PRESERVATION OF PRE-COOKED, UNCURED ROAST BEEF WITH A NATURAL ANTIMICROBIAL AGENT, ESSENTIAL OIL OF HORSERADISH

By

Shannon M. Ward

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
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MASTER OF SCIENCE

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PRESERVATION OF PRE-COOKED, UNCURED ROAST BEEF WITH A NATURAL ANTIMICROBIAL AGENT, ESSENTIAL OIL OF HORSERADISH

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SHANNON M. WARD

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

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Abstract

The antimicrobial properties of gaseous horseradish distillate, composed of ca. 90% allyl isothiocyanate (AIT) and 10% β-phenethyl isothiocyanate (β-PEIT), were evaluated in agar and roast beef model systems. Growth of *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus* and *Serratia grimesii* was completely inhibited on agar by 2 000 nL horseradish distillate / L air at 12°C and 35°C. *Lactobacillus sake* was more resistant to the distillate and 20 000 nL/L was required for complete inhibition at 12°C. Bactericidal activity varied between genera and increased with distillate concentration for each temperature. In the roast beef model system, 20 000 nL/L was required for complete inhibition of bacteria at 12°C while *Lactobacillus sake* was only weakly inhibited at this concentration. Bactericidal activity in the roast beef model system was observed against *Escherichia coli*, *Staphylococcus aureus* and *Serratia grimesii* with 20 000 nL/L at 12°C. Total aerobic, enteric and pseudomonad counts on roast beef were also suppressed by 20 000 nL/L at 4°C over 28 d, however, lactic acid bacteria were resistant to the distillate.

Depletion of residual AIT was observed in both model systems over time. Oxygen did not contribute to depletion while the presence of agar discs slightly increased depletion of residual AIT. The aroma and flavour of roast beef were significantly affected by vaporized horseradish distillate. Roast beef exposed to 20 000 nL/L for three d had high horseradish/irritation aromas and flavours, while the aroma and flavour associated with pre-cooked roast beef stored under air were masked. Suppression of the compounds associated

with fat oxidation in pre-cooked roast beef were also observed by GC analyses. In addition, roast beef exposed to vaporized horseradish distillate had more of a surface pink colour than roast beef stored under air. An increase in pink colour was observed with increasing concentrations of vapour by a sensory panel and by instrumental analysis. The microbiological results suggest that atmospheres containing vaporized horseradish distillate could enhance the safety and shelf-life of pre-cooked, uncured roast beef. However, the observed changes to sensory properties suggest the roast beef would have to be marketed as a novel speciality product.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AIT	allyl isothiocyanate
APC	aerobic plate count
CFU	colony forming unit
ESP	epithiospecifier protein
GC	gas chromatography
GC-MS	gas chromatography - mass spectroscopy
MAP	modified atmosphere packaging
MIC	minimum inhibitory concentration
MID	minimum inhibitory dose
MRS	media devised by J. C. DeMann, M. Rogasa and M. E. Sharpe (1960) for the growth and maintenance of lactic acid bacteria
MRSA	MRS agar
MRSB	MRS broth
MS	mass spectroscopy
MSDS	material safety data sheet
PARC	Pacific Agriculture and AgriFood Research Centre (Summerland, BC)
PB	0.1% peptone buffer
PBS	0.05M phosphate buffered solution (pH 7.2)
PCA	plate count agar
PCFC	pseudomonas agar base supplemented with 0.01% cetrimide, 0.01% fucidin and 0.004% ethanol:water (1:1)
β-PEIT	beta phenethyl isothiocyanate
PROC GLM	general linear models procedure
TSA-YE	trypticase soy agar supplemented with 0.6% yeast extract
TSB	trypticase soy broth
TSB-YE	trypticase soy broth supplemented with 0.6% yeast extract
VRBG	violet red bile glucose agar

List of Appendices

Chapter 1

Introduction

Advances in food technology are primarily gained in response to consumer demands, including those for fresh, minimally processed and safe to consume products that have an extended shelf-life. Pre-cooked, uncured meats fall under the category of minimally processed foods, however, concerns about safety and other microbial defects have limited the availability of these products to food distribution systems with high turnover rates, such as delicatessens or fast food restaurants (Penney *et al.*, 1989). Typically formulated with low concentrations of polyphosphates, salts and spices, pre-cooked, uncured meats serve as ideal substrates for microbial growth. Therefore, to minimize microbial survival and growth refrigeration is a requirement during distribution and storage. While still requiring refrigeration, preservation methods such as modified atmosphere packaging (MAP), including vacuum and carbon dioxide packaging, have been employed for shelf-life extension with these products. However, safety issues still remain a concern. According to Hintlian and Hotchkiss (1987a), this a major reason why the benefits of MAP have not been exploited to their full potential. Recent investigations have shown that pathogenic organisms such as *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* and *Staphylococcus aureus* are able to survive, and may even grow, on MAP pre-cooked, uncured roast beef slices stored at both refrigeration and abusive temperatures (Hintlain and Hotchkiss, 1987b; Michel *et al.*, 1991; Hudson *et al.*, 1994). This provides incentive for those seeking alternative preservation methods.

