixture was allowed to cool and analyzed for ATP using 10 microliter samples. A series of flasks containing only APT broth and eugenol were similarly treated and analyzed in order to compensate for any eugenol-media interference. The same procedure was performed for the NLF culture using 0.0,0.04,0.08 and 0.12%,v/v eugenol with a final cell density of 10^5 CFU/mL.

In order to express the ATP content on a weight basis, 10 mL portions of each culture treatment were removed at each time interval and vacuum filtered (0.45 µm) on preweighed 47 mm filter discs. The filter discs were then dried at 80°C for 2 h and reweighed. The difference in weight of the filter discs was expressed as mg/mL biomass.

The ATP content of the cells was determined using an Integrating Photometer, Model 3000 (SAI Technology Co.,San Diego, CA) with crude firefly lantern extract (FLE-50, Sigma Chemical Co., St. Louis, MO). The firefly extract was used as a source of the enzyme luciferase, and was hydrated in sterile 0.02M Tris buffer,(pH 7.7); a stock solution of 3.34 mg luciferase/mL was then prepared and aged overnight at 7°C prior to use. Ten microliter samples for ATP analysis were reacted with 0.1 mL of the stock luciferase enzyme. The counts per minute (cpm) recorded on the photometer display panel were converted to µg ATP/L using a standard calibration curve relating cpm to ug ATP/mL. All treatments were analyzed in duplicate.

The calibration curve (Appendix Figure 1) was constructed using crystalline ATP (Sigma Chemical Co.) serially diluted in Tris buffer. Standard solutions of ATP (0 to 12.0 µg/L), were analyzed in the photometer, using 10 microliter sample size and 0.1 mL luciferase (3.34 mg/mL) (Cheer et al.,1974).

3.5. CELLULAR LEAKAGE AND GROWTH.

3.5.1. Leakage and lethality in the presence of eugenol.

The LF and NLF organisms were each grown in APT broth for 24 h in a shaker-incubator (150 rpm) at 30°C. The resultant growth was centrifuged (6,000*g for 20 min at 5°C) and the supernatants discarded. The resulting cell pellets were resuspended in 100 mL sterile 0.3 mM phosphate buffer, pH 7.2, by gentle mixing and similarly centrifuged. The pellets were again washed and concentrated by centrifugation. The washed pellets were resuspended

in a final volume of 25 mL sterile phosphate buffer. A series of 250 mL Erlenmeyer flasks each containing 50 mL sterile buffer were standardized with each washed cell suspension to yield a final absorbance (600 nm) of 0.04 to 0.06 using a Spectronic 710 spectrophotometer (Bausch and Lomb, Rochester, NY; Davidson and Branen,1980b).

Eugenol was then added to the standardized cell suspensions such that the final concentrations (%,v/v) ranged from 0.0 to 0.05 and 0.0 to 0.12 for the LF and NLF cell suspensions, respectively. All cell suspensions were incubated at 30°C in a shaker-incubator (150 rpm). At regular time intervals, 5.0 mL portions were removed from each cell suspension and vacuum filtered (0.45 µm). The filtrates were then analyzed for absorbance at 260 and 280 nm. Analysis of filtrates for absorbance employed blanks composed of phosphate buffer containing the corresponding concentration of eugenol. Absorbance for each filtrate (Ab_F) was plotted with time (T), where,

$$Ab_F = Ab_T - Ab_{INITIAL}$$

Growth of the standardized LF and NLF suspensions containing 0.05 and 0.09%,v/v eugenol,respectively was monitored at 0,3,6,9,12 and 24 h. CFU/mL were determined by serial dilution using phosphate buffer (APHA,1978); APT agar was used as the plating medium and incubation was carried out at 30°C for 24 h.

3.5.2. Eugenol-induced protein leakage.

The LF and NLF cultures, grown in APT broth for 24 h at 30°C, were harvested by centrifugation (6,000 * g for 20 min at

5°C). The cell pellets were then washed in sterile phosphate buffer (0.3 mM, pH 7.2) and concentrated by centrifugation two more times. The final cell pellets were each suspended in 1/4 their original volume of phosphate buffer. One milliliter portions of the washed stock cell suspension was added to a series of screw cap bottles containing 100 mL phosphate buffer with 0.0,0.02,0.04 and 0.06%,v/v eugenol, for the LF treatment, and 0.0,0.04,0.08 and 0.12%,v/v eugenol for the NLF treatment. The final cell concentration for both cultures was 10^6 CFU/mL. All cultures were incubated at 30°C on a shaker- incubator (150 rpm). Ten milliliter portions from each culture were then removed at 0,3,6,9,12 and 24 h and vacuum filtered.(0.45 um, Millipore Corp., Bedford, MA). In order to reduce any eugenol interference in the protein assay, 2 mL portions of each filtrate were extracted with 2 mL of chloroform. (Sgaragli et al.,1977) One mL portions of the resulting aqueous phase were assayed for protein using the Folin-Ciocalteau method described by Lowry et al.,(1951). Phosphate buffer blanks containing only the corresponding eugenol concentrations in lieu of extracted filtrate were similarly prepared and chloroform extracted. The resulting aqueous phase was used as a blank.

Absorbance (500 nm) values obtained from each sample were converted to mg/mL protein by the use of a calibration curve (Appendix Figure 2) using bovine serum albumin (Sigma Chemical Co.) as the standard. All treatments were performed in duplicate; absorbance values were determined using a Bausch and Lomb Spectronic 710 spectrophotometer.

3.6. GAS CHROMATOGRAPHIC ANALYSIS OF CELLULAR FATTY ACIDS.

3.6.1. Effect of eugenol on cellular fatty acid type and composition in the LF and the NLF.

Cell preparation.

A series of triplicate 1-L flasks containing 300 mL APT broth and eugenol (0.0,0.02 and 0.04%,v/v) were inoculated with a 5% 24-h LF culture. The flasks were then incubated at 30°C for 60 h in a shaker-incubator (150 rpm). At 12-h intervals of incubation, 150 mL of the growth broth was removed and centrifuged at 6,000*g for 20 min at 5°C. The resultant pellets were suspended in 150 mL of sterile 0.85% saline by gentle mixing and similarly recentrifuged. The final pellets were resuspended in 75 mL 0.85% saline, frozen to -20°C and lyophilized. The lyophilized cells were then transferred to screw capped vials and stored at -20°C until required for analysis. This procedure was repeated for the NLF culture using 0.0,0.02,0.04 and 0.06%,v/v eugenol.

Extraction and methylation of fatty acids.

The fatty acids from 98 to 100 mg portions of lyophilized samples were extracted according to the methods outlined by Supelco Co.(1977) n-Dodecanoic (n-C12:0) acid (1.0 mg/100 mg dry cells) in hexane was added to each sample, as an internal standard, prior to fatty acid extraction (Uchida and Mogi,1973; Herbert,1980 and Brondz et al.,1983). Methyl esters were prepared by initially saponifying each sample for 30 min at 100°C in 5 mL of 0.5N methanolic NaOH. All saponifications were performed in 20 mL Teflon-lined screw-capped tubes. The saponificates were

allowed to cool and then adjusted to a pH of 2.0 with 6N HCl and methylated by the addition of 5 mL of 10-12% boron trifluoride-methanol (BF_3-CH_3OH) reagent at 80°C for 5 min.

The methyl esters from each sample were twice extracted using 10 mL portions of chloroform:hexane (1:4 v/v) after the addition of a few drops of saturated NaCl to enhance separation (Supelco Co., 1977).The solvent extracts containing the fatty acid methyl esters were combined in tubes. A small amount of sodium sulfate was added to remove moisture, and evaporated to a final volume of 0.2 mL under a gentle stream of nitrogen gas. Samples were stored in Teflon-lined, screw-capped, 0.6 mL Reacti-Vials at -20°C until required for gas chromatographic analysis.

Gas liquid chromatography.

A Varian 3700 gas chromatograph fitted with a flame ionization detector (FID) was used to analyze the fatty acid methyl esters. The column (1.83 m * 2 mm, i.d.) was packed with 10% DEGS (diethylene glycol succinate) on 80-100 Chromosorb W mesh (Chromatographic Specialities, Brockville, Ont,) and was linear temperature programmed from 130 to 200°C at 2°C/min; the injector port temperature was 200°C. Nitrogen was used as the carrier gas at a flow rate of 20 mL/min with a column back pressure of 23 psi. A 2 microliter sample was used and the analysis time was 35 min, sufficient to detect fatty acid methyl esters from C12 to C22. The identity of the individual fatty acid methyl esters was determined by comparison of their retention times with those of purified methyl ester standards.(C12:0,I-C13,C14:0,I-C14,C15:0,I-C15,C16:0, I-C16,A-C16,C17:0,C18:0,C18:1,C18:2,C20:0 and C22:0;

Poly Science Corp.,Niles,IL). The relative proportions of the fatty acids were determined by a computing data system.(Varian Vista CDS 401, Varian Canada Inc.,Calgary, Alberta).

3.6.2. Effect of eugenol and culture transfer on cellular fatty acid composition and type.

Cell preparation.

A 1-L flask containing 285 mL APT broth and eugenol (0.03%,v/v) was inoculated with a 5%, 24-h LF culture, previously grown in APT broth at 30°C. After 24 h of incubation at 30°C, two 15 mL portions of the culture broth were removed; one portion was inoculated into a 1-L flask containing fresh sterile APT broth (285 mL,Control-1) while the other 15 mL portion was inoculated into another 1-L flask containing 285 mL fresh sterile APT broth and 0.03% eugenol (APT-eugenol-1). These flasks were incubated at 30°C for 24 h whereupon 2-15 mL portions from the flask designated as APT-eugenol-1, were removed. One portion was added into a second control (Control-2) while the other portion was added into APT broth containing 0.03%,v/v eugenol (APT-eugenol-2).The two flasks were then incubated at 30°C for 24 h. This procedure was repeated three more times for a total of 5 APT broth-eugenol transfers. Control cultures were not transferred.

For each transfer of the control and eugenol culture broths, 150 mL portions were removed and centrifuged at 6,000*g for 20 min at 5°C. The resultant pellets were resuspended in 150 mL of sterile 0.85% saline by gentle mixing and similarly recentrifuged. The final bacterial pellets were resuspended in 75 mL

0.85% saline, frozen to -20°C and lyophilized. The lyophilized cells were then transferred into screw-capped vials and stored at -20°C until required for analysis.

Similar studies using the NLF were performed using an eugenol concentration of 0.05%,v/v. All studies were performed in duplicate. A schematic of the transfer procedure is presented in Figure 2.

Extraction and methylation of fatty acids.

The extraction and methylation of the fatty acids from the lyophilized cells was carried out as previously described (3.6.1.) and stored at -20°C in screw capped Reacti-Vials until required for GLC analysis.

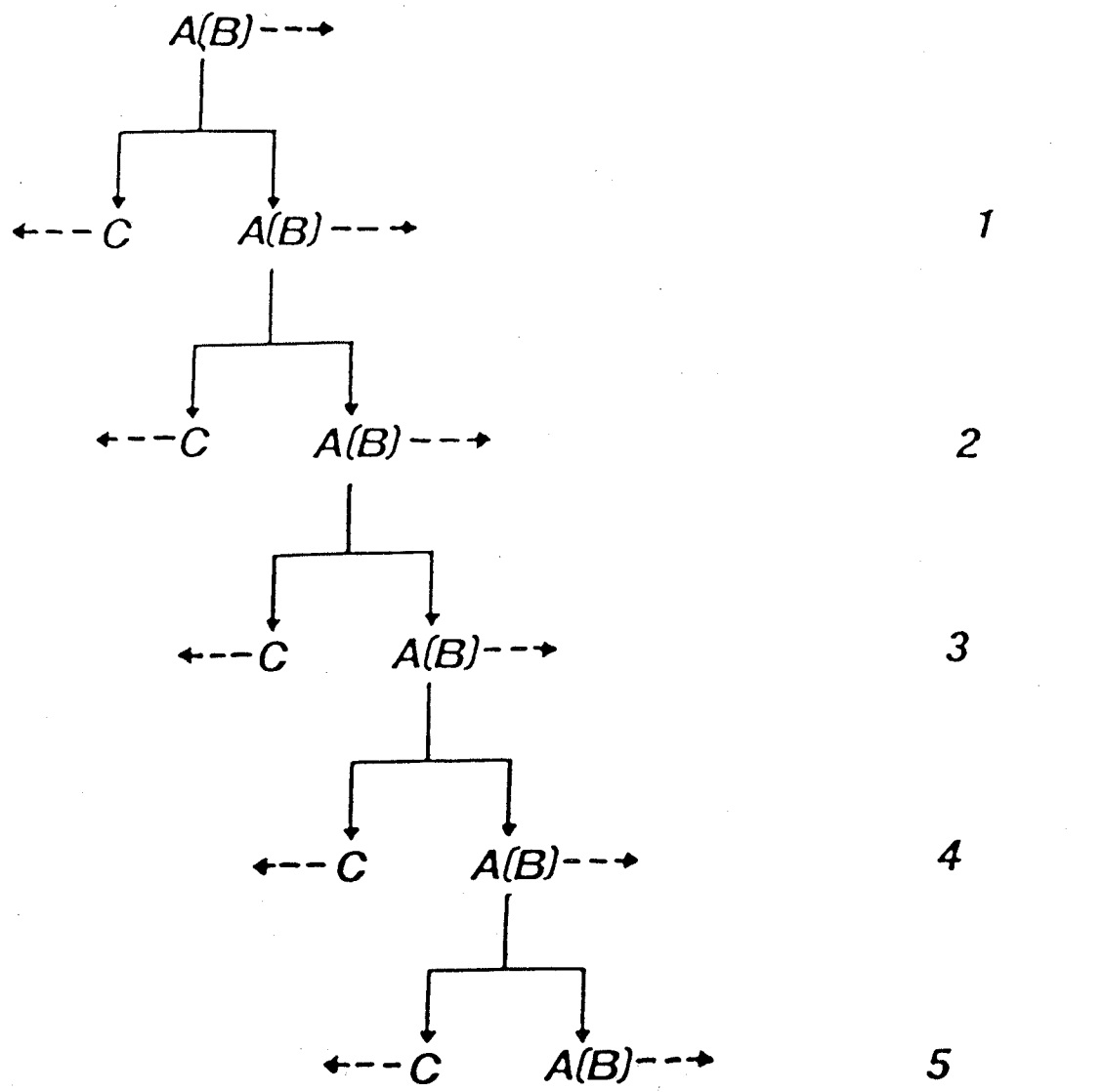
Gas liquid chromatography.

The GLC analysis was performed as previously described (3.6.1.) using the same chromatographic conditions.

3.6.3. Gas chromatographic analysis of the eugenol content of clove powder in APT broth.

Powdered cloves were prepared in APT broth at various concentrations (0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 1.2, and 1.4%,w/v) and steam sterilized for 15 min at 121°C. Following cooling, 5 mL portions of the APT-clove infusion broth were each twice extracted with 10 mL portions of chloroform:hexane (1:4,v/v). The extracts were combined and concentrated to 0.2 mL under a gentle stream of nitrogen gas. The eugenol content was analysed by gas chromatograph, as described in 3.6.1. except that the column was linear temperature programmed from 180 to 200°C at

Figure 2 Schematic of the transfer procedures employed on the effect of culture transfer and eugenol on cellular fatty acid composition.



C - Control.

A - LF + 0.03% eugenol.

B - NLF + 0.05% eugenol.

↓ - 15 mL culture for re-inoculation.

←→ - 150 mL culture for FA analysis.

2°C/min. The injector port temperature was 220°C. The identity of eugenol was determined by comparison of its retention time with that of a previously determined eugenol standard. Eugenol was also determined from cloves added after the basal medium had been steam sterilized.All studies were performed in duplicate, and the results expressed as % eugenol in the clove.

3.7. MICROSCOPIC OBSERVATION STUDIES.

3.7.1. Effect of eugenol on cell morphology.

Microscopic observations of LF and NLF cells grown in the presence/absence of eugenol were performed using a Zeiss phase contrast photomicroscope (Carl Zeiss, West Germany). Cells for microscopic observation were cultured for 24 h, after which they were placed on noble agar microslides (25 * 75 mm) and examined at a magnification of 1000. Photographs of the cells were taken on Kodak Technical Pan film 2415 (ESTAR-AH Base, black and white, ISO 100; Kodak Canada Inc., Toronto, Ont.) and the negatives printed on contact sheets for visual observation and comparison.

4. RESULTS AND DISCUSSION.

4.1. Effect of eugenol on the rate of pH change.

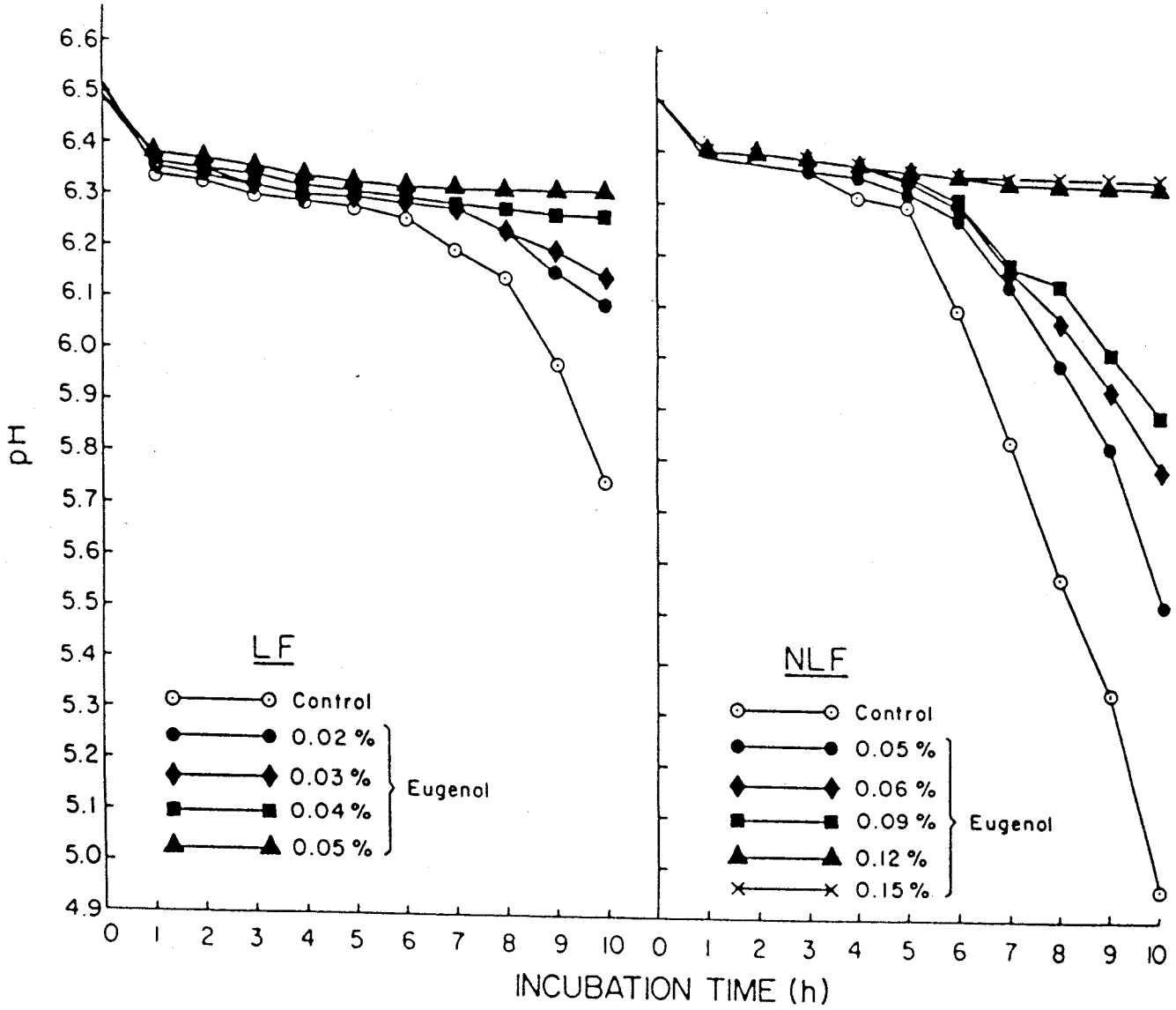
The rate of pH development by the LF and NLF cultures in APT broth containing various concentrations of eugenol (%,v/v) at 30°C is presented in Figure 3. The initial broth pH was 6.52, while the bacterial concentration of both microorganisms was 10^5CFU/mL. Increasing concentrations of eugenol in the growth medium for both organisms progressively decreased the rate of pH development over a 10 h incubation period. This decrease in pH by increasing concentrations of eugenol was found to be significant ($P<0.05$) in both cultures (Appendix Tables 26 and 27). The controls in both the LF and NLF cultures showed the fastest rate of pH development (5.75 and 4.97 after 10 h, respectively). Eugenol in concentrations of 0.04 to 0.05 and 0.12 to 0.15%,v/v, totally inhibited pH development during the incubation period for the LF and NLF cultures respectively.

Although pH development was progressively diminished with increasing eugenol concentrations, the results did not indicate whether lethality or alternate metabolism was occurring. The results indicated however, that the NLF organism was less sensitive to the effects of eugenol as compared to the LF organism, as illustrated by the higher levels of eugenol employed on the NLF organism.

4.2. Effect of eugenol on growth, pH and titratable acidity development with extended incubation.

Time course growth, pH and titratable acidity (TA) develop-

Figure 3 Effect of eugenol on rate of pH change by lactose (LF) and non-lactose (NLF) fermentors at 30°C.



ment studies with the LF and NLF cultures in APT broth containing various concentrations of eugenol are illustrated in Figures 4. and 5., respectively. For the LF culture, both the control and the 0.03% eugenol treatment culture showed similar patterns of pH and TA development. Although maximum growth for these cultures was similar (60 to 80 * 10^7CFU/mL) at 24 h of incubation, the 0.03% eugenol-grown culture displayed an accelerated death phase. Maximum pH (4.6) and TA development (9.6 mL 0.1N NaOH) coincided with logarithmic growth. The LF culture grown in 0.05% eugenol showed only slight growth (26 * 10^6CFU/mL) which occurred at 24 h; pH and TA development were minimal throughout the incubation period (5.84 and 5.5 mL .1N NaOH, respectively) after 96 h of incubation.

Increasing concentrations of eugenol employed with the NLF culture also decreased the rate and extent of growth, pH and TA development. Eugenol employed at 0.08 and 0.12%,v/v completely inhibited growth (30 * 10^7 and 10 * 10^3CFU/mL, respectively at 24 h, and 10 * 10^2CFU/mL for both at 96 h). Minimal TA and pH development occurred with the 0.12% eugenol containing culture, particularly after 24 h of incubation. Both the control and the 0.04% eugenol-containing culture, however, showed similar maximum growth at 24 h (74 to 81 * 10^7CFU/mL), although the latter culture displayed an accelerated death phase after 48 h. Despite the similarities in logarithmic growth between the NLF control and the 0.04% eugenol-grown culture, the rate and extent of pH and TA development was different. This may indicate that in spite of the similarities in growth rate between the control and the

Figure 4 Effect of eugenol on growth, pH and titratable acidity development with extended incubation by the LF.

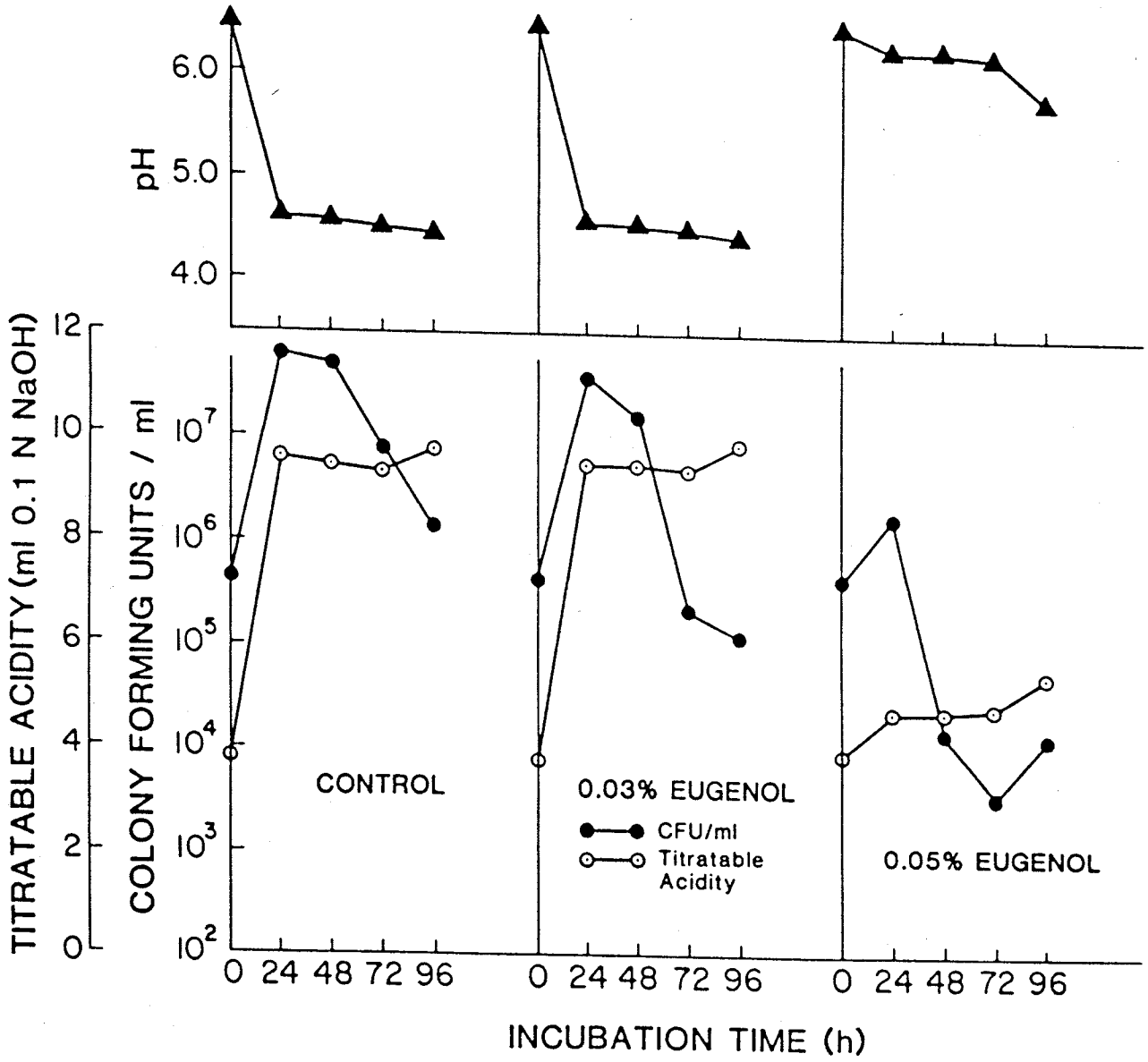
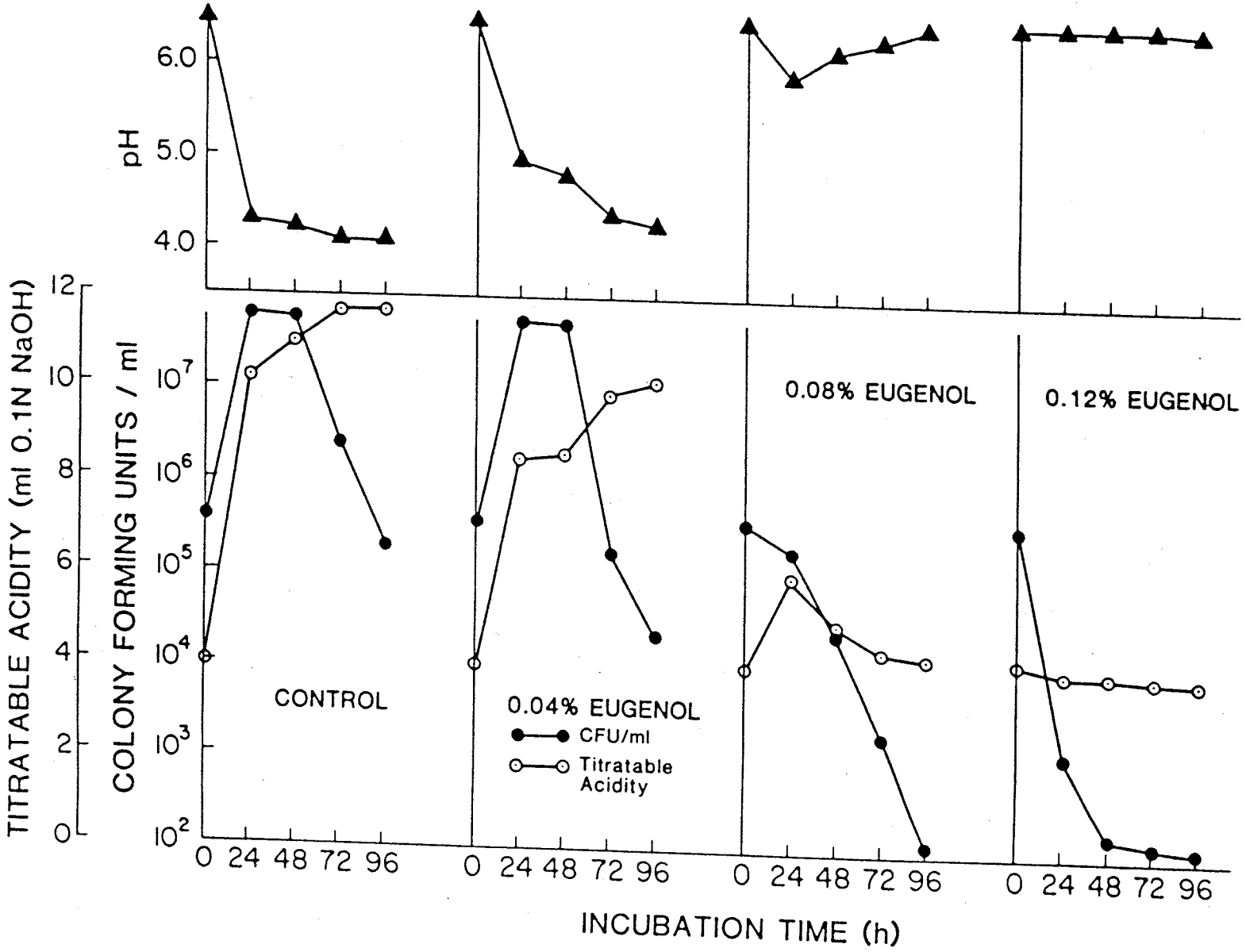


Figure 5 Effect of eugenol on growth, pH and titratable acidity development with extended incubation by the NLF.



relatively low eugenol-containing cultures, metabolic processes may be altered by the presence of eugenol. The concentration of eugenol which was insufficient to affect viable growth may affect metabolism and end-product formation. Several phenolic compounds such as BHA and BHT have already been shown to affect the cellular membrane of organisms (Judis, 1963; Woldringh, 1973; De Smet et al., 1978). It is therefore possible that even though viable growth was not sufficiently depressed or inhibited by relatively lower eugenol concentrations, the activities of some membrane-bound enzymes may have been sufficiently inhibited.

In this investigation therefore, it is evident that the addition of increasing concentrations of eugenol to the growth medium delayed or inhibited growth, pH and TA development in both the LF and NLF cultures. It would appear also from these results that the LF was more sensitive to eugenol than the NLF, as evident in the higher levels of eugenol employed on the NLF to achieve maximum inhibition of these parameters. Al-Jalaly (1985), also reported that the NLF culture appeared to be the least sensitive to individual spices, including cloves. It is also possible that the effect of culture age will influence growth, pH and TA development, since the time course accumulation of acid, and hence decreased pH, might eventually become toxic to the organisms.

4.3. Culture development by the LF and NLF in the presence of eugenol, measured by biomass and absorbance at 600 nm.

The effect of varying concentrations of eugenol on the

growth of the LF and NLF cultures is shown in Figure 6. Although increasing concentrations of eugenol progressively decreased maximum biomass production, sub-lethal concentrations of eugenol (0.02% for the LF and 0.03 to 0.06% for the NLF) in the growth media resulted in biomass maxima higher than those observed in the controls. Since these increases in biomass could not be attributed to increased growth rates (Figures 4 and 5), it seemed likely that sub-lethal concentrations of eugenol increased biomass production through cellular enlargement or swelling, perhaps as a result of cell membrane alteration. Bernheim (1974) reported that phenol caused rapid swelling of Pseudomonas aeruginosa cells, which further substantiated the hypothesis that phenolics react primarily with the phospholipid component of the cell membrane of P. aeruginosa, subsequently causing an increase in the permeability of the cell membrane.

Although absorbance (600 nm) values for these cultures were not totally in agreement with the biomass determinations (Figure 6), they do affirm previous growth study results that increasing concentrations of eugenol cause progressive inhibition of growth in both the LF and the NLF organisms.

4.4. Effect of eugenol on cell morphology.

Phase contrast photomicrographs of LF and NLF cells grown in the presence/absence of eugenol are illustrated in Figures 7 and 8 respectively. Both the LF and NLF cells grown in the absence of eugenol appeared as cocci or small coccobacilli (2.8 to 3.0 μm). The majority of cells appeared opaque and were organized in doub-

Figure 6 Culture development of LF and NLF cells in the presence of eugenol measured by biomass and absorbance at 600nm.

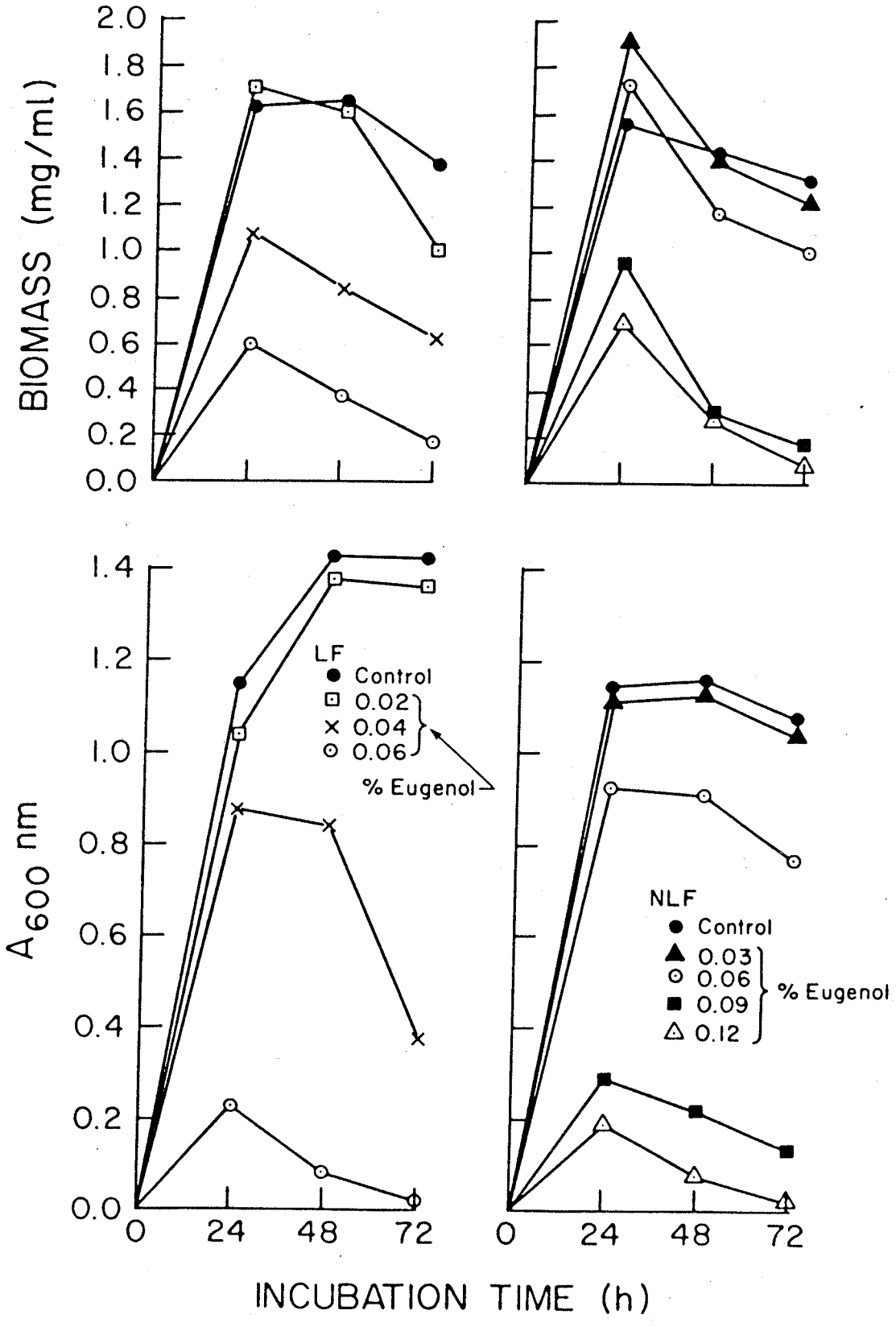
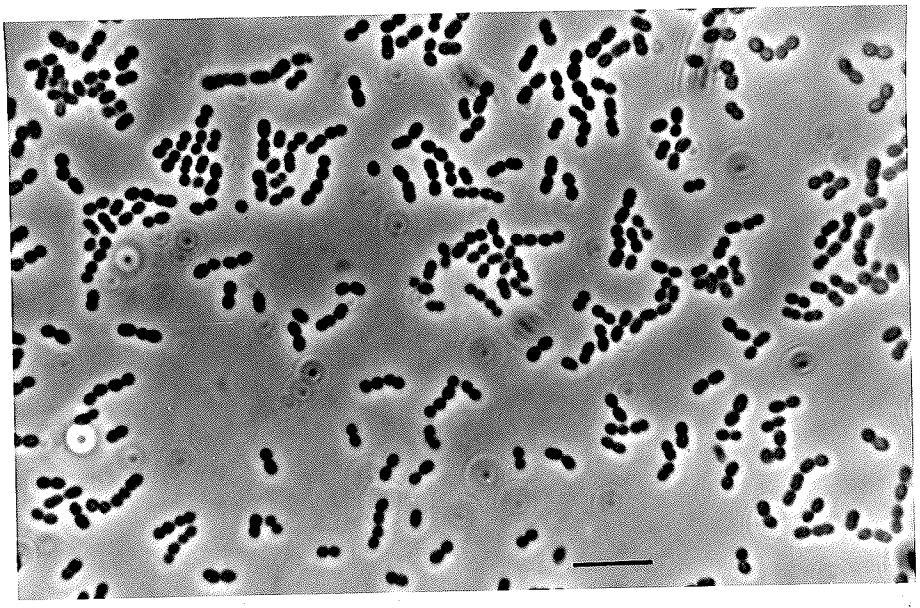
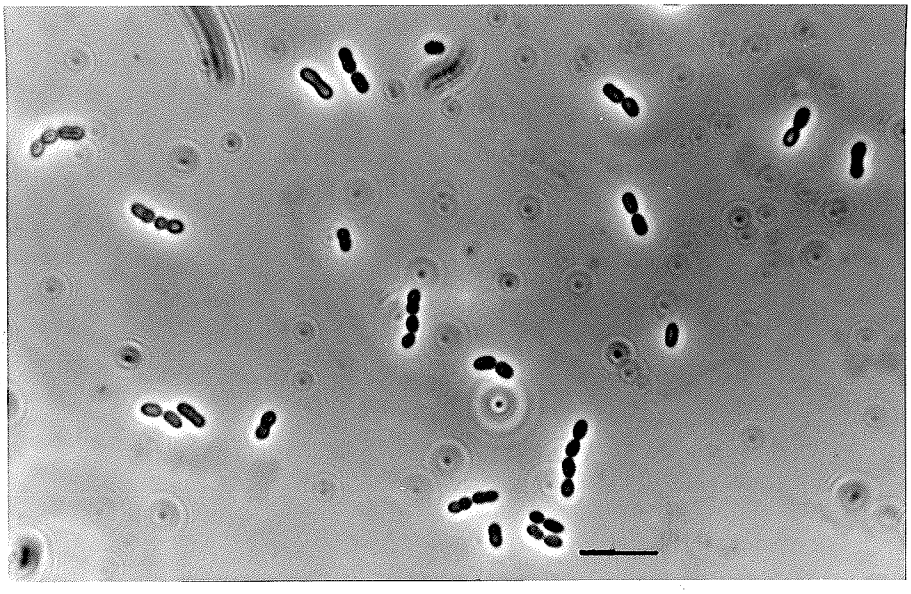


Figure 7 Photomicrographs of LF cells incubated at 30°C for 24 h. in APT broth.