Safety enhancement and shelf-life extension in cured meat products could be achieved with the addition of synthetic antimicrobial agents. However, consumers concerns over synthetic food additives and increasing restrictions governing their use have led to the consideration of alternative natural agents that have antimicrobial activity. The essential oils of horseradish, found primarily in the roots of the plant, have long been known to possess antimicrobial properties. These properties, as well as the pungency associated with this plant are attributed to the isothiocyanates. Allyl isothiocyanate (AIT), the main volatile constituent of horseradish, is reported to be responsible for the antimicrobial activity associated with horseradish extract (Foter, 1940). This compound can exhibit activity in both the aqueous and vapour phase, with the later being more efficient (Inouye *et al.*, 1983; Delaquis and Sholberg, 1997). Preliminary investigations on the use of vaporized AIT from mustard seeds for food preservation have provided encouraging results (Isshiki *et al.*, 1992). Therefore, the use of isothiocyanates from horseradish roots may also prove useful in the preservation of pre-cooked, uncured meats.

The objective of this research was three fold. First, to determine the antimicrobial activity of vaporized horseradish distillate in an agar model system, followed by determination of activity in a pre-cooked, uncured roast beef model system. Second, to determine antimicrobial activity achieved with vaporized horseradish distillate on naturally contaminated pre-cooked, uncured roast beef stored at 4°C for an extended period of time. Finally, to determine sensory changes to the roast beef as a result of exposure to vaporized horseradish distillate. This included changes to flavour, aroma and colour monitored by a sensory panel and by laboratory analysis.

Chapter 2

Literature Review

2.1. Essential Oils of Horseradish

2.1.1. Chemistry

Horseradish (*Amoracia lapthofolia*, Gilib.) belongs to the plant family Cruciferae. All members of this family are known to contain glucosinolates, although in any given species one or two usually predominate (VanEtten *et al.*, 1969). In horseradish, sinigrin is the predominant glucosinolate, followed by gluconasturtiin (VanEtten *et al.*, 1969).

Horseradish glucosinolates are primarily found in the roots of the plant with lesser amounts in the leaves and stems (Mazza, 1984). Disruption of the cell walls upon injury or infection releases the enzyme myrosinase (thioglucoside glucohydrolase EC 3.2.3.1.) which catalyses the hydrolysis of glucosinolates to yield isothiocyanates, glucose and sulphate in the presence of water (Figure 1) (Wogan and Marletta, 1985). Steam distillates of horseradish (defined as essential oils) contain these reaction products. The hydrolysis reaction involves the formation of an unstable aglucon which undergoes a molecular rearrangement called the Lossen rearrangement (Ettlinger and Lundeen, 1957). Although glucose and sulphate are always produced, conditions surrounding the reaction will govern the formation of isothiocyanates (VanEtten *et al.*, 1969). Other products which may be formed instead of, or in combination with, isothiocyanates include nitriles, thiocyanates, oxazolidine-2-thiones, hydroxynitriles and epithionitriles (Fenwick *et al.*, 1982).

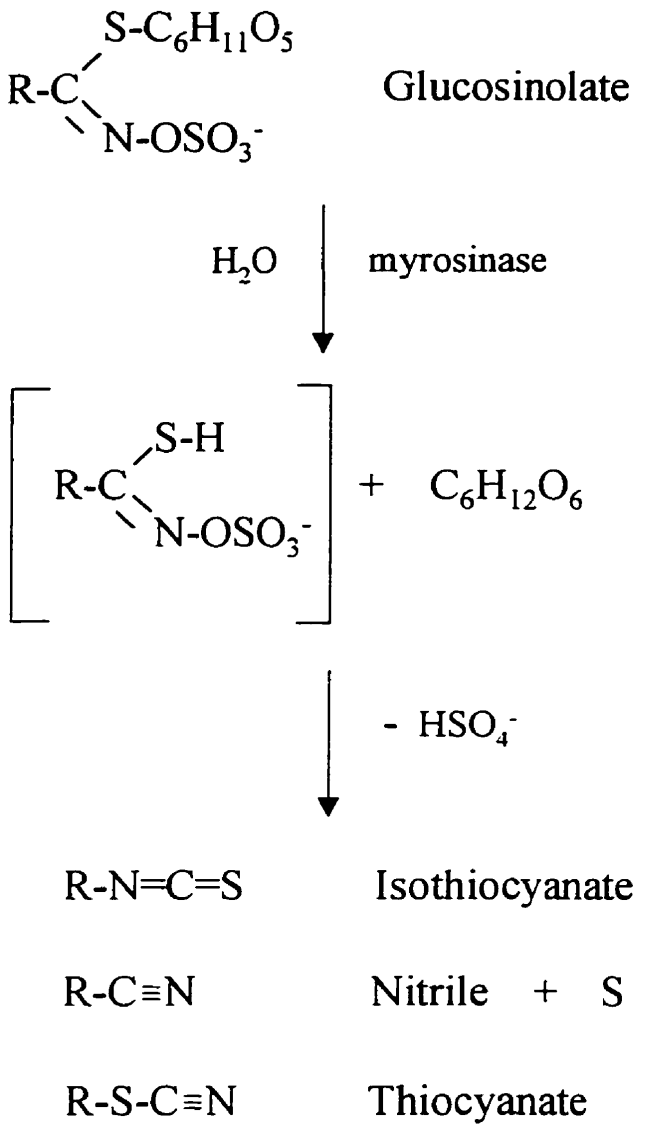


Figure 1. Degradation of glucosinolates via myrosinase hydrolysis (Adapted from Wogan and Marletta, 1985).

Nitrile formation may occur when the reaction medium has a low pH (< pH 5.6) (Virtanen, 1965; Gil and McLeod, 1980b). This reaction proceeds enzymatically without the Lossen rearrangement (Virtanen, 1965). In a recent study focussing on the decomposition products of sinigrin, Borek *et al.* (1994) found that allyl nitrile production reached its maximum at pH 3.0 with production declining from pH 3.0 to 6.0. At pH values of 6.0 and higher, allyl isothiocyanate was the only decomposition product. They also observed that allyl nitrile was produced in larger quantities than allyl isothiocyanate when ferrous ions (Fe^{2+}) were present. This observation was noted at both pH 4.0 and 6.0 (Borek *et al.*, 1994).

Formation of thiocyanate was first thought to be catalysed by a second enzyme (Virtanen, 1965); however, Kirk *et al.* (1964) failed to detect such an enzyme. Instead they suggested that in the presence of heat an equilibrium mixture of isothiocyanate and thiocyanate is formed as a result of chemical rearrangement. Gilbert and Nursten (1972) detected allyl thiocyanate in their analyses of horseradish roots and commented that any heat processed oil that contained allyl isothiocyanate should be expected to contain a proportion of allyl thiocyanate. Mazza (1984) also reported the presence of allyl thiocyanate in horseradish steam distillates. To date, only allyl-, benzyl- and 4-(methylthio)butyl glucosinolates are reported to undergo degradation to thiocyanates (Bones and Rossiter, 1996).

Glucosinolates that have an hydroxyl group at the beta position of the R side chain may form oxazolidine-2-thiones via spontaneous cyclization (Fenwick *et al.*, 1982). This is not of much concern with horseradish since the two predominant glucosinolates, sinigrin and gluconasturtiin, do not have a hydroxyl group at the beta position of the R side chain. Also

of little concern are hydroxynitriles, which have been reported as a product of glucosinolate degradation only when ferrous ions (Fe^{2+}) are present (Fenwick *et al.*, 1982).

Finally, epithionitriles may be formed via glucosinolate hydrolysis. Formation of these compounds requires an epithiospecifier protein (ESP) in addition to the ferrous ions required for hydroxynitriles (Tookey, 1973). Although the exact mechansism is unknown, ESP a small labile protein, has been reported to work in conjunction with myrosinase (Tookey, 1973). It has not been isolated from horseradish (Petroski and Tookey, 1982).

2.1.2. Extraction methods

Steam distillation and solvent extraction are two methods commonly used for the recovery of essential oils from horseradish roots. Both methods involve disruption of the plant cell walls by grinding in order to activate the enzyme myrosinase, and require the addition of water. Sufficient time after the addition of water to the ground sample and before commencement of the distillation should be allowed for the enzyme to react with glucosinolates. Granting this time prior to heating is also important because myrosinase is inactivated at temperatures higher than 70°C (Sahasrabudhe and Mullin, 1980). If steam distillation is the method of choice, it is important to note that the ratio of distilled products will vary with the distillation time. A higher percentage of allyl isothiocyanate has been found to distill off during the first 15-30 min of steam distillation while more β-phenethyl isothiocyanate appears after 30-60 min (Mazza, 1984).

Solvent extraction methods are often employed in sample preparation for determining the types and concentrations of glucosinolate degradation products present in plants.

Commonly used solvents are dichloromethane or a pentane:ether mixture (Daxenbichler *et al.*, 1977; Grob and Matile, 1980; Sahasrabudhe and Mullin, 1980; Gilbert and Nursten, 1972). Using capillary gas chromatography, Grob and Matile (1980) were able to identify 30 glucosinolates and corresponding mustard oils from a solvent extract of horseradish roots. Solvent extraction was also utilized by Sahasrabudhe and Mullin (1980) when determining allyl- and β-phenethyl- isothiocyanate concentrations in horseradish roots that had been subjected to various dehydration treatments.