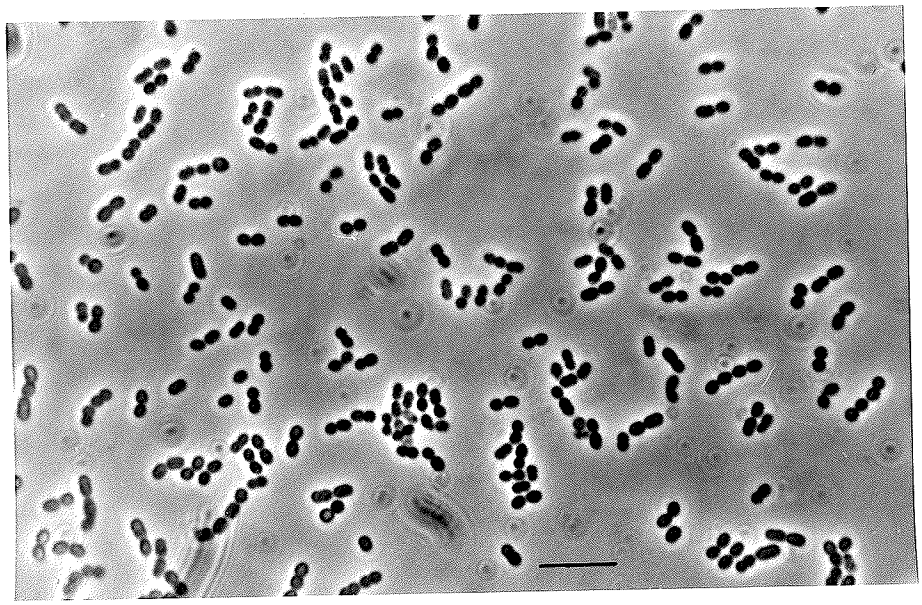


Control. Bar represents 30 µm

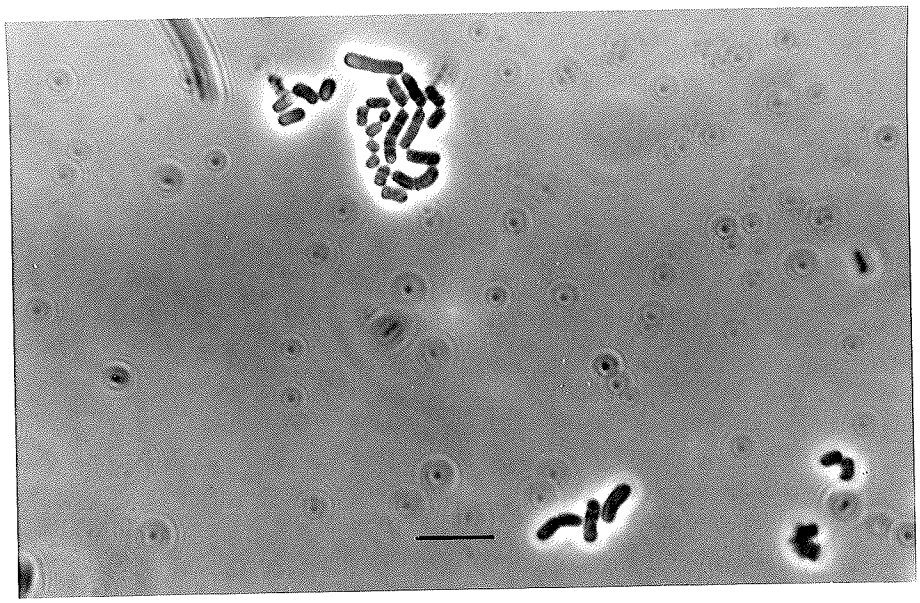


In 0.05%, v/v eugenol. Bar represents 30 µm

Figure 8 Photomicrographs of NLF cells incubated at 30°C for 24 h. in APT broth.



Control. Bar represents 30 µm



In 0.09%, v/v eugenol. Bar represents 30 µm

lets or short chains (2 to 5 cells). LF cells grown in APT broth at 30°C in the presence of 0.05% eugenol appeared more as coccobacilli (3.2 to 3.7 µm). The cells were enlarged as compared to the control, and many showed reticulated cytoplasms, indicating the onset of lysis. The NLF cells grown in APT broth at 30°C in the presence of 0.12% eugenol appeared as short, plump rods (4.0 to 5.0 µm). Cell cytoplasm was reticulated, and in some cells, transparent.

These microscopic observations were consistent with some previous reports in the literature. Bernheim (1974) confirmed that phenol caused rapid swelling of Pseudomonas aeruginosa cells. Toluene was also shown to cause considerable cytoplasmic damage in E. coli cells (Woldringh, 1973; De Smet et al., 1978). It appeared therefore, that eugenol caused some considerable damage to the cell membrane of these organisms, resulting in alteration of the morphology of the cell. The gross enlargement observed with the LF and NLF cells may also partially explain the increased biomass previously described (4.3.).

4.5. Growth, pH and titratable acidity development by the LF and the NLF in clove infusion and eugenol.

Increasing concentrations of clove powder or eugenol in APT broth for both the LF and NLF cultures progressively decreased growth, pH and TA development at 24 h of incubation (Tables 9, 10, 11 and 12). Concentrations of eugenol in excess of 0.05 and 0.08%, v/v were bactericidal for the LF and NLF cultures respectively, while concentrations of clove in excess of 0.3 and

0.6%,w/v were bactericidal for the LF and NLF cultures respectively.

The minimum inhibitory concentration (MIC) for the LF and NLF cultures was 0.3 and 0.5%,w/v clove respectively, while the MIC for the LF and NLF cultures in eugenol was 0.06 and 0.08%,v/v eugenol respectively. The data presented indicated that the LF organism was more sensitive to clove and eugenol than the NLF culture. Assuming eugenol is the main bacteriostatic or bactericidal agent in cloves, it would appear that ca. 10 times more of the spice was required as compared to the pure essential oil, in order to attain the MIC value.

The data presented in Tables 13 and 14 show the percentage inhibition (PI) of both the LF and NLF organisms by eugenol respectively. Generally, PI by eugenol increased as both eugenol concentration increased, and as the length of incubation was prolonged. It was noted that 0.06% eugenol produced ca. the same PI in the LF as 0.12% eugenol for the NLF. This further confirmed the higher sensitivity of the LF to eugenol as compared to the NLF. Since both organisms were from the same genus, having similar characteristics, it might be expected that they would also have similar susceptibility to eugenol. In all cases, however, the NLF was less sensitive than the LF to growth inhibition (as well as pH and TA development) by eugenol. Davidson and Branen (1980a) suggested that the differences in susceptibility to BHA of closely related microorganisms could be related to metabolic or structural differences. It would therefore appear that differences in susceptibility to eugenol by the LF and the NLF organ-

Table 9 Growth, pH and titratable acidity development by the LF in clove infusion.

Cloves (%,w/v)	Time (h) 0			24		
	CFU/mL	pH	TA	CFU/mL	pH	TA
0	$58x10^5$	6.56	3.4	$71x10^8$	4.69	9.4
0.1	$58x10^5$	6.54	3.6	$46x10^7$	4.70	9.2
0.2	$58x10^5$	6.52	3.8	$19x10^6$	5.32	8.4
0.3[a]	$58x10^5$	6.50	4.0	$38x10^5$	5.80	7.1
0.4	$58x10^5$	6.48	4.3	$26x10^4$	6.41	5.0
0.5	$58x10^5$	6.45	4.6	$43x10^2$	6.43	4.9
0.6	$58x10^5$	6.42	4.8	$16x10^1$	6.43	4.9

[a] Minimum inhibitory concentration.

Table 10 Growth, pH and titratable acidity development by the NLF in clove infusion.

Cloves (%,W/v)	Time (h) 0 CFU/mL	pH	TA	24 CFU/mL	pH	TA
0	$49x10^5$	6.56	3.4	$64x10^8$	4.15	11.4
0.2	$49x10^5$	6.54	3.7	$40x10^7$	4.23	10.5
0.4	$49x10^5$	6.48	4.2	$29x10^6$	5.17	9.3
0.5[a]	$49x10^5$	6.45	4.5	$80x10^5$	5.64	8.6
0.6	$49x10^5$	6.42	4.7	$64x10^5$	5.84	7.5
0.8	$49x10^5$	6.40	5.0	$15x10^4$	6.23	6.0
1.0	$49x10^5$	6.38	5.4	$58x10^3$	6.41	5.0
1.2	$49x10^5$	6.35	5.8	$52x10^2$	6.45	4.5
1.4	$49x10^5$	6.33	6.1	$10x10^1$	6.45	4.5

[a] Minimum inhibitory concentration.

Table 11 Growth, pH and titratable acidity development by the LF in the presence of eugenol.

Eugenol (%,v/v)	Time (h) 0 CFU/mL	pH	TA	24 CFU/mL	pH	TA
0	$52x10^5$	6.52	3.8	$84x10^8$	4.53	9.60
0.02	$52x10^5$	6.52	3.8	$80x10^8$	4.71	9.20
0.03	$52x10^5$	6.51	3.8	$64x10^7$	4.88	9.00
0.04	$52x10^5$	6.50	3.9	$29x10^6$	5.21	8.10
0.05	$52x10^5$	6.50	3.9	$26x10^6$	5.65	7.30
0.06[a]	$52x10^5$	6.49	4.0	$50x10^3$	6.37	4.60
0.07	$52x10^5$	6.48	4.1	$10x10^1$	6.37	4.60

[a] Minimum inhibitory concentration.

Table 12 Growth, pH and titratable acidity development by the NLF in the presence of eugenol.

Eugenol (%, v/v)	Time (h) 0			24		
	CFU/mL	pH	TA	CFU/mL	pH	TA
0	58×10^5	6.52	3.9	88×10^8	4.29	10.20
0.02	58×10^5	6.52	3.9	63×10^8	4.58	9.30
0.04	58×10^5	6.51	3.9	74×10^7	4.97	8.60
0.06	58×10^5	6.51	3.9	53×10^6	5.34	8.10
0.08[a]	58×10^5	6.50	4.0	33×10^5	5.90	6.80
0.10	58×10^5	6.49	4.1	40×10^4	6.23	5.10
0.12	58×10^5	6.48	4.2	11×10^2	6.50	4.00
0.14	58×10^5	6.47	4.3	43×10^1	6.50	5.00

[a] Minimum inhibitory concentration.

Table 13 Percentage Inhibition (PI) of the lactose fermentor (LF) by eugenol.

Eugenol (%, v/v)	Percentage Inhibition[a] Time (h) 0	24	48	72
Control	-	-	-	-
.02	-	1.14	3.29	4.35
.04	-	23.54	41.15	73.75
.06	-	80.19	94.05	98.74

[a]Tested in APT Broth, inhibition determined at 30°C for 3 days at 600 nm. Determined using:

$$PI = \frac{\text{Control OD} - \text{Eugenol Treatment OD}}{\text{Control OD}} \times 100$$

Table 14 Percentage Inhibition (PI) of the non-lactose fermentor (NLF) by eugenol.

Eugenol (%, v/v)	Percentage Inhibition[a] Time (h) 0	24	48	72
Control	-	-	-	-
.03	-	2.70	2.84	3.70
.06	-	18.83	21.79	29.07
.09	-	74.19	80.96	87.31
.12	-	84.35	93.37	98.06

[a]Tested in APT Broth, inhibition determined at 30 C for 3 days at 600 nm. Determined using:

$$PI = \frac{\text{Control OD} - \text{Eugenol Treatment OD}}{\text{Control OD}} \times 100$$

isms was related to the differences in their metabolic activity.

The higher PI at 48 and 72 h observed for both cultures may not be totally due to the effects of eugenol since the accumulation of end-products of metabolism viz. lactic acid are also known to influence growth. Klindworth et al. (1979), reported that culture age played an important role in the susceptibility of Clostridium perfringens to BHA, and concluded that young or old cultures had increased susceptibility to BHA, the susceptibility being independent of cell number and related to the metabolic rate and toxic end-product formation. These findings may then partially explain the increased susceptibility of the LF compared to the NLF. In both cases, 24-h old cultures containing similar numbers of cells (10^5CFU/mL) were used in all inhibition tests, which minimized the effects of cell number. As evident in the initial growth curves (Figures 4 and 5) for both organisms, the LF exhibited a faster growth rate than the NLF in APT broth. The LF culture may therefore have been in a more susceptible stage of growth when inoculated into the test medium.

Differences in the membrane structure could also be responsible for the difference in sensitivity. Since the basic structure of Lactobacillus species is expected to be similar, these differences would be subtle or minimal. Judis (1963) and Juven et al.(1972), reported that phenolic compounds act on the cytoplasmic membrane. The organization of proteins or lipids in the cytoplasmic membranes of the LF and NLF could therefore confer more or less resistance to the effects of eugenol.

Gas chromatographic analysis of the clove powder used,

yielded a mean eugenol concentration of 16.15%, which was reduced to 10.64% following steam sterilization (Appendix Table 25). This concentration of eugenol (added after media sterilization) is consistent with previous data (Merory, 1960; Pruthi, 1980) which reported that clove contains essential oil ranging from 15 to 20%, of which 85 to 95% is eugenol. It would be expected then that one gram of ground cloves would yield 0.15 to 0.20 g of essential oil, of which 0.13 to 0.19 g is eugenol. In this study, 16.15% eugenol content for clove falls within this range. Since most essential oils of spices are obtained by either steam or water distillation, or a combination of both (Pruthi, 1980), and considering that distillation often employs high temperatures, which will result in the loss of volatile materials, some loss of eugenol in the total yield of the essential oil fraction was expected. Therefore, steam sterilization of the APT broth-clove system (clove infusion) would reduce the volatile or essential oil content of cloves (which is known to contain a relatively high level of volatile oil) by heat degradation and/or volatilization. It is probable that this reduction in essential oil content decreased the antimicrobial activity of ground cloves.

4.6. Effect of eugenol and culture transfer on growth pH and titratable acidity development.

The routine transfer and growth of the LF and NLF cultures in fresh APT broth containing eugenol is illustrated in Figures 9 and 10 respectively. Acclimatization of both organisms through five repeated transfers (each representing a 24 h incubation

Figure 9 Effect of eugenol and culture transfer on growth, pH and titratable acidity development by the LF.

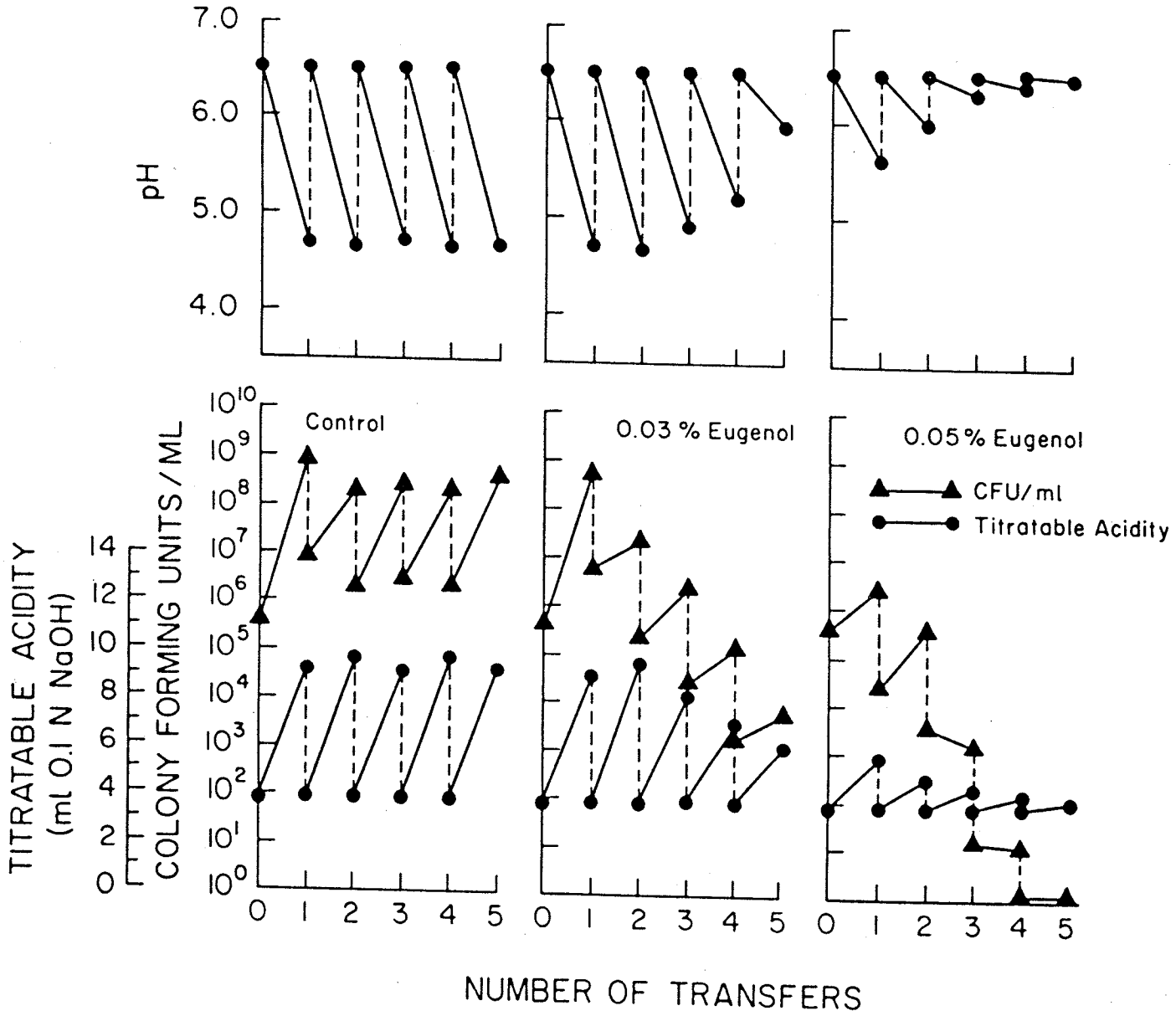
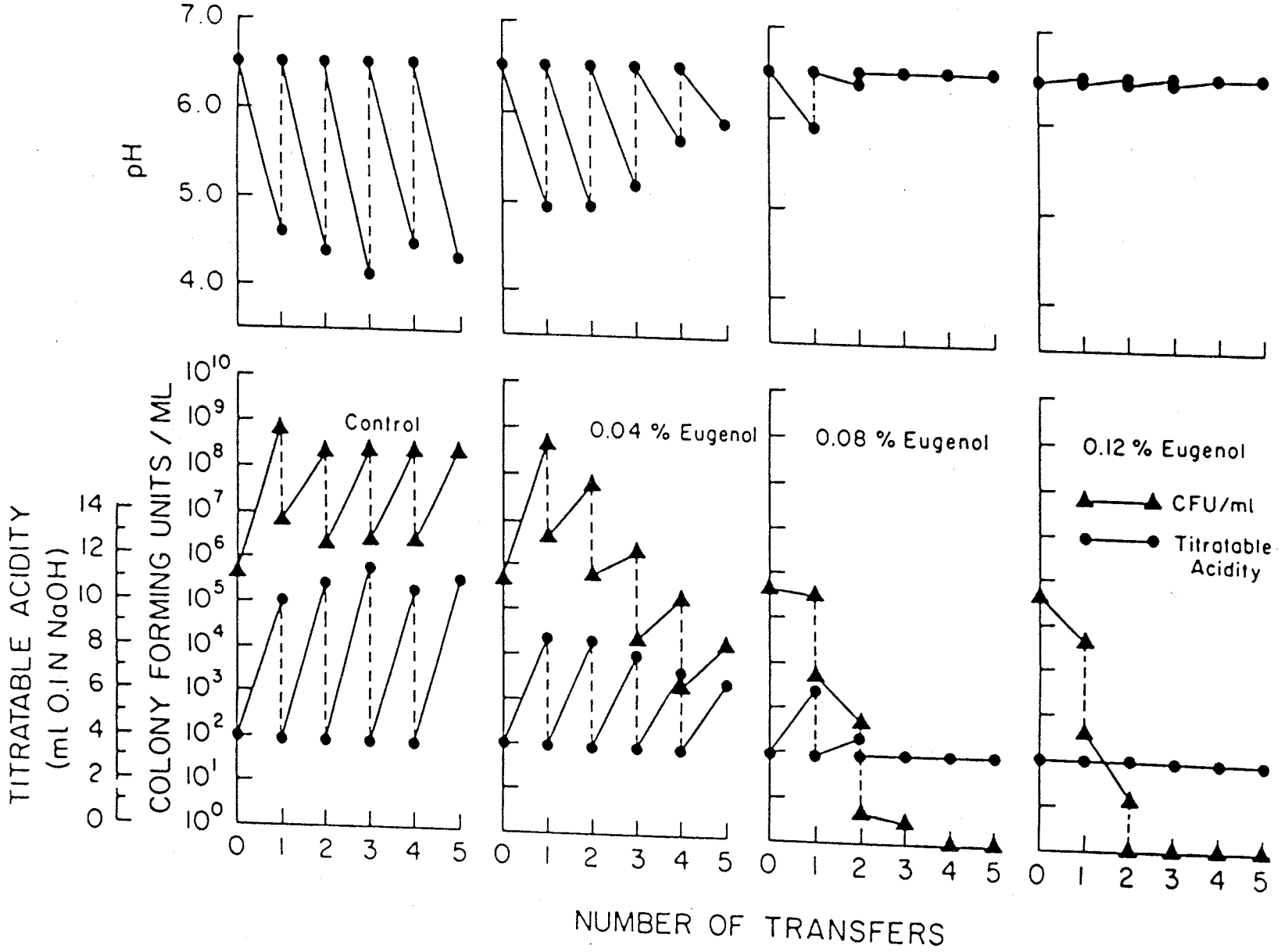


Figure 10 Effect of eugenol and culture transfer on growth, pH and titratable acidity development by the NLF.