2.1.3. Isothiocyanates

2.1.3.1. Properties of isothiocyanates

The predominant glucosinolates in horseradish, sinigrin and gluconasturtiin, are the precursors for the isothiocyanates allyl isothiocyanate (AIT) and β-phenethyl isothiocyanate (β-PEIT), respectively. AIT is a strong lachrymator and skin irritant (Clark, 1992). At 20°C, its specific gravity (1.013) is similar to that of water (Budavari *et al.*, 1989). Although the boiling point for AIT ranges from 148°C to 154°C, it is extremely volatile at lower temperatures (Budavari *et al.*, 1989). Sekiyama *et al.* (1994b) reported AIT to be only slightly soluble in water, yet readily soluble in fats and oils, as well as in alcohol. These investigators have also found that the evaporation behaviour of AIT remains the same under varying levels of humidity.

Physical properties of β-PEIT are not as easily found in the literature. According to the material safety data sheet (MSDS) provided by Sigma-Aldrich Canada, Ltd (Oakville,

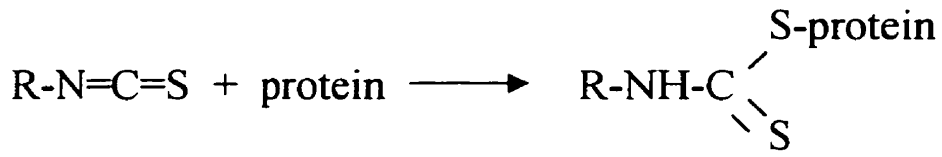
ON) β-PEIT, as previously noted with AIT, is a lachrymator and irritant. It has a specific gravity of 1.094 (20°C) and a boiling point range of 139°C to 140°C.

Over two decades ago, Gilbert and Nursten (1972) conducted a study on the volatile constituents of horseradish and found that the pungency and lachrymatory properties of the plant, as well as the characteristic horseradish aroma, could be attributed to AIT. β-PEIT, on the other hand, was found to produce a fresh watercress aroma that is masked in freshly ground horseradish. Furthermore, the odour thresholds for these compounds were investigated by Buttery *et al.* (1976) and were found to be 375 ppb and 6 ppb (in water) for AIT and β-PEIT, respectively.

2.1.3.3. Isothiocyanate-protein interaction

Information on isothiocyanate interactions with food components is scarce, and only a few studies on interactions with proteins have been reported over the past 25 years. These studies have shown that isothiocyanates have a strong affinity towards thiol, sulphydryl and terminal amino groups of proteins (Kawakishi and Kaneko, 1985; Kroll and Rawel, 1996). The interaction between isothiocyanates and proteins involves covalent bonding and results in the formation of protein derivatives, mainly thiourea and dithiocarbamate derivatives (Kroll *et al.*, 1994; Rawel and Kroll, 1995) (Figure 2).

A.



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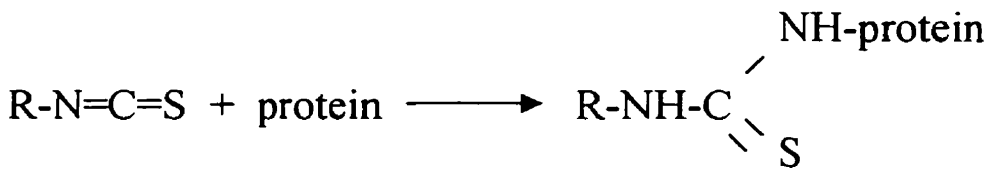

Figure 2. Formation of protein derivatives via isothiocyanate and protein interaction (A - dithiocarbamate derivative; B - thiourea derivative) (Adapted from Kroll *et al.*, 1994).

Björkman (1976) noted that the isothiocyanate-protein interaction occurred more readily with basic low molecular weight proteins which were rich in lysine and terminal amino acids. A more pronounced reaction with low molecular weight proteins was also observed by Kishore Kumar Murthy and Narasinga Rao (1986). Changes in the physicochemical properties of the protein also occurs with the formation of protein derivatives. Most notable are a decrease in solubility, an increase in hydrophobicity and an increase in electrophoretic mobility (Björkman, 1976; Kishore Kumar Murthy and Narasinga Rao, 1986; Rawel and Kroll, 1995). Since isothiocyanates are strong electrophilic agents they are capable of attacking at the nucleophilic sites of the protein, causing an increase in the total negative charge (Kroll *et al.*, 1994).

2.1.3.2. Stability of allyl isothiocyanate (AIT) in aqueous solutions

The stability of AIT in aqueous solutions is of importance since many food systems have high moisture contents. Kawakishi and Namiki (1969) proposed a degradation scheme (Figure 3) for this compound in aqueous solutions. They found that the decomposition was not based on a hydrolysis reaction as may be expected, but rather on the addition of water or other decomposition products to the isothiocyanate (Kawakishi and Namiki, 1969). More recently, Ohta *et al.* (1995a) found that nucleophilic attack of water molecules and hydroxide ions on the AIT molecule (concentration < 1.6 mM) followed the first order rate equation.