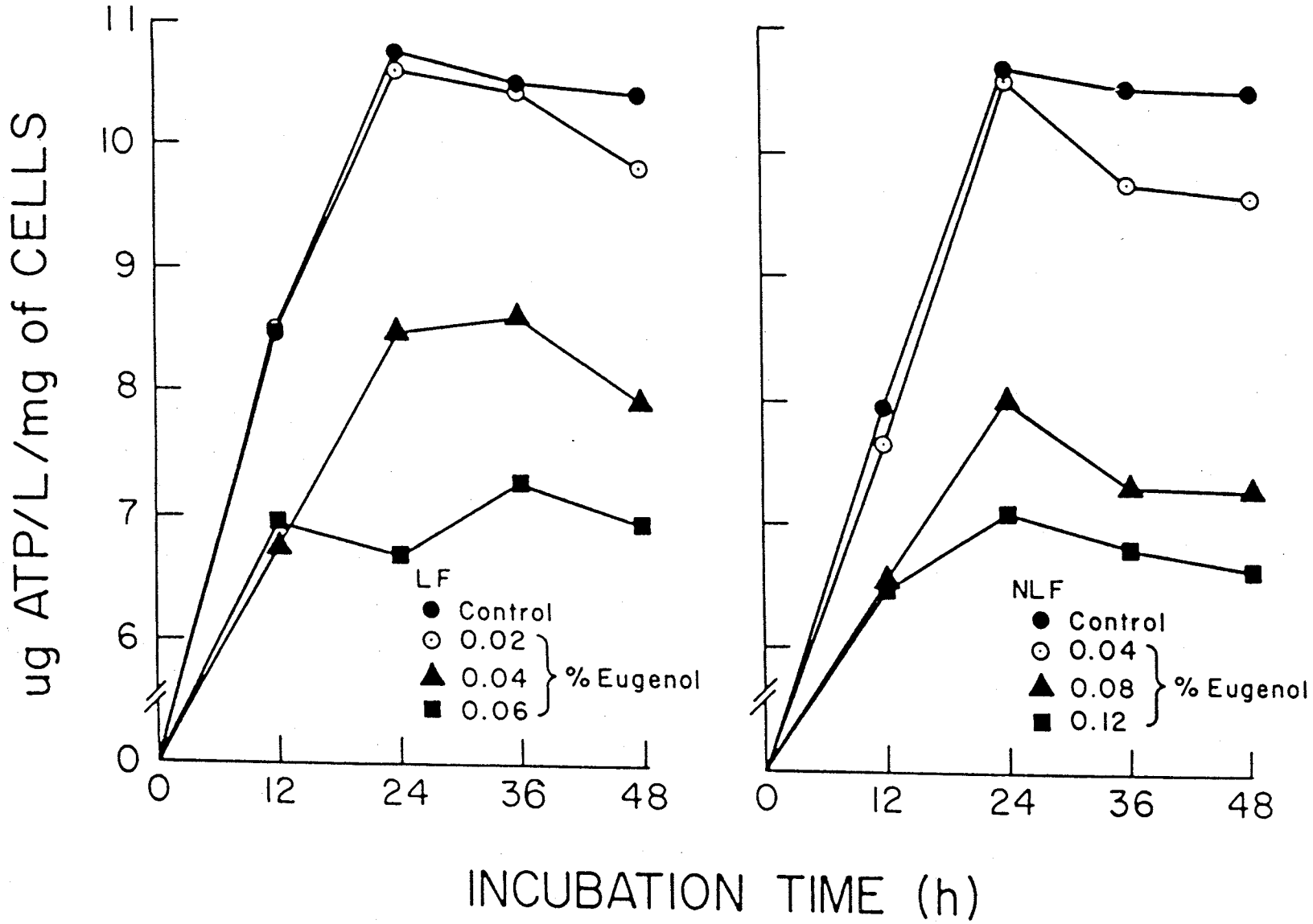
period) into APT both containing eugenol was not observed. A gradual loss of cell viability with a concomitant decrease in pH and TA development was observed throughout the 96 h transfer period in both organisms. Since the initial cell population from each subsequent transfer decreased with a corresponding decrease in maximum growth, it is also highly improbable that any individual wild cells underwent acclimatization.

4.7. Adenosine triphosphate content of LF and NLF cells grown in the presence of eugenol.

The adenosine triphosphate (ATP) content of the LF and NLF cells grown in the presence of various concentrations (%,v/v) of eugenol is presented in Figure 11. Increasing the concentrations of eugenol generally resulted in smaller levels of cellular ATP at each time interval for both organisms. Cell cultures showed maximum accumulation of ATP at 24 h. The LF and NLF cultures grown in 0.02 and 0.04% eugenol respectively, showed similar levels of cellular ATP accumulation with their respective controls, particularly during the first 24 h of cultivation. Although smaller levels of ATP were accumulated in the eugenol-treated cells as compared to the controls, ATP synthesis did not appear to be completely inhibited by eugenol. Statistical analysis indicated that eugenol had a significant ($P<0.05$) effect on the ATP levels of both the LF and NLF organisms (Appendix Tables 28 and 30).

The results of ATP accumulation in the LF and NLF cells agree with the findings of Juven et al. (1972) who reported

Figure 11 Adenosine triphosphate content of LF and NLF cells grown in the presence of eugenol.



smaller levels of ATP in Lactobacillus plantarum cells treated with oleuropein, a phenolic glycoside in green olives. These researchers concluded that while oleuropein did not inhibit ATP synthesis, it did affect plasmatic membranes with subsequent leakage of ATP or phosphates from the bacterial cells. Reduction in the ATP content of the cell would therefore cause less energy to be available for growth and metabolism. It was further concluded that since glycolysis was not inhibited by oleuropein, it would seem unlikely that ATP synthesis would also be inhibited, since glycolysis appears to be the only source of energy and ATP production in L. plantarum (Juven et al., 1972).

Eugenol did not appear to inhibit ATP synthesis, as demonstrated by the smaller but increasing levels of ATP in eugenol-treated LF and NLF cells. The lower levels of ATP found in these cells could be a consequence of a perturbing effect of eugenol on the cellular membrane, causing a release or leakage of ATP or phosphate from the cells, and thereby allowing less energy to be made available for growth and metabolism. This leakage could also be attributed to the damage of the regulatory mechanisms controlling the permeability of the cell membrane.

The data presented on the effect of eugenol on ATP levels in LF and NLF cells suggest that eugenol acts in a similar fashion to that of other phenolic compounds and surface active substances like oleuropein.

4.8. Leakage and lethality in the presence of eugenol.

The data presented in Figures 12 and 13 indicate an increase

in UV absorbing material from the filtrates of both the LF and NLF cells treated with eugenol for varying time. The LF culture filtrate showed maximum absorbance at 260 nm (Figure 12), while the NLF showed maximum absorbance at 280 nm (Figure 13). Cell filtrates from both cultures showed a progressive increase in absorbance at both wavelengths with increasing time of eugenol exposure and concentration. Judis (1963), Juven et al. (1972) and Davidson and Branen (1980b) also reported that phenolic compounds cause leakage of intracellular constituents from various bacteria. Degre' and Sylvestre (1983) suggested that the increase in absorbance at 260 nm was due to the leakage of intracellular nucleotides, while the increase in absorbance at 280 nm was due to leakage of cytoplasmic or periplasmic proteins (Davidson and Branen, 1980b). At 24 h, the increase in A_{260} material from the LF cells treated with 0.05%,v/v eugenol was ca. the same as that for NLF cells treated with 0.12% eugenol. Both the LF and the NLF filtrates reached a maximum absorbance of 0.22. This is consistent with the difference in susceptibility of the two organisms to eugenol as shown in growth and acid production studies. At $A_{280\ nm}$ however, NLF filtrates with 0.12% eugenol appeared to lose more UV material than the LF filtrates with 0.05% eugenol.

The LF and NLF filtrates, at both 260 and 280 nm, showed the largest increase in UV absorbance within the first 3 h of exposure to eugenol. This may represent an attempt by these organisms to recover from the initial effects of eugenol. It appears that eugenol rapidly attacks the cell membrane, causing disruption, and that eugenol, being a phenolic compound, reacts with the cel-

Figure 12 Leakage of UV (260 nm) material from LF and NLF cells grown in the presence of eugenol.

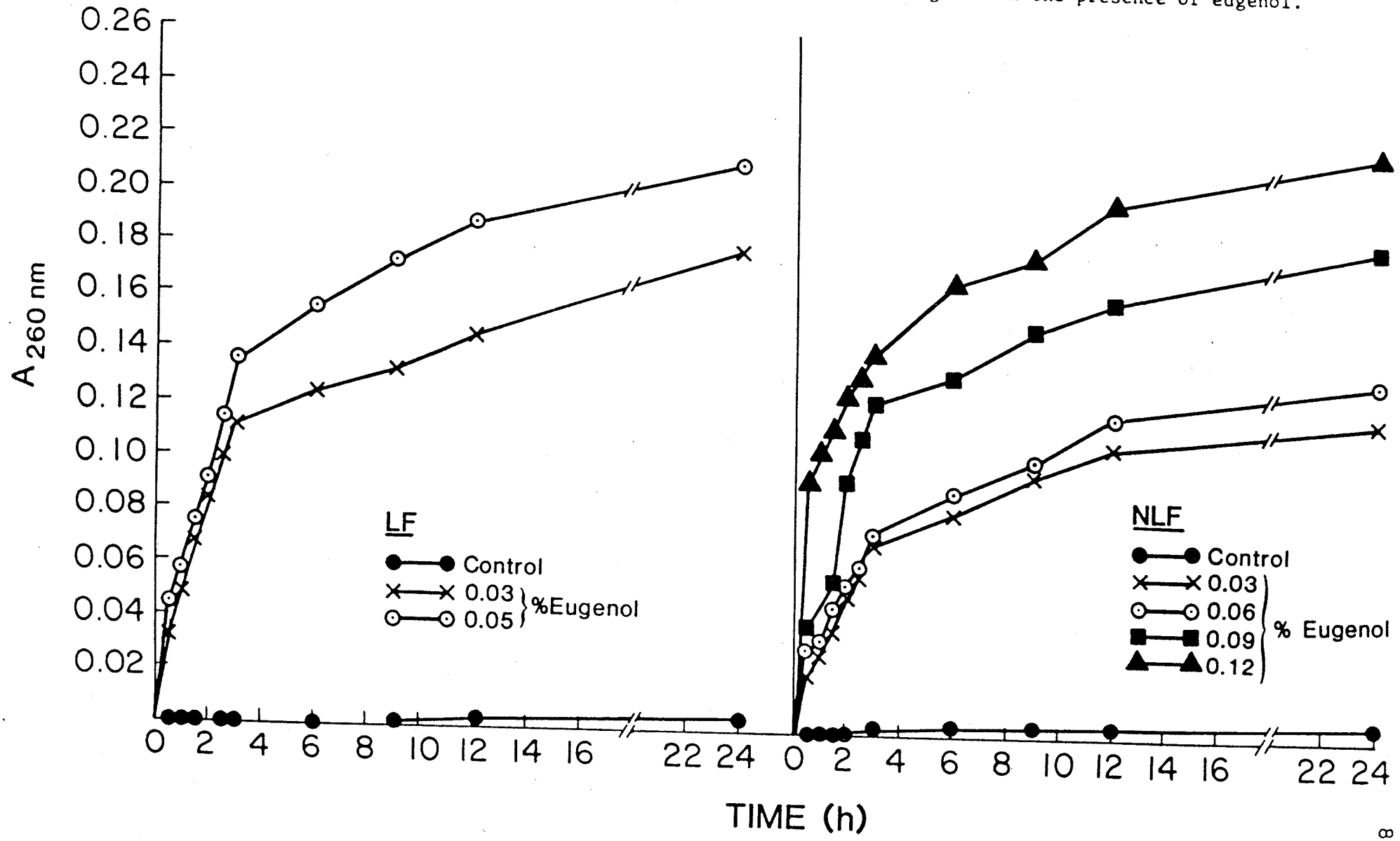
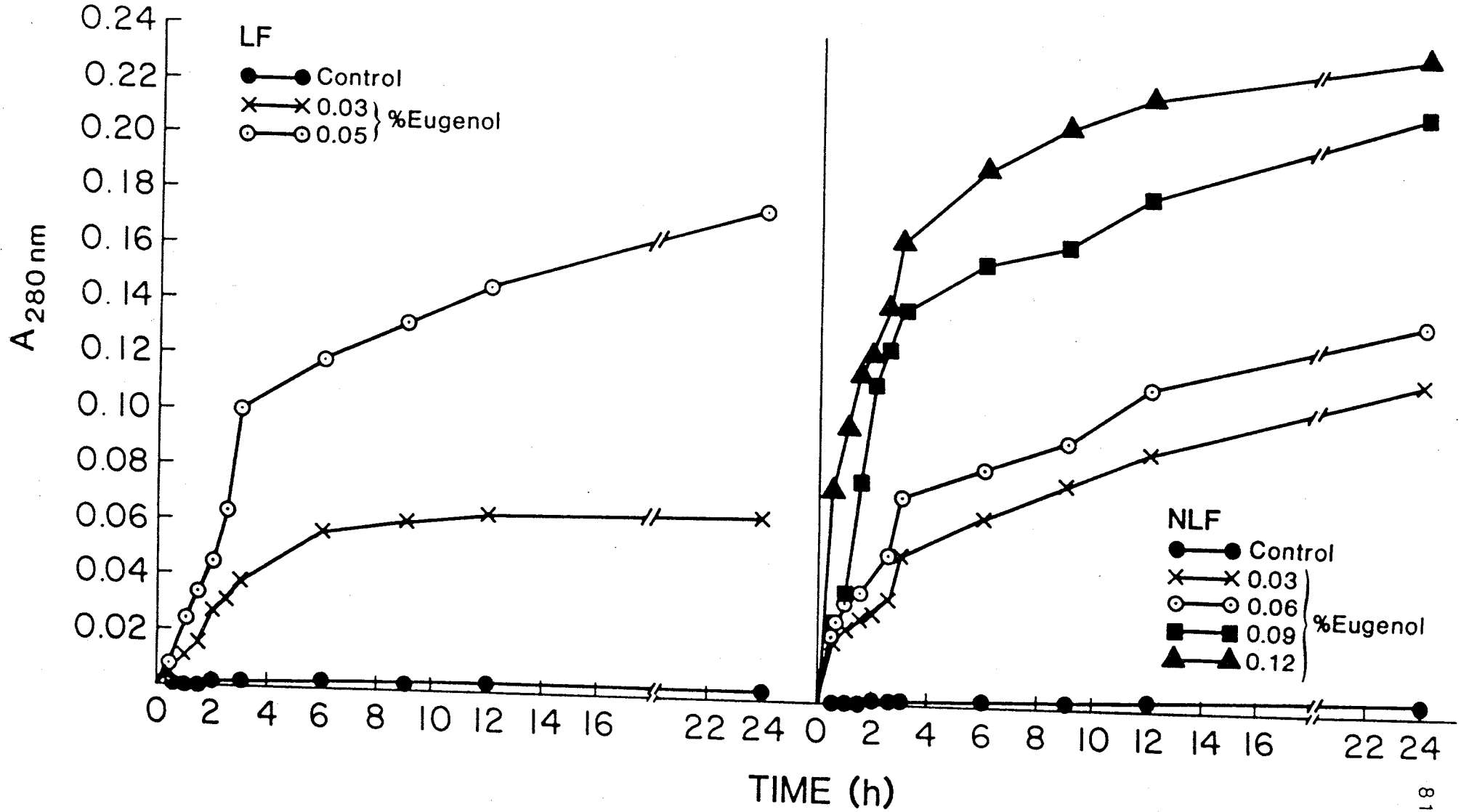


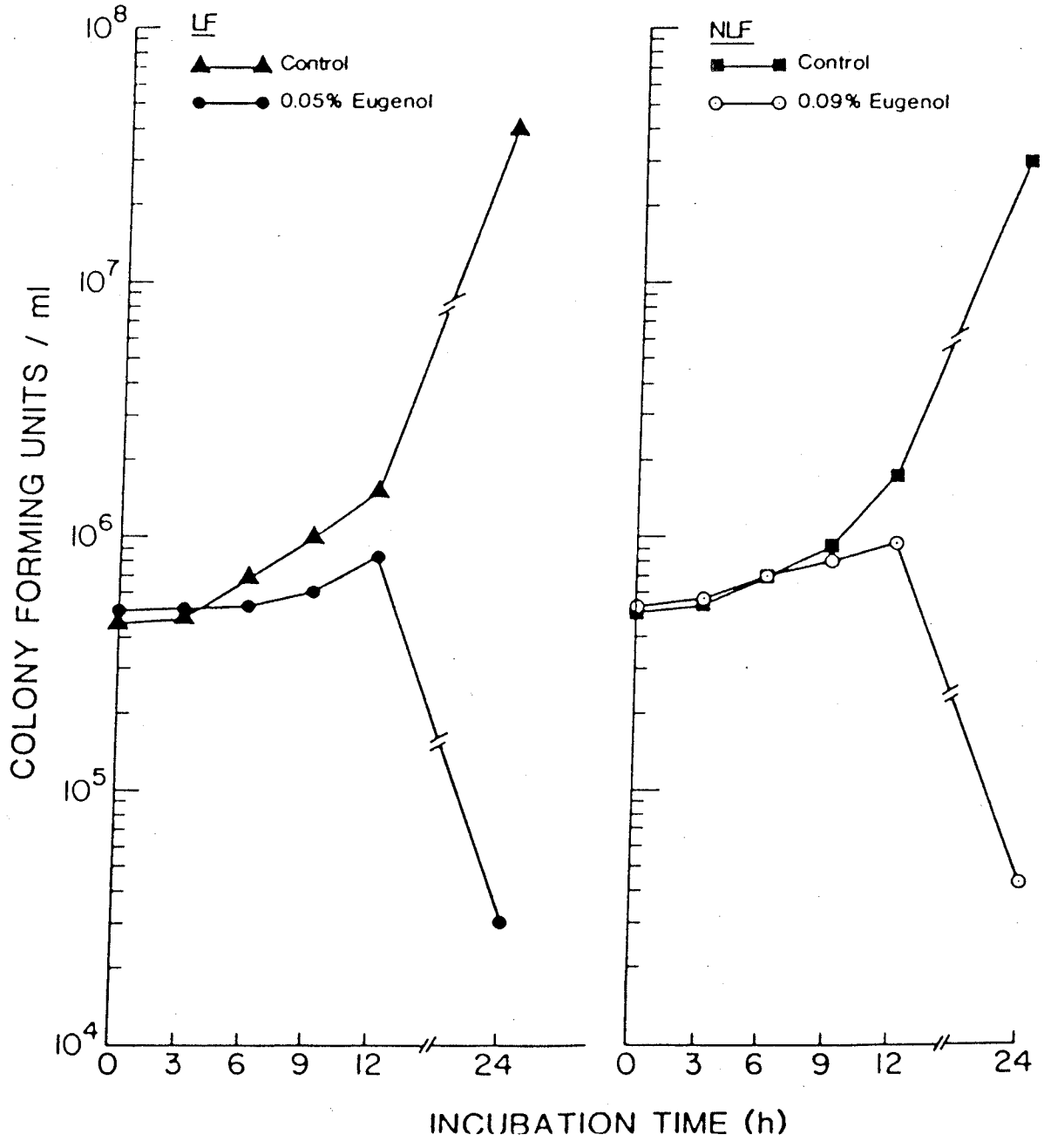
Figure 13 Leakage of UV (280 nm) material from LF and NLF cells grown in the presence of eugenol.



lular membrane, impairing both its function and integrity. This is consistent with previous reports on phenolic compounds causing a weakness in the permeability barrier of the cell membrane and thereby cause release of intracellular components (Judis, 1963; Juven et al., 1972; Davidson, 1979; Davidson and Branen, 1980b; Degre' and Sylvestre, 1983). However, it appears that eugenol is not as lethal as BHA or BHT, when compared with the investigations of Davidson and Branen (1980b), who reported lethality of BHA within 30 min in Pseudomonas fluorescens and P. fragi cells. Nevertheless, there exists a possibility that this difference in susceptibility to eugenol and BHA is actually dependent on microbial species or strain, and/or on cell concentration, and/or on the type and concentration of the phenolic compound employed.

The leakage of UV absorbable material did not appear to be directly related with the loss of cell viability (Figure 14). Enumeration of both the LF and NLF cells treated with eugenol (0.05 and 0.09%,v/v respectively) indicated that although the filtrates showed considerable UV absorbable leakage material, the cell count remained fairly constant and even slightly increased during the first 12 h of exposure. The increase in UV absorbance was therefore detected prior to any lethal effects on the cells per se, and is consistent with the findings of Davidson and Branen (1980b). The results presented in Figure 14 suggest that lethality may be partially a result of leakage but not vice versa, and that lethality is not the total result of leakage. Since leakage results from the disruption of the cell membrane, it is possible that some membrane-bound enzymes could be affected

Figure 14 Lethality of LF and NLF cells grown in the presence of eugenol.



by phenolic compounds, which would also contribute to the death of the organism (Davidson and Branen, 1980b). However, the leakage of intracellular materials probably does not totally account for the inhibitory powers of eugenol.