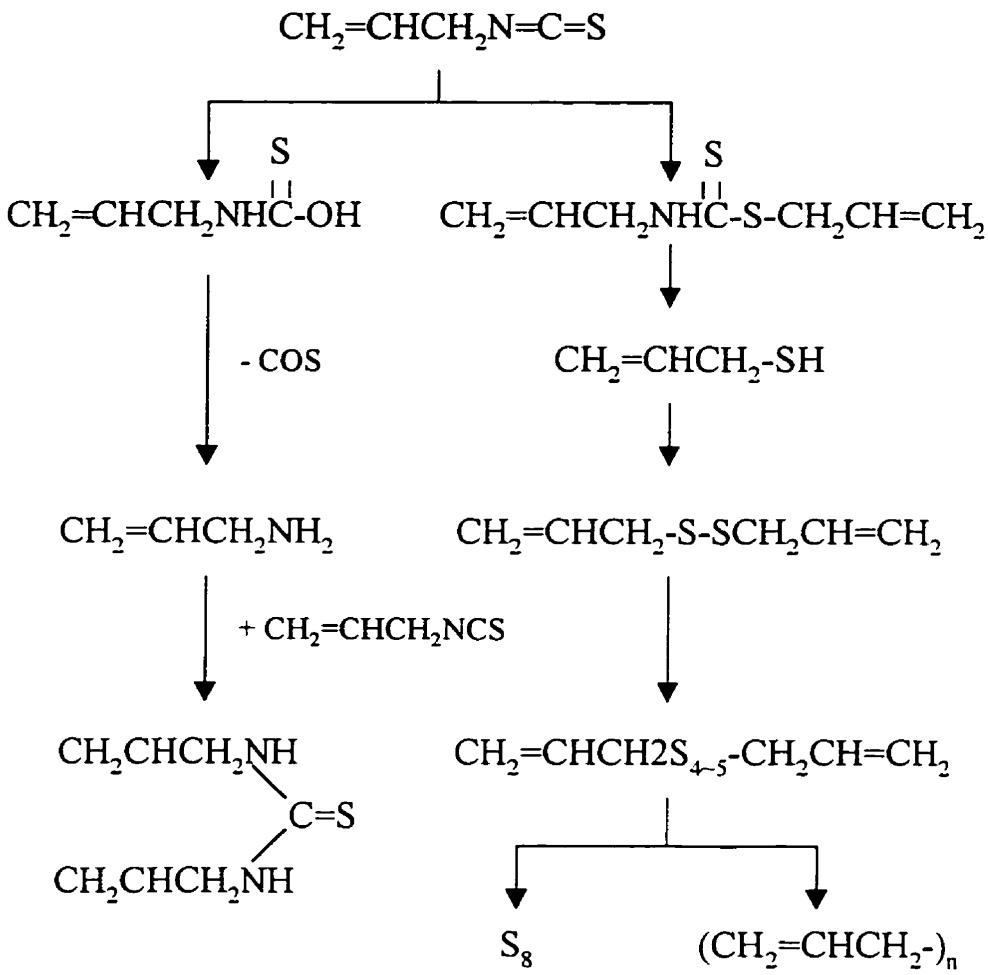

Figure 3. Degradation of AIT in aqueous solution (Adapted from Kawakishi and Namiki, 1969).

They also noted that while ionic strength did not factor into the decomposition of AIT in aqueous solutions, pH and temperature did. In order to suppress decomposition, low pH and low temperatures are necessary (Ohta *et al.*, 1995a). In a follow-up study, Ohta *et al.* (1995b) reported that dextrins and polysaccharides suppressed decomposition of AIT in aqueous solutions. They believe that an inclusion complex is formed when AIT is bound and stabilized in the cavities of α-cyclodextrin. Therefore, it is difficult for water molecules and hydroxide ions to attack the AIT nucleophilically.

2.1.3.4. Antimicrobial activity of isothiocyanates

2.1.3.4.1. Antibacterial activity

Essential oils of horseradish have long been known to possess both antibacterial and antifungal activity. Antibacterial activity was described early in the century by Foter and Golick (1938) who determined that crushed horseradish vapours inhibited the growth of several bacteria, including *Serratia marcescens*, *Bacillus subtilis* and *Escherichia coli*. A follow-up study attributed this property to the main volatile constituent of horseradish, AIT (Foter, 1940). Blum and Fabian (1943) also reported that AIT had significant antibacterial activity, although this observation was made using an extract from mustard seeds. Most studies on the antimicrobial properties of naturally occurring AIT have been conducted using mustard extract as the source of this isothiocyanate. In parallel with horseradish, AIT comprises over 90% of the volatile components present in the extract of mustard seeds. Indeed, the terms mustard oil and AIT are often used interchangeably in the literature.