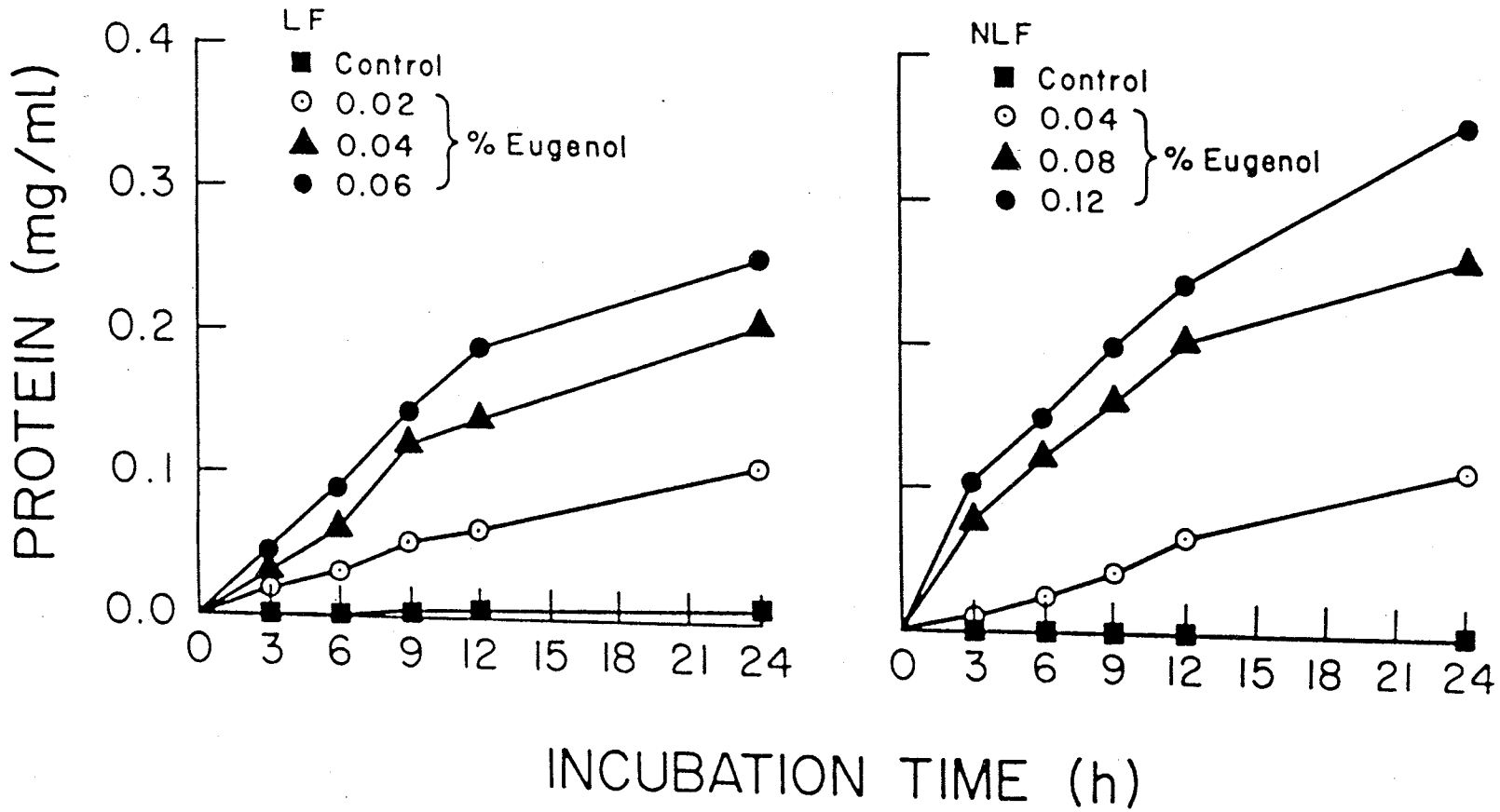
Although viability of both the LF and the NLF cell cultures was maintained during the period of maximum UV absorbable material loss, the degree of injury in these cultures is not known. Cells injured during the first 12 h of eugenol exposure may have undergone repair in the plating medium. Since the nature and adequacy of the recovery medium is vital, there exists a possibility that lethality, to a certain degree, may be dependent on the recovery medium.

4.9. Eugenol-induced protein leakage from LF and NLF cells.

Protein leakage from the LF and NLF cultures induced by various concentrations of eugenol is shown in Figure 15. Increasing concentrations of eugenol (%,v/v) progressively increased protein leakage; the LF cell filtrate (0.06% eugenol) was analysed to contain 0.25 mg/mL protein at 24 h, which was substantially higher than the control (0.1 mg/mL). The NLF filtrate (0.12% eugenol) showed 0.34 mg/mL protein leakage at 24 h while the control filtrate contained no detectable protein.

Protein leakage, as a result of the presence of increasing concentrations of phenolic compounds was also reported by Sgaragli et al. (1977). These researchers found that monocyclic compounds like BHA and BHT caused protein solubilization, thereby resulting in leakage of proteins from rat liver mitochondria-ly-

Figure 15 Eugenol-induced protein leakage from LF and NLF cells.



sosomes suspensions. The results presented on protein leakage suggest that eugenol exerts a perturbing action on the cellular membrane, causing a weakening or disruption of the permeability barrier, which allows proteins to be released or leaked from the cell. Protein is thus confirmed as one of the constituents involved in intracellular leakage from the cells of both the LF and the NLF organisms.

4.10. Effect of eugenol on cellular fatty acid composition.

The fatty acid types identified from the LF and NLF cells grown in the presence of varying concentrations of eugenol are presented in Tables 16 and 17. Fifteen fatty acid methyl esters were identified in each organism (Table 15; Appendix Tables 17 and 18). Most of the fatty acid methyl esters found in the control samples of the two organisms are in agreement with the reported fatty acids normally found in Lactobacillus species (Thorne and Kodicek, 1962; O'Leary, 1965; Veerkamp, 1971; Uchida and Mogi, 1973) although some reported fatty acids such as n-C16:1, n-C17:1, a-C15:0 and C19-cyclopropane (lactobacillic acid) were not identified since standards for these fatty acids could not be obtained. The actual percentages of these fatty acids were different however, from those reported in the literature,and may represent variations in the environmental growth conditions (Davidson and Branen, 1980b). In addition, contrary to previous investigations, which reported only trace amounts of n-C12:0 (n-dodecenoic (lauric) acid) in Lactobacillus strains, this fatty acid, after taking into consideration the concentra-

Table 15 Fatty acids observed in the lactic acid bacteria, Lactobacillus spp (lactose (LF) and non-lactose (NLF) fermentors).

No. of carbon atoms	Systematic name	Common name	Relative retention time[1] (min)
n-C12:0	n-dodecenoic	lauric	4.39
n-C14-0	n-tetradecenoic	myristic	7.78
n-C15:0	n-pentadecenoic	-	9.84
n-C16:0	n-hexadecenoic	palmitic	12.58
n-C17:0	n-heptadeconoic	margaric	15.35
n-C18:0	n-octadecenoic	stearic	18.42
n-C20:0	n-eicosanoic	arachidic	24.66
n-C22:0	n-docosanoic	behenic	30.92
n-C18:1	9-octadecenoic	oleic	19.76
n-C18:2	9,12-octadecenoic	linoleic	22.22
i-C13:0	iso-tridecenoic	-	6.48
i-C14:0	iso-tetradecenoic	-	8.77
i-C15:0	iso-pentadecenoic	-	10.90
i-C16:0	iso-hexadecenoic	-	13.89
a-C16:0	anteiso-hexadecenoic	-	14.34

n - normal
i - iso-branched
a - anteiso-branched

[1]Chromatographic Conditions: 10% DEGS column (1.83 m x 2 mm, i.d.) on 80/100 Chromosorb W mesh; 130°-200°C at 2°C/min; N_2 carrier gas at flow rate of 20 mL/min; 23 psi. column back pressure; flame ionization detector.

Table 16 Relative percentage of fatty acid types in LF cells grown in the presence of various concentrations of eugenol.

Time (h)	Eugenol (%, v/v)	Fatty acid type (%, w/w)				Ratio Saturated: Unsaturated
		Saturated[a]	Unsaturated[b]	Branched[c]	UI[d]	
	0	55.60	44.40	0	-	1.26
12	.02	49.71	47.93	2.30	0.06	1.04
	.04	50.50	47.00	2.46	0.04	1.08
	0	64.24	35.71	0.02	0.03	1.80
24	.02	43.39	51.29	3.24	0.08	0.89
	.04	40.32	55.66	3.91	0.11	0.73
	0	66.75	32.39	0.80	0.06	2.06
36	.02	40.72	53.93	5.29	0.06	0.76
	.04	32.77	61.96	5.24	0.03	0.53
	0	70.20	28.19	1.56	0.05	2.49
48	.02	34.80	58.52	6.59	0.09	0.60
	.04	23.10	69.63	7.21	0.06	0.34
	0	69.38	28.43	2.30	-	2.44
60	.02	30.00	67.08	8.64	-	0.45
	.04	13.83	77.11	8.99	0.07	0.18

[a]Saturated FA - C12:0; C14:0; C15:0; C16:0; C17:0; C18:0; C20:0 and C22:0

[b]Unsaturated FA - C18:1 and C18:2

[c]Branched FA - I-C13; I-C14; I-C15; I-C16 and A-C16

[d]UI - unidentified fatty acids

Table 17 Relative percentage of fatty acid types in NLF cells grown in the presence of various concentrations of eugenol.

Time (h)	Eugenol (%, v/v)	Fatty acid type (%, w/w)				Ratio Saturated: Unsaturated
		Saturated[a]	Unsaturated[b]	Branched[c]	UI[d]	
12	0	69.78	29.90	0.25	0.07	2.34
	.02	69.55	30.15	0.21	0.09	2.31
	.04	70.41	29.15	0.38	0.06	2.42
	.06	67.32	32.21	0.40	0.05	2.09
24	0	72.77	26.64	0.51	0.08	2.74
	.02	72.84	26.38	0.80	0.02	2.77
	.04	68.99	27.89	2.99	0.13	2.48
	.06	63.61	32.90	3.38	0.11	1.94
36	0	63.78	19.38	0.66	16.18	3.29
	.02	74.27	24.46	1.20	0.07	3.02
	.04	65.42	30.85	3.64	0.09	2.12
	.06	59.50	35.91	4.48	0.11	1.66
48	0	80.53	18.26	1.12	0.09	4.41
	.02	74.78	23.29	1.86	0.07	3.22
	.04	59.35	35.45	4.97	0.23	1.67
	.06	54.43	39.92	5.60	0.05	1.36
60	0	64.60	20.76	1.47	13.17	3.43
	.02	72.32	24.88	2.77	0.03	2.92
	.04	52.13	41.59	6.19	0.09	1.26
	.06	47.34	45.49	7.09	0.08	1.04

[a]Saturated FA - C12:0; C14:0; C15:0; C16:0; C17:0; C18:0; C20:0 and C22:0

[b]Unsaturated FA - C18:1 and C18:2

[c]Branched FA - I-C13; I-C14; I-C15; I-C16 and A-C16

[d]UI - unidentified fatty acids

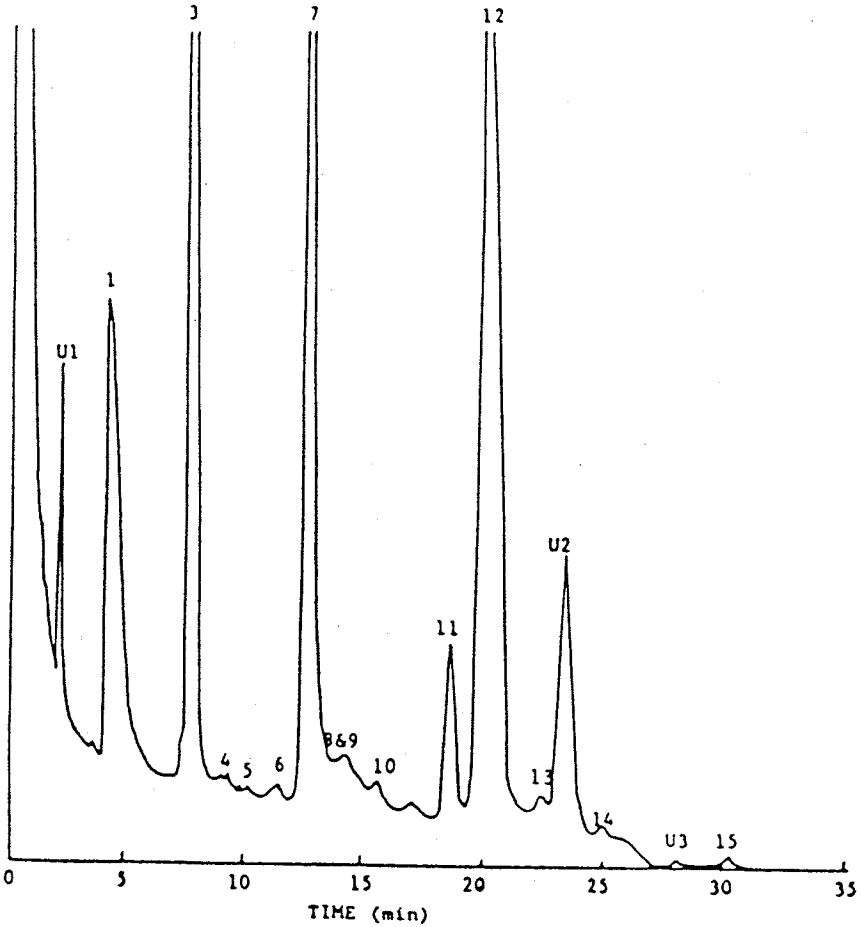
tion used as an internal standard (Uchida and Mogi, 1973) was relatively high in both organisms (up to 3.46% in the LF and 28% in the NLF).

Eight saturated fatty acids were identified (Figures 16 and 17; Table 15). n-C16:0 (n-hexadecenoic (palmitic) acid) appeared to be the dominant saturated fatty acid in both organisms, comprising 27 to 37% of total fatty acids identified; followed by n-C14:0 (n-tetradecenoic (myristic) acid) in the LF (20-28%) and n-C12:0 in the NLF (21-31%), and n-C18:0 (n-octadecenoic (stearic) acid) in both organisms (6-12%). Small, but significant amounts of n-C20:0 (n-eicosanoic (arachidic) acid) and n-C22:0 (n-docosanoic (behemic) acid) were also identified in both organisms; the latter fatty acids have not been previously reported to exist in the lactobacilli. Two unsaturated fatty acids, n-C18:1 (oleic acid) and n-C18:2 (linoleic acid) were identified, with the former fatty acid present in the higher concentration (ca. 43% in the LF, and 28% in the NLF). n-C18:2 had not been previously reported in the literature, but was found in lesser amounts in the controls. The occurrence of these previously unreported fatty acids in Lactobacillus cannot be explained, but may be due to the cultural conditions employed.

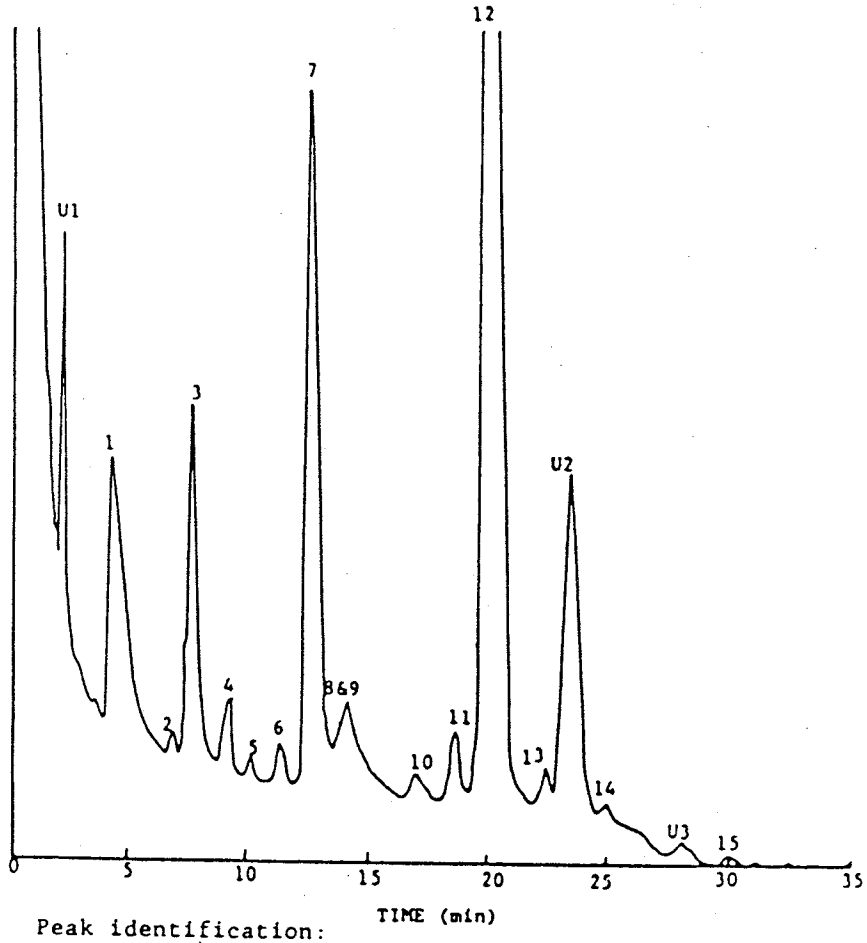
Generally, increasing concentrations of eugenol appeared to cause a significant ($P<0.05$) decrease in the saturated to unsaturated (S/U) fatty acid ratio in both organisms (Tables 16 and 17; Appendix Tables 30 and 31). Increased amounts of unsaturated fatty acids (n-C18:1 and n-C18:2) appeared to be synthesized in the presence of eugenol, with a concomitant decrease in the

Figure 16 Representative gas chromatograms of the cellular fatty acid methyl esters from saponified whole cells of the LF.

LF (Control) grown for 24 h.



LF grown in 0.04%, v/v eugenol for 24 h.



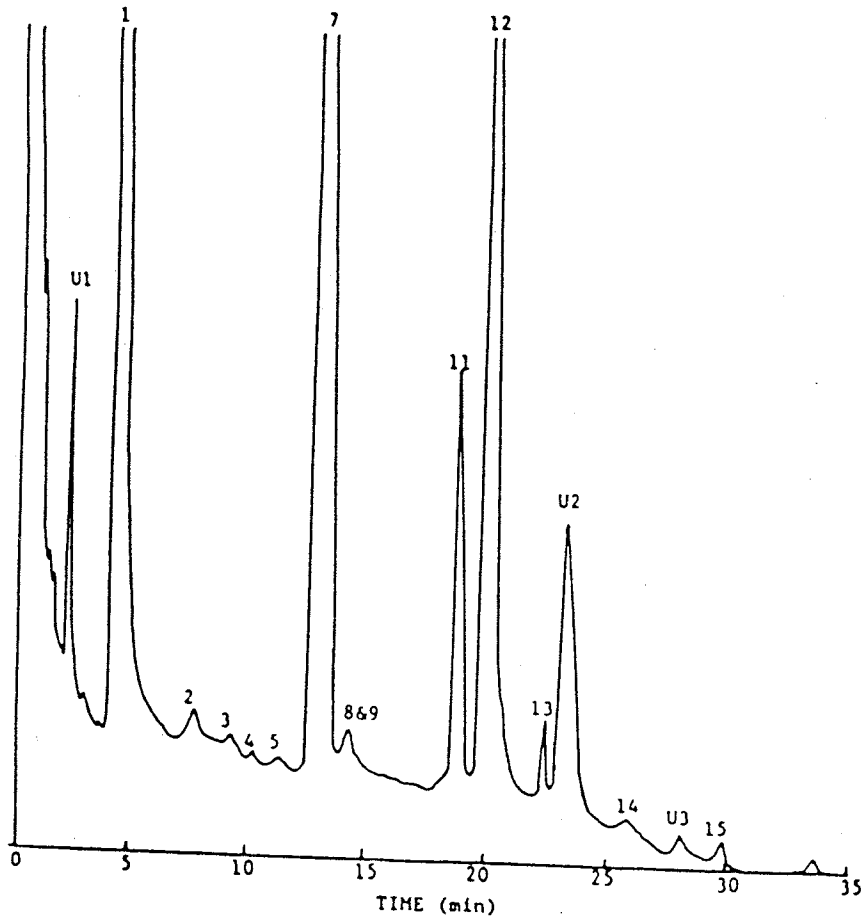
Chromatographic conditions:
Varian Model 3700 Gas Chromatograph; detector: FID; column: 10% DEGS(1.83 m *2 mm,i.d.) on 80/100 Chromosorb W mesh; N_2 flow rate: 20 mL/min; column temperature: 130 -200°C at 2°C/min; column backpressure: 23 psi; sample size: 2 µl.

Peak identification:
(1)*n-C12:0;(2) i-C13:0;(3) n-C14:0;(4) i-C14:0;
(5) n-C15:0;(6) i-C15:0;(7) n-C16:0;(8) i-C16:0;
(9) a-C16:0;(10) n-C17:0;(11) n-C18:0;(12) n-C18:1;
(13) n-C18:2;(14) n-C20:0;(15) n-C22:0;
(U1, U2 and U3) unidentified fatty acids.

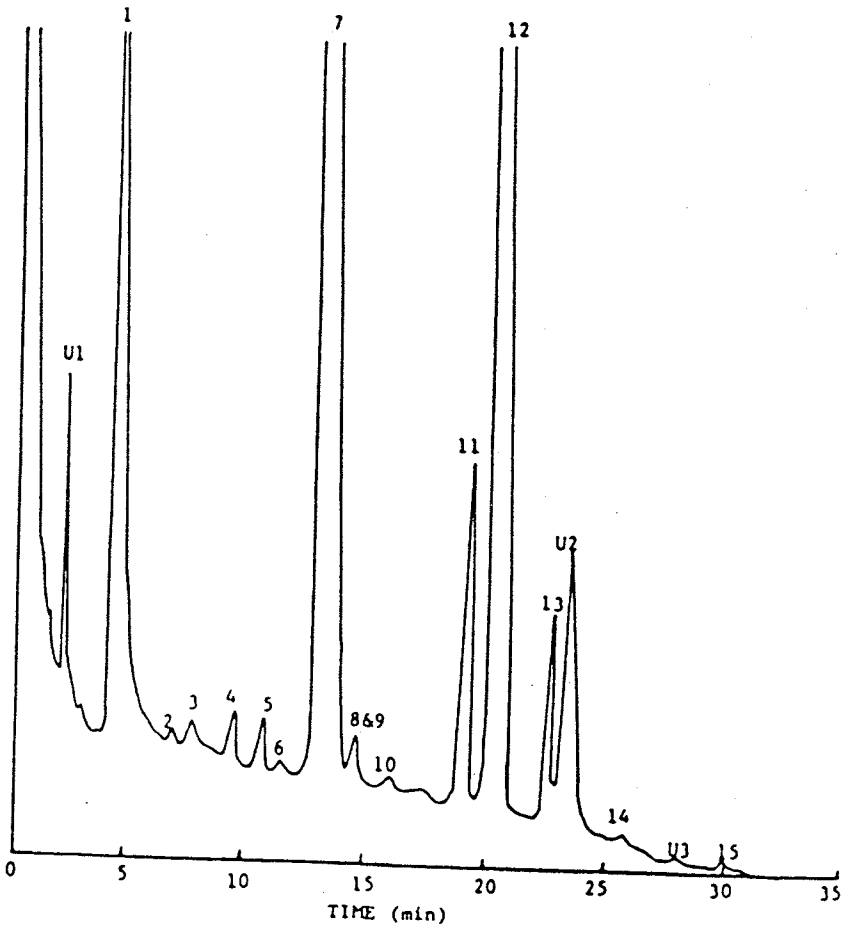
*n-C12:0 is an internal standard.

Figure 17 Representative gas chromatograms of the cellular fatty acid methyl esters from saponified whole cells of the NLF.

NLF (Control) grown for 24 h.



NLF grown in 0.06%, v/v eugenol for 24 h.



Chromatographic conditions:
Varian Model 3700 Gas Chromatograph; detector: FID; column: 10% DEGS(1.83 m *2 mm,i.d.) on 80/100 Chromosorb W mesh; N_2 flow rate: 20 mL/min; column temperature: 130 -200°C at 2°C/min; column backpressure: 23 psi; sample size: 2 µl.

Peak identification:
(1)*n-C12:0;(2) i-C13:0;(3) n-C14:0;(4) i-C14:0; (5) n-C15:0;(6) i-C15:0;(7) n-C16:0;(8) i-C16:0; (9) a-C16:0;(10) n-C17:0;(11) n-C18:0;(12) n-C18:1; (13) n-C18:2;(14) n-C20:0;(15) n-C22:0; (U1, U2 and U3) unidentified fatty acids.
*n-C12:0 is an internal standard.

syntheses of saturated fatty acids. Although it was expected that the organisms, in the presence of phenolic compounds, would synthesize increased amounts of saturated fatty acids in order to maintain proper cell membrane "fluidity" (Eletr et al., 1974; Singer and Wan, 1977), the opposite effect was found in the LF and NLF organisms, compared to their respective controls. The S/U fatty acid ratio was higher in the NLF than in the LF (Tables 16 and 17). Sgaragli et al (1977) reported that BHA and BHT caused structural modifications in biomembranes, as a result of perturbations of the lipid alkyl chains in the membranes. When the lipid alkyl chains were perturbed, they were shown to lose their ordered state and became more "fluid" (Eletr et al.,1974; Singer and Wan, 1977). Davidson and Branen (1980b) reported that conversely to Pseudomonas fluorescens grown in the presence of 50 mg/L BHA, P. fragi cultures grown in BHA showed a decrease in S/U fatty acid ratios over the controls. This phenomenon was also observed with the LF and NLF cultures, and suggested that these organisms may not necessarily respond to phenolics only by synthesizing increased amounts of saturated fatty acids.

Eletr et al.(1974), reported that compounds which perturb the lipids of membranes also cause decreased activity of membrane-bound enzymes. Therefore, the LF and NLF organisms may have changed the organization of their membranes to minimize the effect of eugenol on these enzymes. The data on fatty acid types and composition also indicated a possible reason for the difference in susceptibility to eugenol between these two organisms. Both the NLF control and treatment cultures had much higher S/U

fatty acid ratios compared to those for the LF control and treatment samples (Tables 16 and 17). This may suggest that membranes high in saturated fatty acids are more resistant to the initial effects of phenolic compounds, as observed with the NLF, which also had higher amounts of saturated fatty acids than the LF, and which from previous studies, showed higher resistance to eugenol. Davidson and Branen (1980b), postulated that this phenomenon is probably due to a higher degree of organization or packing of the lipid alkyl chains within the membrane of such organisms.

The presence of eugenol also appeared to cause the syntheses of some fatty acids not normally found in Lactobacillus species. These included branched chain fatty acids (i-C13:0, i-C14:0, i-C15:0, i-C16:0 and a-C16:0), as well as two saturated, but odd-numbered carbon fatty acids (n-C15:0 and n-C17:0) (Table 15; Figures 16 and 17). The occurrence of these unusual fatty acids in the LF and NLF organisms treated with eugenol may be due to the operation of different metabolic pathways brought about as a result of the presence of eugenol, in an attempt to minimize toxic effects. Ingram (1977) reported that Escherichia coli synthesized several unusual fatty acids, including n-C15:0 and n-C17:0, when grown in the presence of various organic solvents and food additives. This could also explain the occurrence of branched-chain fatty acids in the LF and NLF organisms.

The synthesis of these unusual fatty acids in the LF and NLF organisms could also be a result of the stress placed on the organisms by eugenol, and may represent a distinctive effort or attempt by the cells to maintain a homeoviscous membrane (Sine-

sky, 1974; Cronan and Gelman, 1975). The concentration of these unusual fatty acids was observed to increase with increasing eugenol concentrations, and with the length of incubation (Figures 18 and 19). However, the presence of n-C15:0 and n-C17:0, which are also saturated fatty acids, contributed to an increase in the overall amount of saturated fatty acids found in both organisms. The quantity of these unusual fatty acids in the controls were, however, very small compared to the treatments in both organisms. The amount of unidentified fatty acids present in the control were found to be generally higher than in the treatments (Tables 16 and 17), although the significance of this cannot be established.

The length of incubation at 30°C also resulted in altered fatty acid type and composition in both organisms, although this was not confirmed by reports in the literature. It is quite plausible, however, to expect that organisms in their maximal stationary and death phases will have altered fatty acid compositions, probably in response to adverse cultural and environmental conditions. In the LF and NLF cultures, maximum growth was always achieved at 24 h, and extended incubation beyond 24 h up to 60 h would be expected to result in decreased growth and growth rate, due to substrate limitation and accumulation of end-products, in this case lactic acid, which may itself inhibit growth and metabolism of the organisms. In both organisms, extended incubation up to 60 h was found to significantly ($P<0.05$) affect the S/U ratios. Incubation time, in conjunction with the effect of eugenol, was similarly found to significantly ($P<0.05$) affect the S/U

Figure 18 Effect of eugenol on total cellular branch-chained (iso - and ante-iso -) fatty acid contents in the LF and NLF cells.

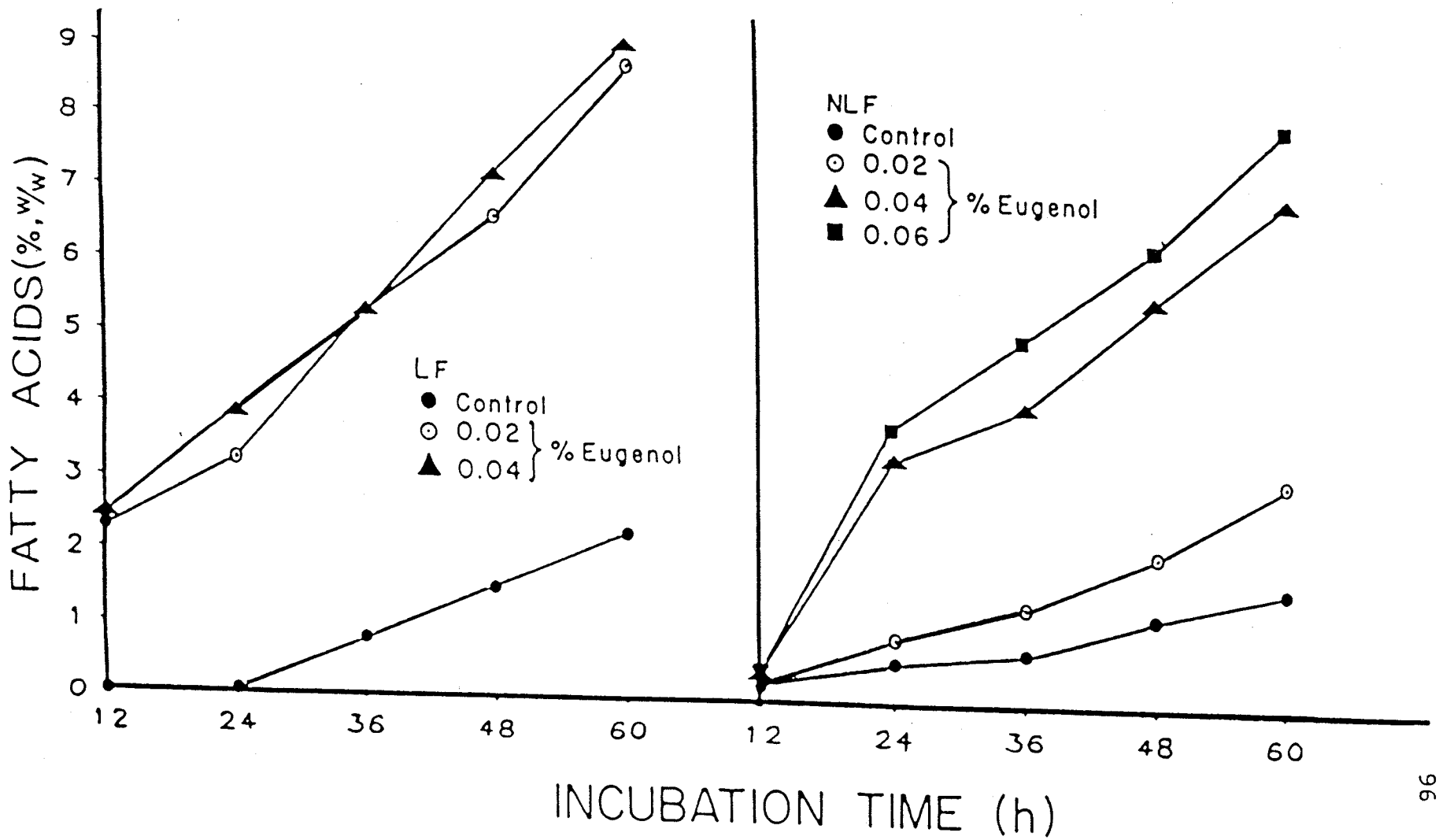
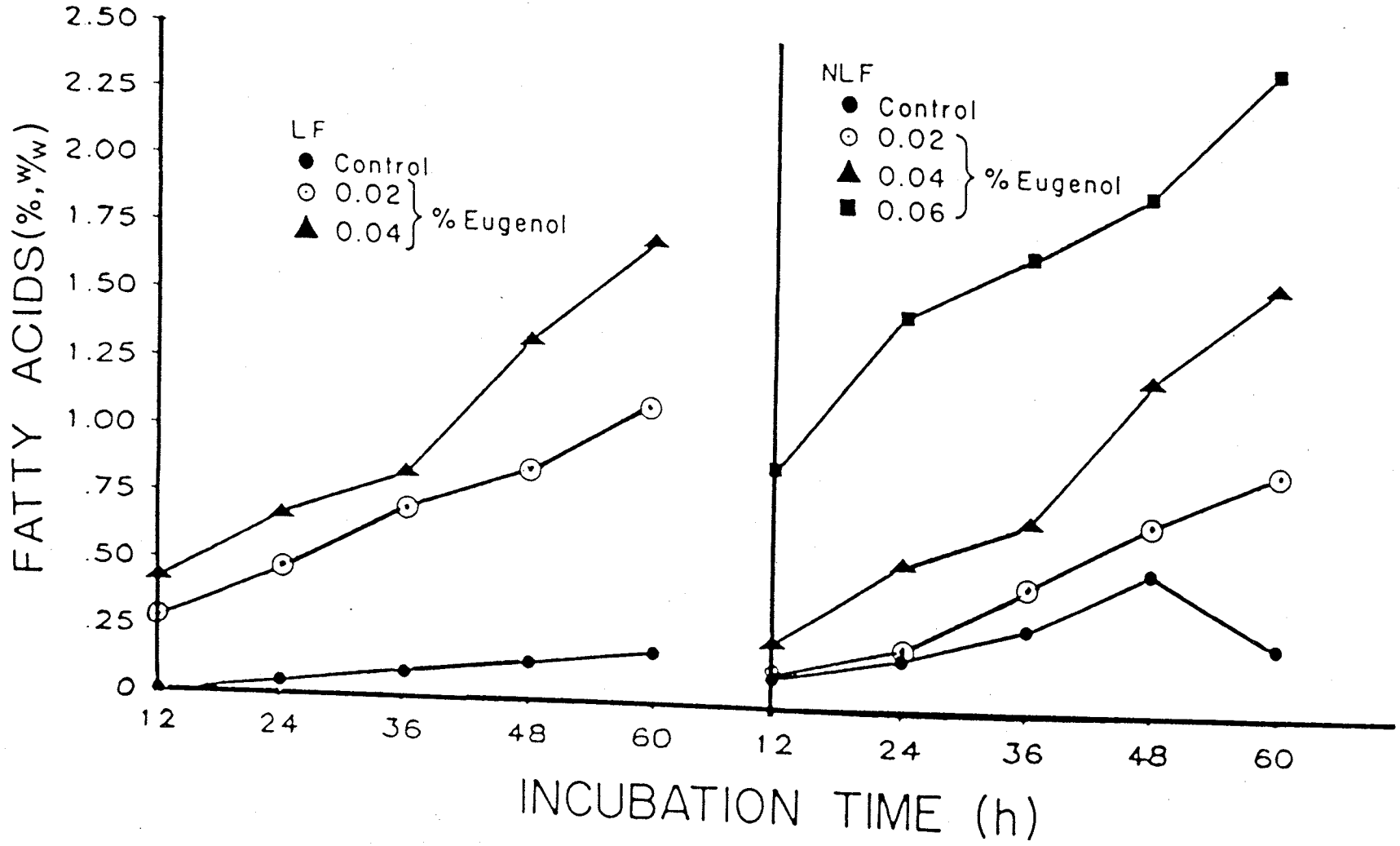


Figure 19 Effect of eugenol on total cellular saturated odd-numbered carbon fatty acid contents in LF and NLF cells.



fatty acid ratios in both the LF and NLF organisms (Appendix Tables 30 and 31).

4.11. Effect of culture transfer and eugenol on cellular fatty acid composition.

When the LF and NLF organisms were grown in 0.03 and 0.05%,v/v eugenol respectively, and then transfered after each 24 h incubation period into fresh APT broth, with or without the addition of the corresponding concentration of eugenol, the same fatty acids previously identified (4.9.) were observed and quantitated (Tables 18 and 19). When eugenol cultured LF and NLF cells were transfered into fresh APT broth devoid of eugenol (Figure 2), and the transfer repeated four additional times, the S/U fatty acid ratio in both organisms progressively increased. In the LF organism, this ratio increased from 0.51 (1st transfer) to 1.21 (5th transfer); in the NLF organism, from 3.21 (1st transfer) to 7.16 (5th transfer). It thus appeared that everytime eugenol-treated cells were transfered into a medium without eugenol, the cells were able to recover from the initial effects of eugenol by synthesizing more of the saturated fatty acids. As mentioned earlier in the literature, the S/U fatty acid ratio is expected to increase in the presence of phenolic compounds, and conversely, a decrease in the absence of these compounds (Sgaragli et al., 1977; Davidson and Branen, 1980b). Again, the opposite effect was found in both test organisms.

Based on these observations, the increase in the S/U fatty acid ratio in LF and NLF cells transferred from eugenol-based

Table 18 Effects of culture transfer and eugenol on the relative percentage of fatty acid types found in LF cells.

No of transfers	Eugenol (%, v/v)	Fatty acid type (%, w/w)				Ratio Saturated: Unsaturated
		Saturated[a]	Unsaturated[b]	Branched[c]	UI[d]	
	.03	23.26	73.73	2.86	0.15	0.32
1	0	33.69	65.82	0.43	0.06	0.51
	.03	27.30	68.91	3.70	0.09	0.40
2	0	41.19	58.15	0.58	0.08	0.71
	.03	26.54	67.59	5.70	0.17	0.39
3	0	45.89	53.36	0.65	0.10	0.86
	.03	25.02	67.27	7.39	0.32	0.37
4	0	50.57	48.52	0.81	0.10	1.04
	.03	21.93	69.43	8.30	0.34	0.32
5	0	54.26	44.68	1.00	0.06	1.21
	.03	16.89	74.17	8.49	0.45	0.23

[a]Saturated FA - C12:0; C14:0; C15:0; C16:0; C17:0; C18:0; C20-0 and C22:0

[b]Unsaturated FA - C18:1 and C18:2

[c]Branched FA - I-C13; I-C14; I-C15; I-C16 and A-C16

[d]UI - unidentified fatty acids

Table 19 Effects of culture transfer and eugenol on the relative percentage of fatty acid types found in NLF cells.

No of transfers	Eugenol (%, v/v)	Fatty acid type (%, w/w)				Ratio Saturated: Unsaturated
		Saturated[a]	Unsaturated[b]	Branched[c]	UI[d]	
	.05	71.71	27.62	0.60	0.07	2.50
1	0	75.83	23.62	0.50	0.05	3.21
	.05	63.74	34.89	1.32	0.05	1.83
2	0	81.82	17.71	0.43	0.04	4.62
	.05	59.39	38.57	2.00	0.04	1.54
3	0	83.28	16.25	0.44	0.03	5.13
	.05	54.51	42.40	3.04	0.05	1.29
4	0	85.56	14.19	0.21	0.04	6.03
	.05	45.49	49.39	5.10	0.02	0.92
5	0	87.66	12.24	0.08	0.02	7.16
	.05	41.04	53.47	5.47	0.02	0.77

[a]Saturated FA - C12:0; C14:0; C15:0; C16:0; C17:0; C18:0; C20:0 and C22:0

[b]Unsaturated FA - C18:1 and C18:2

[c]Branched FA - I-C13; I-C14; I-C15; I-C16 and A-C16

[d]UI - unidentified fatty acids

media into non-eugenol media, may indicate a successful attempt by the organisms to recover from the initial effects of eugenol. It was noted that transfers into non-eugenol containing broths was carried out from treated cells, and thus, the organisms were repeatedly subjected to the same concentrations of eugenol to test their adaptability to this compound. Also, the absence of eugenol in the culture broth enabled these organisms to multiply and those which were injured to undergo repair; this may have actually contributed to the increase in the S/U fatty acid ratios. However, since new cell growth apparently occurred from the initial eugenol-treated cells, and sufficiently recovered from the effects of eugenol, it would appear that a lingering effect of eugenol on the organisms existed. This would contribute to a slower growth rate of the cells in the untreated broth. Also, the concentrations of eugenol used (0.03 and 0.05% for the LF and NLF respectively) were not totally inhibitory towards the growth of these organisms, therefore some recovery would be expected to occur.

With successive transfer of these organisms into fresh eugenol-based media (Figure 2), the S/U fatty acid ratio decreased; this ratio was again higher in the NLF organism than in the LF organism (Tables 18 and 19). This again may indicate an attempt by these organisms to synthesize more unsaturated instead of saturated fatty acids. This phenomenon involving decreased synthesis or occurrence of saturated fatty acids was again contrary to previous investigations by Eletr et al., (1974) and Singer and Wan (1977). However, altered fatty acid compositions in both organ-

isms is consistent with the literature (Ingram, 1977; Singer and Wan, 1977; Davidson and Branen, 1980b).

A difference in the amount of unusual fatty acids (branched and odd-number carbon) was also observed in these transfer cultures. Transfer of LF and NLF cultures from eugenol-based media to non-eugenol based media resulted in a decrease in the amount of unusual fatty acids in the NLF (Table 19) and an increase in the LF (Table 18) as the number of transfers was increased (up to the 5th transfer). According to previous findings in this study, a decrease in the amount of these fatty acids in non-treated NLF cultures was expected, however, the reason for the increase in unusual fatty acids in the LF cannot be adequately explained. The probability exists that the amount of unusual fatty acids may be a result of differences existing in their cell membranes, which in turn influenced their susceptibility to eugenol. Transfers from eugenol-based media to fresh eugenol-based media resulted in increased levels of unusual fatty acids in both organisms as transfers were continued (Tables 18 and 19), while the amount of unidentified fatty acids appeared to increase in the LF, but remained more or less constant in the NLF organism. It is suggested that interconversion of fatty acids might be occurring to compensate for the lower amounts of saturated fatty acids. The significance of these unusual fatty acids in the regulation of metabolism in the lactic acid bacteria is yet to be established.

Overall, it appeared that when eugenol-treated LF and NLF cells were transfered into fresh medium devoid of eugenol, the organisms adjusted or altered their fatty acid compositions to,

at least, near normal levels. This may indicate an attempt by the organisms to recover from the initial effects of eugenol. Transfer of eugenol-treated cells to a corresponding eugenol-based medium appeared not to result in any adaptability of the organisms to eugenol, at least as far as the organization of their cellular membranes is concerned.