Interest in AIT declined after these early studies but was rekindled by Inouye *et al.* (1983) who reported that AIT has a broader spectrum of antibacterial activity than the components of lemon oil. This activity was reported to be bactericidal towards Gram negative bacteria and bacteriostatic towards Gram positive bacteria. These investigators also found the activity of AIT to be more efficient in the gaseous phase than in the liquid phase (Inouye *et al.*, 1983).

The number of investigations on the antibacterial effects of both naturally occurring and synthetically produced AIT increased in the 1990's. Kanemaru and Miyamoto (1990) reported that resistance toward AIT from mustard seed was greater in *Staphylococcus aureus* and *Escherichia coli* than *Pseudomonas aeruginosa* in a liquid medium at 30°C. The antibacterial activity of AIT in a liquid medium was also investigated by Brabban and Edwards (1995). Sinigrin, the precursor of AIT, was reported to have little effect upon the growth of *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Bacillus stearothermophilus* and *Streptomyces thermoviolaceus*. Conversely, the hydrolysis products of sinigrin, primarily AIT, were completely inhibitory towards growth of all microorganisms tested. The concentrations required for complete inhibition were also found to be species dependent (Brabban and Edwards, 1995).

Isshiki *et al.* (1992) also investigated the antibacterial effectiveness of naturally occurring AIT from mustard but in the vapour phase. At 37°C, bacteria were inhibited by concentrations ranging from 34-110 ng/mL. Yeasts and molds were more susceptible to the vapour and only 16-62 ng/mL were required for inhibition at 25°C (Isshiki *et al.*, 1992). Similarly, the antibacterial effects of mustard extract in the vapour phase were reported by

Sekiyama *et al.* (1994a). Over a 24 h storage period at 30°C, bacteriostatic effects were achieved against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium*, while a bactericidal effect was observed against *Vibrio parahaemolyticus*. No theories were provided for these observations but a depletion in AIT concentration (180 ppm to 30 ppm) was observed over the experimental period. In a follow up study, Sekiyama *et al.* (1996) reported that volatile mustard extract was bacteriostatic towards the vegetative forms of spore-forming bacteria but not the spores. Further investigations on the antibacterial effects of vaporized AIT were carried out by Delaquis and Sholberg (1997) who studied the effects of synthetic AIT on the growth of microorganisms at varying temperatures. The pathogenic bacteria, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella typhimurium*, were completely inhibited by a gaseous concentration of 1500 ug AIT/L air over a temperature range of 25°C-40°C. Under atmospheres containing lower AIT concentrations, Delaquis and Sholberg (1997) reported that the degree of inhibition achieved became species specific. Gram negative, spoilage organisms were more sensitive towards gaseous AIT being completely inhibited under 500 ug AIT/L over a temperature range of 5°C-30°C.

2.1.3.4.2. Antifungal activity

Concurrent with investigations on the antibacterial activity of AIT, the antifungal activity associated with this compound has also been studied. Activity against *Colletrotrichum circinans*, *Botrytis allii*, *Aspergillus niger*, *Aspergillus alliaceus* and the yeast *Saccharomyces ellipsoideus* were reported by Walker *et al.* (1937), Hooker *et al.*

(1943) and Kosker *et al.* (1952). As observed with bacteria, sinigrin, the precursor of AIT, was not found to exhibit antifungal properties (Walker *et al.*, 1937).

The other isothiocyanate associated with horseradish, β-PEIT, has also been investigated for antifungal activity. Hooker *et al.* (1943) reported that the antifungal activity of this isothiocyanate was similar to that of AIT in liquid media, although activity towards *Aspergillus alliaceus* was greater. It is also interesting to note that β-PEIT has no activity in the vapour phase (Hooker *et al.*, 1943). These investigators attributed this phenomenon to differences in vapour pressures between β-PEIT and AIT.

Research on the antifungal properties of both naturally occurring and synthetically produced isothiocyanates has continued through the past several decades with similar observations (Drobnica *et al.*, 1967; Lewis and Papavizas, 1971; Hejtmankova *et al.*, 1979; Goi *et al.*, 1985; Mari *et al.*, 1993; Tsunoda, 1994; Mari *et al.*, 1996; Sekiyama *et al.*, 1996). A more detailed review on this subject has been presented by Delaquis and Mazza (1995).

2.1.3.4.3. Mechanism of inhibition

Although information on the antimicrobial effectiveness of AIT is available, theories on the mechanism(s) behind this activity are scarce. Zsolnai (1966) was among the first to theorize on the mechanism suggesting that isothiocyanates should be regarded as enzyme inhibitors, since they appear to inactivate several enzymes. Kojima and Ogawa (1971), while investigating the inhibition of oxygen uptake by yeast cells, suggested that the isothiocyanate is active after it passes through the cell membrane. These researchers also observed inhibition of cytochrome c oxidase activity by AIT. Brabban and Edwards (1995) suggested that in

light of a direct relationship between the concentration of actively growing microbial cells and the concentration of sinigrin hydrolysis products (primarily AIT), these compounds most likely act by binding to a site(s) within the organism. Delaquis and Sholberg (1997) also suggested that actively growing cells may be more sensitive than slow-growing or resting cells.