6. CONCLUSIONS.

Although the antimicrobial effects of spices are well documented, relatively little information is available regarding their mechanism of microbial inhibition. In this investigation, preliminary data indicated that eugenol, a phenolic component of cloves, exhibited antimicrobial properties similarly described for BHA and BHT. Clearly, as with other antimicrobials, the concentration of the active compound in relation to the bacterial load and its environment were important in determining target sites. The leakage of protein and ATP content from eugenol-treated cells as well as the shift of saturated to unsaturated fatty acids indicates, at least in part, that cell membranes are a target site. Growth, pH and titratable acidity development in cultures grown with increasing levels of eugenol indicate varying susceptibilities. Since to be effective, the antimicrobial agent must be adsorbed/absorbed by the cell, it would seem plausible that the ease for this to occur would, at least in part, be dependent on the nature of the cell wall and/or cell membrane. At this particular point, there is no reason to believe that the cell membrane is the primary target site and/or is responsible for lethality.

Further work is recommended to elucidate the inhibitory mechanism of this compound, including its effect on the physiology and metabolism of microorganisms. It is also recommended that such future investigations be carried out with pathogenic and/or food spoilage organisms in food systems, rather than in laboratory media.

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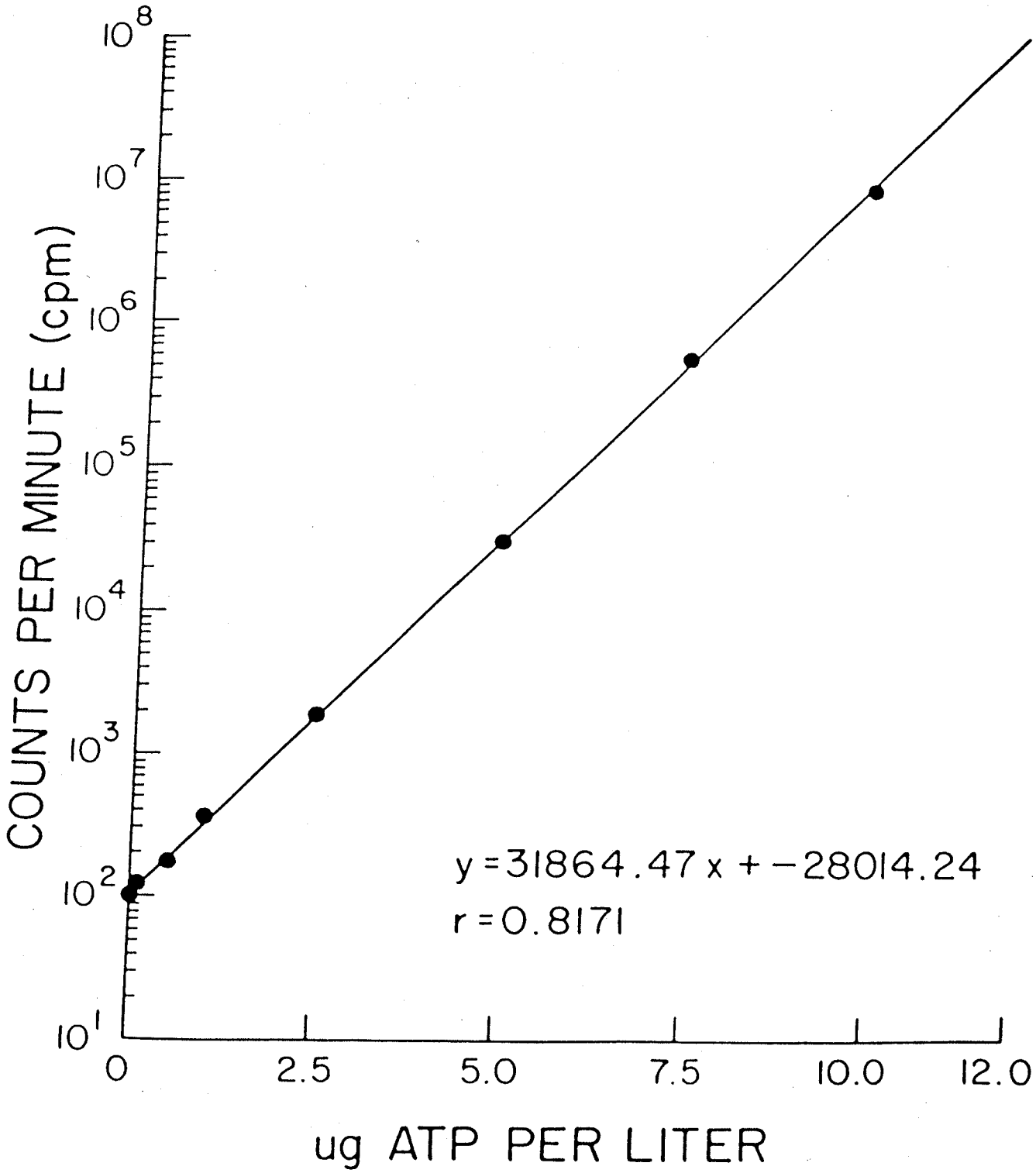
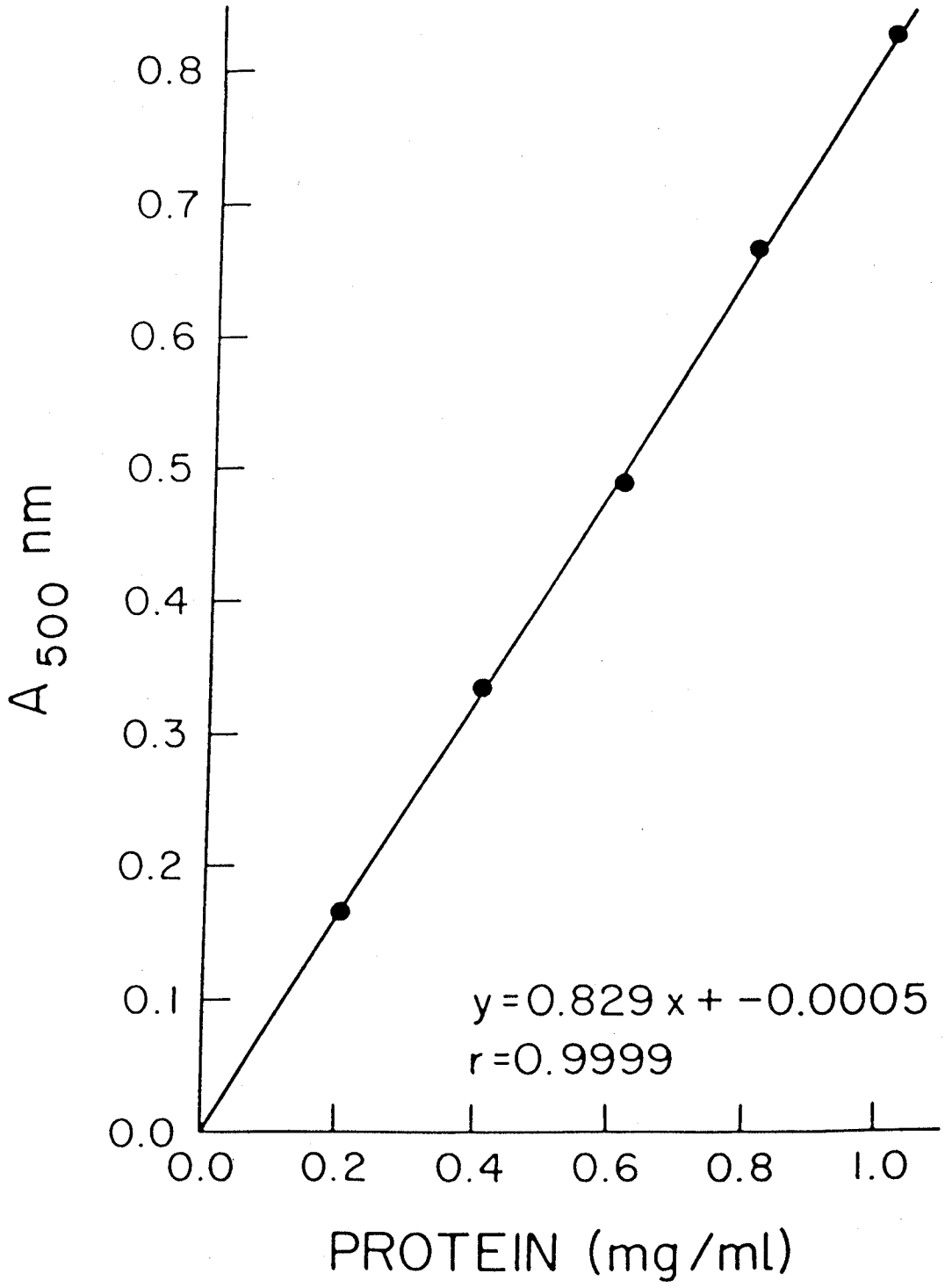
Appendix Figure 1 Standard ATP calibration curve relating light emission (in counts per minute) to ATP concentration in µg/L.



Appendix Figure 2 Standard protein calibration curve relating absorbance at 500 nm to protein concentration in mg/mL.



Appendix Table 1 Effect of eugenol on the rate of pH change by LF cells at 30°C.

Time (h)	pH				
	Eugenol (%,v/v)				
	0	.02	.03	.04	.05
0	6.52	6.52	6.50	6.50	6.50
1	6.34	6.35	6.35	6.35	6.37
2	6.33	6.36	6.34	6.35	6.36
3	6.30	6.32	6.32	6.34	6.35
4	6.29	6.30	6.31	6.32	6.34
5	6.28	6.30	6.30	6.31	6.33
6	6.26	6.29	6.29	6.30	6.32
7	6.20	6.28	6.28	6.29	6.32
8	6.16	6.24	6.24	6.28	6.32
9	5.99	6.16	6.20	6.27	6.31
10	5.75	6.10	6.15	6.27	6.31

Appendix Table 2 Effect of eugenol on the rate of pH change by NLF cells at 30°C.

Time	pH					
	Eugenol (%,v/v)					
(h)	0	.03	.06	.09	.12	.15
0	6.51	6.51	6.51	6.50	6.50	6.50
1	6.39	6.39	6.40	6.40	6.41	6.41
2	6.38	6.38	6.40	6.40	6.40	6.40
3	6.37	6.37	6.39	6.39	6.39	6.39
4	6.32	6.36	6.38	6.38	6.38	6.38
5	6.30	6.33	6.35	6.35	6.37	6.37
6	6.10	6.28	6.30	6.31	6.36	6.36
7	5.85	6.15	6.18	6.19	6.35	6.36
8	5.58	6.00	6.08	6.15	6.35	6.36
9	5.35	5.84	5.95	6.02	6.35	6.36
10	4.97	5.53	5.79	5.93	6.34	6.36

Appendix Table 3 Growth, pH and titratable acidity development by the LF in the presence of eugenol with extended incubation.

Time (h)	Eugenol (%, v/v)	pH	Titratable Acidity (mL 0.1N NaOH)	Growth (CFU/mL)
0	0	6.52	3.80	65×10^5
	.03	6.51	3.80	64×10^5
	.05	6.50	3.90	65×10^5
24	0	4.60	9.60	80×10^7
	.03	4.57	9.50	60×10^7
	.05	6.27	4.80	26×10^6
48	0	4.58	9.50	70×10^7
	.03	4.56	9.50	20×10^7
	.05	6.26	4.80	20×10^4
72	0	4.53	9.40	90×10^6
	.03	4.52	9.40	36×10^5
	.05	6.21	4.90	55×10^3
96	0	4.48	9.80	16×10^6
	.03	4.45	9.90	15×10^5
	.05	5.84	5.50	15×10^4

Appendix Table 4 Growth, pH and titratable acidity development by the NLF in the presence of eugenol with extended incubation.

Time (h)	Eugenol (%, v/v)	pH	Titratable Acidity (mL 0.1N NaOH)	Growth (CFU/mL)
0	0	6.52	3.90	56×10^5
	.04	6.51	3.90	54×10^5
	.08	6.50	4.00	56×10^5
	.12	6.48	4.20	57×10^5
24	0	4.30	10.20	81×10^7
	.04	4.99	8.50	74×10^7
	.08	5.90	6.00	30×10^5
	.12	6.50	4.00	10×10^3
48	0	4.22	11.00	74×10^7
	.04	4.81	8.60	70×10^7
	.08	6.21	4.90	40×10^4
	.12	6.50	4.00	22×10^2
72	0	4.11	11.70	40×10^6
	.04	4.38	9.90	23×10^5
	.08	6.32	4.40	30×10^3
	.12	6.51	3.90	14×10^2
96	0	4.10	11.70	30×10^5
	.04	4.29	10.20	37×10^4
	.08	6.46	4.30	10×10^2
	.12	6.52	3.90	10×10^2

Appendix Table 5 Culture development of LF cells in the presence of eugenol measured by biomass and absorbance at 600 nm.

Time (h)	Eugenol (%,v/v)	Biomass (mg/ml)	$A_{600\ nm}$
0	0	0.02	.011
	.02	0.02	.010
	.04	0.02	.008
	.06	0.02	.007
24	0	1.62	1.151
	.02	1.70	1.138
	.04	1.08	.880
	.06	0.60	.229
48	0	1.65	1.429
	.02	1.60	1.382
	.04	0.84	.841
	.06	0.38	.085
72	0	1.38	1.425
	.02	1.00	1.362
	.04	0.62	.374
	.06	0.18	.018

Appendix Table 6 Culture development of NLF cells in the presence of eugenol measured by biomass and absorbance at 600 nm.

Time (h)	Eugenol (%,v/v)	Biomass (mg/ml)	$A_{600\ nm}$
0	0	0.02	.014
	.03	0.02	.014
	.06	0.02	.013
	.09	0.02	.012
	.12	0.02	.013
24	0	1.56	1.147
	.03	1.91	1.116
	.06	1.72	.931
	.09	0.96	.296
	.12	0.70	.191
48	0	1.44	1.161
	.03	1.40	1.128
	.06	1.18	.908
	.09	0.32	.221
	.12	0.28	.077
72	0	1.32	1.080
	.03	1.22	1.040
	.06	1.01	.767
	.09	0.18	.137
	.12	0.09	.021

Appendix Table 7 Effect of eugenol and culture transfer on growth, pH and titratable acidity development by the LF.

Transfer Number	Eugenol (%, v/v)	CFU/ml Time (h) 0	CFU/ml Time (h) 24	pH Time (h) 0	pH Time (h) 24	TA (ml 0.1N NaOH) Time (h) 0	TA (ml 0.1N NaOH) Time (h) 24
0	0	61×10^5	88×10^8	6.52	4.69	3.8	9.1
	.03	61×10^5	75×10^8	6.52	4.69	3.8	9.1
	.05	61×10^5	40×10^6	6.51	5.63	3.8	5.8
1	0	88×10^6	28×10^8	6.52	4.66	3.8	9.7
	.03	75×10^6	35×10^7	6.52	4.66	3.8	9.7
	.05	40×10^4	58×10^5	6.51	6.00	3.8	4.9
2	0	28×10^6	44×10^8	6.52	4.72	3.8	9.0
	.03	35×10^5	40×10^6	6.52	4.90	3.8	8.2
	.05	58×10^3	18×10^3	6.51	6.33	3.8	4.5
3	0	44×10^6	33×10^8	6.52	4.66	3.8	9.7
	.03	40×10^4	18×10^5	6.52	5.20	3.8	7.1
	.05	18×10^1	10×10^1	6.51	6.41	3.8	4.2
4	0	33×10^6	61×10^8	6.52	4.68	3.8	9.2
	.03	18×10^3	78×10^3	6.51	5.97	3.8	6.1
	.05	10×10^1	11×10^0	6.51	6.50	3.8	4.0

Appendix Table 8 Effect of eugenol and culture transfer on growth, pH and acidity development by the NLF.

Transfer Number	Eugenol (%, v/v)	CFU/ml Time (h) 0	CFU/ml Time (h) 24	pH Time (h) 0	pH Time (h) 24	TA (ml 0.1N NaOH) Time (h) 0	TA (ml 0.1N NaOH) Time (h) 24
	0	$63x10^5$	$84x10^8$	6.52	4.59	3.8	10.0
0	.04	$63x10^5$	$62x10^8$	6.51	4.94	3.8	8.7
	.08	$63x10^5$	$50x10^5$	6.50	5.90	3.9	6.8
	.12	$63x10^5$	$59x10^4$	6.48	6.50	3.9	3.9
	0	$84x10^6$	$34x10^8$	6.52	4.38	3.8	10.8
1	.04	$62x10^6$	$79x10^7$	6.51	4.95	3.8	8.6
	.08	$50x10^3$	$64x10^2$	6.50	6.38	3.9	4.6
	.12	$59x10^2$	$12x10^1$	6.48	6.50	3.9	3.9
	0	$34x10^6$	$42x10^8$	6.52	4.12	3.8	11.6
2	.04	$79x10^5$	$31x10^6$	6.51	5.21	3.8	8.0
	.08	$64x10^0$	$50x10^0$	6.50	6.52	3.9	3.8
	.12	$12x10^0$	-	6.48	6.52	3.9	3.8
	0	$42x10^6$	$38x10^8$	6.52	4.47	3.8	10.6
3	.04	$31x10^4$	$30x10^5$	6.51	5.67	3.8	7.3
	.08	-	-	6.50	6.52	3.9	3.8
	.12	-	-	6.49	6.52	3.9	3.8
	0	$38x10^6$	$43x10^8$	6.52	4.33	3.8	11.1
4	.04	$30x10^3$	$28x10^4$	6.52	5.88	3.8	6.8
	.08	-	-	6.51	6.52	3.9	3.8
	.12	-	-	6.50	6.52	3.9	3.8

Appendix Table 9 Adenosine triphosphate content of LF cells grown in the presence of eugenol.

Time (h)	Eugenol (%, v/v)	Biomass (mg/mL)	cpm	µg ATP/L/mg of cells
0	0	0.02	-	-
	.02	0.02	-	-
	.04	0.02	-	-
	.06	0.02	-	-
12	0	0.95	10.20×10^{6}	8.47
	.02	0.95	10.43×10^{6}	8.49
	.04	0.52	21.13×10^{5}	6.75
	.06	0.25	26.60×10^{5}	6.95
24	0	1.67	18.63×10^{7}	10.74
	.02	1.63	13.56×10^{7}	10.62
	.04	1.05	10.45×10^{6}	8.50
	.06	0.62	19.16×10^{5}	6.68
36	0	1.64	8.52×10^{7}	10.49
	.02	1.60	7.42×10^{7}	10.45
	.04	0.93	15.85×10^{6}	8.62
	.06	0.56	39.87×10^{5}	7.30
48	0	1.62	6.85×10^{7}	10.43
	.02	1.56	64.73×10^{6}	9.84
	.04	0.81	83.38×10^{5}	7.95
	.06	0.41	26.56×10^{5}	6.95

Appendix Table 10 Adenosine triphosphate content of NLF cells grown in the presence of eugenol.

Time (h)	Eugenol (%, v/v)	Biomass (mg/mL)	cpm	µg ATP/L/mg of cells
0	0	0.02	-	-
	.04	0.02	-	-
	.08	0.02	-	-
	.12	0.02	-	-
12	0	1.02	91.13×10^5	7.95
	.04	1.15	61.69×10^5	7.66
	.08	0.66	15.80×10^5	6.55
	.12	0.35	10.84×10^5	6.48
24	0	1.68	18.33×10^7	10.70
	.04	1.78	16.19×10^7	10.60
	.08	0.99	89.00×10^5	8.03
	.12	0.69	32.20×10^5	7.10
36	0	1.53	9.70×10^7	10.52
	.04	1.62	61.20×10^6	9.76
	.08	0.80	40.01×10^5	7.32
	.12	0.48	22.91×10^5	6.82
48	0	1.43	9.77×10^7	10.50
	.04	1.30	54.58×10^6	9.66
	.08	0.53	39.02×10^5	7.30
	.12	0.26	18.50×10^5	6.65

Appendix Table 11 Leakage of UV material from LF cells grown in the presence of eugenol ($A_{260\ nm}$ and $A_{280\ nm}$).

Eugenol (%.v/v)	A_{260}[a] Time (h)									
	.5	1	1.5	2	2.5	3	6	9	12	24
0	-	-	-	-	-	-	.001	.002	.002	.004
.03	.032	.048	.067	.084	.099	.111	.124	.133	.146	.179
.05	.044	.058	.075	.091	.114	.135	.154	.173	.198	.211

Eugenol (%.v/v)	A_{280}[a] Time (h)									
	.5	1	1.5	2	2.5	3	6	9	12	24
0	-	.001	.001	.001	.001	.002	.002	.002	.002	.003
.03	.005	.011	.015	.027	.031	.038	.057	.061	.064	.066
.05	.008	.024	.034	.044	.064	.100	.119	.133	.147	.177

[a] $Ab_F = Ab_T - Ab_{INITIAL}$

where Ab_F = Filtrate absorbance

and Ab_T = absorbance at time, T.

Appendix Table 12 Leakage of UV material from NLF cells grown in the presence of eugenol ($A_{260\ nm}$ and $A_{280\ nm}$).

Eugenol	A_{260}[a] Time (h)									
(%.v/v)	.5	1	1.5	2	2.5	3	6	9	12	24
0	-	-	-	.001	.001	.002	.003	.003	.003	.005
.03	.022	.029	.039	.052	.059	.071	.083	.098	.108	.119
.06	.031	.034	.048	.056	.062	.074	.090	.102	.120	.133
.09	.040	.047	.057	.094	.111	.125	.135	.151	.162	.183
.12	.094	.105	.114	.126	.133	.141	.168	.178	.199	.218

Eugenol	A_{280}[a] Time (h)									
(%.v/v)	.5	1	1.5	2	2.5	3	6	9	12	24
0	-	-	.001	.001	.001	.002	.002	.002	.003	.004
.03	.022	.028	.031	.033	.038	.054	.069	.081	.093	.120
.06	.024	.038	.040	.049	.054	.075	.086	.096	.117	.141
.09	.030	.040	.080	.116	.129	.143	.160	.167	.185	.216
.12	.077	.101	.120	.127	.144	.167	.194	.210	.221	.237

[a] $Ab_F = Ab_T - Ab_{INITIAL}$

where Ab_F = Filtrate absorbance

and Ab_T = absorbance at time, T.

Appendix Table 13 Lethality of LF cells grown in the presence of eugenol.

Time	CFU/mL	
(h)	Control	0.05%,v/v Eugenol
0	45.0×10^5	50.0×10^5
3	47.0×10^5	51.0×10^5
6	68.0×10^5	53.0×10^5
9	99.0×10^5	60.0×10^5
12	10.0×10^6	83.0×10^5
24	30.5×10^7	30.0×10^4

Appendix Table 14 Lethality of NLF cells grown in the presence of eugenol.

Time	CFU/mL	
(h)	Control	0.09%,v/v Eugenol
0	50.0×10^5	53.0×10^5
3	53.0×10^5	55.0×10^5
6	70.0×10^5	70.0×10^5
9	93.0×10^5	81.0×10^5
12	15.0×10^6	94.0×10^5
24	30.0×10^7	44.0×10^4

Appendix Table 15 Eugenol-induced protein leakage in the LF.

Eugenol (%, v/v)	A_{500} Time (h)					
	0	3	6	9	12	24
0	.000	.000	.001	.002	.004	.007
.02	.000	.015	.027	.044	.052	.090
.04	.002	.026	.051	.100	.113	.171
.06	.004	.038	.075	.120	.155	.211

Appendix Table 16 Eugenol-induced protein leakage in the NLF.

Eugenol (%, v/v)	A_{500} Time (h)					
	0	3	6	9	12	24
0	.000	.000	.000	.000	.000	.000
.04	.000	.009	.020	.035	.055	.095
.08	.004	.063	.101	.133	.168	.215
.12	.005	.084	.121	.164	.200	.293

Appendix Table 17 Cellular fatty acid spectra of LF cells grown in the presence of eugenol with extended incubation.

Incubation Time (h)	Eugenol (%, v/v)	Fatty Acids (%, w/w) Saturated							
		n-C12:0	n-C14:0	n-C15:0	n-C16:0	n-C17:0	n-C18:0	n-C20:0	n-C22:0
12	C	0.79	20.41	0.06	27.02	-	6.05	0.83	0.50
	.02	1.17	8.43	0.16	32.02	0.12	4.72	1.87	1.26
	.04	1.03	11.90	0.23	31.48	0.21	3.65	1.03	1.01
24	C	1.22	27.56	0.03	28.10	0.02	5.59	0.76	0.98
	.02	1.87	8.92	0.31	27.46	0.16	4.54	1.14	1.05
	.04	0.88	11.13	0.42	23.25	0.26	2.77	0.73	0.95
36	C	1.56	28.43	0.02	28.56	0.08	5.56	1.15	1.41
	.02	2.16	8.65	0.49	23.52	0.22	3.86	1.06	0.61
	.04	0.67	8.22	0.51	19.63	0.33	2.11	0.63	0.70
48	C	2.35	28.68	0.02	30.50	0.13	6.21	0.69	1.67
	.02	1.61	6.47	0.60	21.83	0.27	3.15	0.36	0.56
	.04	0.55	5.21	0.80	12.87	0.55	2.13	0.53	0.49
60	C	3.46	26.23	0.03	31.12	0.16	5.49	0.37	2.30
	.02	0.88	4.39	0.79	18.79	0.33	2.34	0.33	0.38
	.04	0.31	5.03	1.01	4.88	0.72	1.29	0.26	0.38

n - normal

Appendix Table 17 (contd.) Cellular fatty acid spectra of LF cells grown in the presence of eugenol with extended incubation.

Incubation Time (h)	Eugenol (%, v/v)	Fatty Acids (%, w/w) Unsaturated		Branched			
		n-C18:1	n-C18:2	i-C13:0	i-C14:0	i-C15:0	i & a-C16:0
	C	43.58	0.83	-	-	-	-
12	.02	47.27	0.68	-	1.26	0.38	0.68
	.04	46.46	0.55	-	1.35	0.48	0.64
	C	34.94	0.78	-	-	0.03	-
24	.02	50.34	0.96	-	1.62	0.51	1.11
	.04	54.72	0.97	0.22	1.76	0.73	1.23
	C	31.63	0.78	-	0.37	0.03	0.41
36	.02	52.74	1.19	0.29	2.67	0.85	1.68
	.04	60.60	1.37	0.27	2.66	0.63	1.86
	C	27.30	0.90	0.16	0.51	0.10	0.78
48	.02	56.87	1.68	0.43	3.13	0.89	2.15
	.04	67.58	2.04	0.51	2.91	0.82	3.00
	C	27.71	0.72	0.24	0.95	0.18	1.03
60	.02	61.16	1.94	0.49	4.53	1.08	2.56
	.04	74.58	2.54	1.13	3.62	0.98	3.28

n - normal
i - iso-branched
a - anteiso-branched

Appendix Table 18 Cellular fatty acid spectra of NLF cells grown in the presence of eugenol with extended incubation.

Incubation Time (h)	Eugenol (%, v/v)	Fatty Acids (%, w/w) Saturated							
		n-C12:0	n-C14:0	n-C15:0	n-C16:0	n-C17:0	n-C18:0	n-C20:0	n-C22:0
12	C	21.48	0.75	0.13	32.88	-	11.99	1.26	1.34
	.02	18.51	0.60	0.14	36.88	-	11.01	1.10	1.40
	.04	20.61	0.89	0.18	37.14	0.08	10.10	0.56	0.87
	.06	14.10	1.95	0.82	37.72	0.08	11.06	0.68	0.82
24	C	26.61	1.55	0.21	31.21	-	10.83	1.26	1.14
	.02	26.68	0.85	0.24	31.80	-	10.94	1.02	1.22
	.04	27.57	0.60	0.40	30.31	0.16	8.88	0.46	0.67
	.06	16.65	0.93	1.20	33.49	0.27	9.95	0.55	0.63
36	C	24.67	1.43	0.33	26.71	-	8.60	1.19	0.88
	.02	27.35	1.46	0.34	33.43	0.14	9.46	1.17	0.94
	.04	24.01	1.28	0.49	29.89	0.22	8.86	0.39	0.32
	.06	14.08	0.67	1.31	33.53	0.39	8.96	0.33	0.28
48	C	31.52	3.01	0.45	33.97	0.08	9.47	1.24	0.82
	.02	27.75	2.38	0.51	33.43	0.19	8.59	1.33	0.64
	.04	17.44	1.60	0.90	30.56	0.34	8.41	0.15	-
	.06	11.26	0.47	1.42	31.97	0.50	8.67	0.17	-
60	C	28.13	4.54	0.19	28.61	0.08	8.18	0.95	0.59
	.02	22.57	5.54	0.61	33.04	0.29	8.68	1.14	0.49
	.04	11.66	2.38	1.06	29.48	0.53	7.08	-	-
	.06	7.32	0.28	1.70	31.03	0.68	6.40	-	-

n = normal

Appendix Table 18 (contd.) Cellular fatty acid spectra of NLF cells grown in the presence of eugenol with extended incubation.

Incubation Time (h)	Eugenol (%, v/v)	Fatty Acids (%, w/w) Unsaturated		Branched			
		n-C18:1	n-C18:2	i-C13:0	i-C14:0	i-C15:0	i & a-C16:0
12	C	27.99	1.99	-	-	-	0.27
	.02	28.15	2.00	-	-	-	0.22
	.04	26.25	2.91	0.10	-	0.08	0.23
	.06	29.39	2.82	0.11	-	0.06	0.39
24	C	24.78	1.86	-	0.07	0.05	0.43
	.02	25.12	1.27	0.15	0.19	0.12	0.39
	.04	24.99	2.93	0.19	0.95	0.07	1.81
	.06	29.74	3.17	0.25	1.09	0.14	1.94
36	C	18.12	1.25	-	0.18	0.04	0.46
	.02	23.65	0.82	0.18	0.37	0.18	0.50
	.04	27.54	3.31	0.25	1.11	0.12	2.20
	.06	31.83	4.04	0.36	1.34	0.31	2.53
48	C	17.29	0.99	0.06	0.23	0.08	0.78
	.02	22.76	0.54	0.19	0.49	0.26	0.94
	.04	31.26	4.33	0.36	1.24	0.18	3.22
	.06	34.97	4.98	0.53	1.58	0.42	3.06
60	C	20.16	0.61	1.11	0.40	0.15	0.83
	.02	24.46	0.38	0.29	0.63	0.29	1.59
	.04	36.38	5.23	0.53	1.53	0.26	3.90
	.06	39.93	5.56	0.77	1.89	0.56	3.86

n - normal
i - iso-branched
a - anteiso-branched

Appendix Table 19 Effect of eugenol and culture transfer on the cellular fatty acid spectra of LF cells.

Number of Transfer	Eugenol (%, v/v)	Fatty Acids (%, w/w) Saturated							
		n-C12:0	n-C14:0	n-C15:0	n-C16:0	n-C17:0	n-C18:0	n-C20:0	n-C22:0
	.03	1.90	3.76	2.61	3.83	0.29	7.19	2.90	0.79
1	0	2.34	3.39	0.20	15.89	-	9.73	1.40	0.75
	.03	1.76	3.36	4.56	8.19	0.37	6.26	2.13	0.67
2	0	2.74	3.60	0.29	23.19	0.06	9.04	1.50	0.86
	.03	1.43	3.02	4.93	8.63	0.52	5.74	1.76	0.52
3	0	2.78	4.39	0.40	26.80	0.08	8.61	1.66	1.18
	.03	1.13	2.23	5.55	8.90	0.78	4.48	1.54	0.41
4	0	3.12	4.31	0.51	30.56	0.13	8.75	1.90	1.29
	.03	0.74	1.41	5.60	7.87	0.96	3.71	1.31	0.32
5	0	3.04	4.20	0.14	35.27	0.07	8.51	1.86	1.16
	.03	0.44	1.26	4.79	4.89	0.95	3.13	1.22	0.20

n - normal

Appendix Table 19 (contd.) Effect of eugenol and culture transfer on the cellular fatty acid spectra of LF cells.

Number of Transfer	Eugenol (%, v/v)	Fatty Acids (%, w/w) Unsaturated		Branched			
		n-C18:1	n-C18:2	i-C13:0	i-C14:0	i-C15:0	i & a-C16:0
	.03	73.19	0.68	0.21	-	0.68	1.97
1	0	65.14	0.68	0.23	-	0.10	0.16
	.03	68.11	0.80	0.63	0.33	0.80	2.03
2	0	57.63	0.52	0.14	0.12	0.14	0.17
	.03	66.52	1.04	0.68	1.10	1.72	2.37
3	0	53.01	0.35	0.13	0.27	0.24	0.11
	.03	65.30	1.97	0.88	1.35	2.92	2.57
4	0	48.24	0.28	0.13	0.36	0.30	0.13
	.03	67.22	2.21	0.70	1.63	3.46	2.85
5	0	44.54	0.14	0.12	0.45	0.38	0.12
	.03	71.79	2.38	0.54	2.45	3.47	2.48

n - normal
i - iso-branched
a - anteiso-branched

Appendix Table 20 Effect of eugenol and culture transfer on the cellular fatty acid spectra of NLF cells.

Number of Transfer	Eugenol (%, v/v)	Fatty Acids (%, w/w) Saturated							
		n-C12:0	n-C14:0	n-C15:0	n-C16:0	n-C17:0	n-C18:0	n-C20:0	n-C22:0
	.05	21.44	0.83	0.16	35.63	0.11	12.07	0.59	0.88
1	0	23.40	1.32	0.27	35.98	0.08	13.18	0.69	0.90
	.05	13.38	0.91	0.37	34.22	0.55	12.89	0.67	0.76
2	0	25.13	1.69	0.25	38.87	0.06	13.91	0.87	1.04
	.05	12.00	0.79	0.53	31.20	0.86	12.76	0.56	0.69
3	0	25.28	2.15	0.20	39.02	0.03	14.07	1.37	1.17
	.05	10.81	0.70	0.77	28.47	1.12	11.51	0.49	0.63
4	0	27.02	2.46	0.08	38.69	0.03	14.33	1.67	1.29
	.05	10.84	0.59	1.73	20.34	1.46	9.62	0.35	0.55
5	0	27.74	2.73	-	39.14	-	14.54	1.98	1.53
	.05	8.57	0.46	2.08	19.04	1.70	8.57	0.21	0.42

n - normal

Appendix Table 20 (contd.) Effect of eugenol and culture transfer on the cellular fatty acid spectra of NLF cells.

Number of Transfer	Eugenol (%, v/v)	Fatty Acids (%, w/w)					
		Unsaturated		Branched			
		n-C18:1	n-C18:2	i-C13:0	i-C14:0	i-C15:0	i & a-C16:0
	.05	26.93	0.70	0.27	-	0.16	0.29
1	0	23.02	0.60	0.33	-	-	0.22
	.05	33.85	1.04	0.43	0.27	0.30	0.37
2	0	17.12	0.59	0.22	0.08	-	0.17
	.05	37.05	1.52	0.53	0.40	0.50	0.63
3	0	15.75	0.50	0.17	0.14	0.03	0.14
	.05	40.01	2.39	0.74	0.67	0.70	0.98
4	0	13.78	0.41	0.11	0.03	0.03	0.08
	.05	45.61	3.78	1.14	1.58	1.06	1.34
5	0	11.83	0.40	0.05	-	0.03	0.03
	.05	48.90	4.57	1.50	1.21	1.21	1.62

n - normal
i - iso-branched
a - anteiso-branched

Appendix Table 21 Weight of lyophilized LF cells used in gas chromatographic analysis of cellular fatty acid composition[a].

Incubation Time (h)	Eugenol (%, v/v)	Weight of dry cells[b] (mg)
12	0	100.3
	.02	99.8
	.04	100.1
24	0	100.1
	.02	99.9
	.04	100.0
36	0	100.0
	.02	99.8
	.04	99.8
48	0	99.9
	.02	100.1
	.04	100.2
60	0	100.1
	.02	100.1
	.04	100.1
Mean Weight		100.02
Standard Deviation		0.152
Total of Weights		1500.3
Variance		0.0216

[a]Effect of eugenol on cellular fatty acid composition
[b]Mean of duplicate samples

Appendix Table 22 Weight of lyophilized NLF cells used in gas chromatographic analysis of cellular fatty acid composition[a].

Incubation Time (h)	Eugenol (% v/v)	Weight of dry cells[b] (mg)
12	0	100.5
	.02	100.1
	.04	99.6
	.06	99.9
24	0	99.9
	.02	100.1
	.04	100.2
	.06	100.0
36	0	100.0
	.02	100.0
	.04	99.8
	.06	100.1
48	0	100.1
	.02	100.0
	.04	99.9
	.06	99.9
60	0	99.9
	.02	100.3
	.04	100.1
	.06	100.0
Mean Weight		100.02
Standard Variation		0.188
Total of Weights		2000.4
Variance		0.0336

[a]Effect of eugenol on cellular fatty acid composition
[b]Mean of duplicate samples

Appendix Table 23 Weight of lyophilized LF cells used in gas chromatographic analysis of cellular fatty acid composition[a].

Number of Transfer	Eugenol ((%, v/v)	Weight of dry cells[b] (mg)
	.03	100.1
1	0	100.1
	.03	100.2
2	0	99.6
	.03	99.8
3	0	99.9
	.03	100.1
4	0	100.1
	.03	99.8
5	0	100.3
	.03	100.0
Mean Weight		100.0
Standard Deviation		0.205
Total of Weights		1100.0
Variance		0.038

[a]Effect of eugenol and culture transfer on cellular fatty acid composition
[b]Mean of duplicate samples

Appendix Table 24 Weight of lyophilized NLF cells used in gas chromatographic analysis of cellular fatty acid composition[a].

Number of Transfer	Eugenol (%, v/v)	Weight of dry cells[b] (mg)
	.05	98.9
1	0	99.9
	.05	100.1
2	0	100.1
	.05	100.3
3	0	100.2
	.05	100.0
4	0	100.1
	.05	99.8
5	0	99.9
	.05	100.1
Mean Weight		99.95
Standard Deviation		0.375
Total of Weights		1099.4
Variance		0.128

[a]Effect of eugenol and culture transfer on cellular fatty acid composition
[b]Mean of duplicate samples

Appendix Table 25 Gas chromatographic determination of eugenol concentration in ground cloves.

Clove concentration %, w/v	mg Cloves/100 mL APT Broth	% eugenol in cloves[1]	
		Without sterilization[2]	After sterilization[3]
0	-	-	-
.2	200	16.46	10.94
.3	300	16.12	10.67
.4	400	16.25	10.78
.5	500	16.33	11.18
.6	600	16.68	10.09
.8	800	15.17	10.94
1.0	1000	15.12	10.11
1.2	1200	16.28	10.67
1.4	1400	15.95	10.39
Mean		16.15	10.64
Standard Deviation		.160	.377

[1]Mean of duplicate samples.
[2]Ground cloves added after steam sterilization of the APT broth, and let stand for 30 mins.
[3]Clove-infusion broth; ground clove added to APT broth and then steam sterilized at 121°C for 15 mins. at 15 p.s.i., allowed to cool at room temperature for 30 mins.

Appendix Table 26 Analysis of Variance (ANOVA) of the effect of eugenol on the rate of pH change by LF cells grown at 30°C.

Source	d.f.	S S	M S	F value
Eugenol	4	0.2241	0.0560	280.00*
Time	10	1.0041	0.1004	502.00*
Rep.	1	0.0018	0.0018	9.00ns
Eug x Time	40	0.4062	0.0102	51.00*
Error	54	0.0100	0.0002	
Total	109	1.6462		

* significantly different at P<0.05
ns not significantly different at P<0.05

Appendix Table 27 Analysis of Variance (ANOVA) of the effect of eugenol on the rate of pH change by NLF cells grown at 30°C.

Source	d.f.	S S	M S	F value
Eugenol	5	2.1251	0.4250	2125.00*
Time	10	5.2654	0.5265	2632.50*
Rep.	1	0.0001	0.0001	0.50ns
Eug x Time	50	3.3088	0.0662	331.00*
Error	65	0.0095	0.0002	
Total	131	10.7089		

* significantly different at P<0.05
ns not significantly different at P<0.05

Appendix Table 28 Analysis of Variance (ANOVA) of the effect of eugenol on total ATP content of LF cells.

Source	d.f.	S S	M S	F value
Eugenol	3	42.5619	14.1873	47291.00*
Time	4	496.8535	124.2134	414044.67*
Rep.	1	0.0001	0.0001	0.33ns
Eug x Time	12	15.3593	1.2799	4266.33*
Error	19	0.0062	0.0003	
Total	39	554.7810		

* significantly different at P<0.05
ns not significantly different at P<0.05

Appendix Table 29 Analysis of Variance (ANOVA) of the effect of eugenol on total ATP content of NLF cells.

Source	d.f.	S S	M S	F value
Eugenol	3	46.1198	15.3733	51244.33*
Time	4	462.8088	115.7022	385674.00*
Rep.	1	0.0015	0.0015	5.00ns
Eug x Time	12	17.1692	1.4308	4769.33*
Error	19	0.0056	0.0003	
Total	39	526.1050		

* significantly different at P<0.05
ns not significantly different at P<0.05

Appendix Table 30 Analysis of Variance (ANOVA) of the saturated to unsaturated fatty acid ratio in the LF.

Source	d.f.	S S	M S	F value
Eugenol	2	12.3374	6.1687	7710.87*
Time	4	0.0589	0.0147	18.38*
Rep.	1	0.00001	0.00001	0.01ns
Eug x Time	8	3.4087	0.4261	532.63*
Error	14	0.0105	0.0008	
Total	29	15.8117		

* significantly different at P<0.05
ns not significantly different at P<0.05

Appendix Table 31 Analysis of Variance (ANOVA) of the saturated to unsaturated fatty acid ratio in the NLF.

Source	d.f.	S S	M S	F value
Eugenol	3	16.8861	5.6287	3752.47*
Time	4	1.2736	0.3184	212.27*
Rep.	1	0.00001	0.00001	0.01ns
Eug x Time	12	8.2424	0.6869	457.93*
Error	19	0.0282	0.0015	
Total	39	26.4304		

* significantly different at P<0.05
ns not significantly different at P<0.05