2.1.3.5. Beneficial and harmful properties of isothiocyanates

Isothiocyanates have been identified as one of the fourteen classes of phytochemicals believed to possess anticarcinogenic properties (Caragay, 1992). Supporting evidence for the beneficial properties associated with isothiocyanates has been reported by a multitude of researchers. The formation of α-hydroxylation products of nitrosamines has been found to be inhibited by both AIT and β-PEIT; an important finding since this reaction is believed to yield a reactive species that can damage DNA (Chung *et al.*, 1984). β-PEIT has also been found to inhibit nitrosamine-induced esophageal cancer (Stoner *et al.*, 1991; Stoner *et al.*, 1994). Morse *et al.* (1989) reported inhibition of nitrosamine-induced lung tumorigenesis by β-PEIT while Tawfig *et al.* (1995) found that β-PEIT induced quinone reductase activity, an anticarcinogenic phase II marker enzyme. The mechanism(s) behind these findings are yet to be determined. According to Stoner *et al.* (1991) these compounds may act in two ways: the first, based on the reactivity of isothiocyanates towards proteins, is that β-PEIT may prevent nitrosamine-induced cancer by binding to the cytochrome P450 isozymes responsible for activation of a powerful carcinogen, nitrosobenzylmethylamine (NBMA). The second is that β-PEIT may inhibit the oxidative metabolism of NBMA.

Conversely, isothiocyanates have also been found to possess mutagenic and carcinogenic properties. Yamaguchi (1980) reported that isothiocyanates exhibited mutagenicity against *Salmonella typhimurium* TA100 with AIT having the highest level of potency. Rihova (1982) also reported that AIT, with metabolic activation, exerted a mutagenic effect against *E. coli* WP67. In terms of cytotoxicity, β-PEIT and AIT were found to be the third and fifth most active compounds of six tested against HeLa cells (Horakova, 1966). Musk and Johnston (1993) found β-PEIT to be clastogenic or capable of inducing chromosome aberrations in an Indian mutjac cell line. AIT, however did not exhibit this property. In a later study Musk *et al.* (1995) reported that isothiocyanates were more than three orders of magnitude more cytotoxic than their corresponding glucosinolates (w/w basis). These researchers cautioned against increasing normal dietary levels of isothiocyanates until the issue of cancer prevention vs cancer induction is resolved. Caragay (1992) also mentioned that foods containing biologically active phytochemicals may be toxic when consumed in quantities greater than obtained through a normal diet. In addition, some isothiocyanates have been shown to be goitrogenic, however this is dependent on the iodine content of the diet. The thiocyanate ion formed via glucosinolate hydrolysis and deemed the causative agent in 'cabbage-goiter' is reported to act as an iodine competitor (Fenwick *et al.*, 1982). Oxazolidine-2-thiones, also products of glucosinolate hydrolysis, have been deemed causative agents in 'Brassica seed-goiter' by interfering with thyroxine synthesis (Fenwick *et al.*, 1982).

2.2. Pre-cooked Uncured Meat Products

2.2.1. Definition

Meat products may be defined as being cooked or pasteurized, in microbiological terms, if an internal temperature of 60-75°C has been achieved. This temperature range is reported to inactivate most bacteria, yeasts, molds, parasites and viruses but not spores (Ingram and Simonsen, 1980). These products may be further classified as uncured when chemical curing agents such as nitrites or nitrates have not been added. However, low concentrations of polyphosphates may be used as humectants plus salts and other spices as flavouring agents.

2.2.2. Microbiology

Available data on the microbiological characteristics of pre-cooked, uncured meat products is limited and focussed primarily on modified atmosphere packaged pre-cooked meats. Plate counts for freshly pre-cooked, uncured meats have been reported to be 2.0 $\log_{10}$ cfu/g or less (Johnston and Tompkin, 1992). Mol *et al.* (1971) and Anderson *et al.* (1989) have reported initial aerobic plate counts for pre-cooked, uncured meats in vacuum packages to range from 1.6 - 3.0 $\log_{10}$ cfu/g. Although these microorganisms may be present as a result of inadequate heat processing, the primary cause of contamination in pre-cooked, uncured meats is reported to originate during post processing handling (Cooksey *et al.*, 1993). Mol *et al.* (1971) reported that the highest incidence of contamination on sliced cooked meats originated from the slicing and packaging line. Holley (1997) also reported that slicing, and

more importantly co-slicing, contaminates the surface of cooked meats. Since slicing would normally be conducted in a refrigerated room, contamination by psychrotrophic organisms would be of particular concern (Ingram and Simonsen, 1980).

Psychrotrophic Gram negative bacteria, primarily *Pseudomonas* spp., tend to dominate the microflora of cooked meats stored aerobically at refrigeration temperatures (Penney *et al.*, 1993). While the initial microflora of pre-cooked, vacuum packaged meats also tends to be dominated by *Pseudomonas* spp., prolonged refrigerated storage results in the microflora becoming dominated by lactic acid bacteria (Anderson *et al.*, 1989; Penney *et al.*, 1993). Anderson *et al.* (1989) observed that the initial microflora of vacuum packaged roast beef slices was made up of 54% pseudomonads and 39% lactobacilli, with the remaining 7% consisting of *Staphylococcus* spp., *Streptococcus* spp., *Micrococcus* spp., *Moraxella-Acinetobacter* spp., *Enterobacter* spp. and *Hafnia alvei*. Lactobacilli dominated the microflora (99%) after 28 d of storage at 1°C. At 5°C and 10°C, however, *Hafnia alvei* made up 7% and 53% of the microflora after 28 d of storage. *Serratia* ssp. were also present, albeit to a lesser degree, after 28 d of storage at 5°C and 10°C (Anderson *et al.*, 1989).

Penney *et al.* (1993) also observed that the spoilage microflora of vacuum and CO_2 packaged roast beef slices was dominated by the lactobacilli after as little as four d storage at 10°C and after one week at 3°C, respectively. In addition, *Brochothrix* spp. were reported to make up a significant portion of the microflora of the vacuum packaged roast beef slices after one week of refrigerated storage. This group of microorganisms also appeared as part of the microflora of the CO_2 packaged slices, but not to the same extent as with the vacuum

packaged slices (Penney *et al.*, 1993). In contrast, Anderson *et al.* (1989) only observed *Brochothrix thermosphacta* once during the course of their study and it accounted for less than 0.1% of the spoilage microflora after 14 d of storage at 5°C. These microorganisms are also reported to have unique effects on the odour of meat. Penney *et al.* (1993) detected a 'sharply acidic' flavour when *Lactobacillus* ssp. dominated the spoilage microflora of vacuum or CO_2 packaged roast beef. However, a 'sweaty' flavour appeared when *Brochothrix* spp. were dominant

2.2.3. Pathogens associated with pre-cooked, uncured roast beef

Concern over pathogenic survival and proliferation in pre-cooked, uncured roast beef can be attributed to many factors. The composition of pre-cooked roast beef is important as it offers an ideal substrate for microbial growth. Also, the cooking process destroys most of the spoilage microflora, including species that may provide protection through competitive inhibition. Finally, temperature abuse during processing, storage or distribution could also promote the survival and growth of pathogenic microorganisms (Ingram and Simonsen, 1980; Johnston and Tompkin, 1992).

The psychrotrophic bacterium *Listeria monocytogenes*, has been the most extensively studied pathogen in pre-cooked, uncured roast beef. The presence of this microorganism on heat processed meats has been suggested to be a result of post processing contamination since the decimal reductions achieved with heating should be sufficient to destroy the low numbers of listeriae normally found on raw meat (Mackey *et al.*, 1990; Beumer *et al.*, 1996). This Gram positive, nonsporing rod was shown to survive and proliferate in vacuum packaged

roast beef at storage temperatures of 3°C-4.4°C (Glass and Doyle, 1989; Michel *et al.*, 1991; Cooksey *et al.*, 1993). At temperatures as low as –1.5°C, evidence of growth in vacuum packaged roast beef has been presented by Hudson *et al.* (1994). In addition to being able to proliferate at low temperatures, *Listeria monocytogenes* may also exhibit heat resistance. If present in high levels, this species may survive cooking temperatures up to 70°C (Boyle *et al.*, 1990). A more extensive review on the presence, survival and proliferation of *Listeria monocytogenes* in meat and meat products has been compiled by Johnson *et al.* (1990).

Salmonella typhimurium is also known to survive refrigerated storage on pre-cooked roast beef. Although this microorganism is usually cited in literature as having a minimum growth temperature of 5°C, survival at 3°C for as long as 70 d in vacuum packaged roast beef has been documented (Michel *et al.*, 1991). *Staphylococcus aureus* and *Escherichia coli* are also often associated with meat and meat products. Roast beef was implicated as the vehicle for *Escherichia coli* O157:H7 transmission in a 1990 foodborne outbreak (Anon., 1992). Although *Staphylococcus aureus* and *Escherichia coli* do not proliferate at low temperatures, survival at 3°C and 4°C on modified atmosphere packaged roast beef has been reported (Michel *et al.*, 1991; Hintlian and Hotchkiss, 1987b).

Pathogenic sporeformers such as *Clostridium perfringens* are also of concern because their spores can survive the cooking process. Since *Clostridium perfringens* is not capable of growth at refrigeration temperatures (Hintlian and Hotchkiss, 1987b), this pathogen is only of concern once temperature abuse occurs. In vacuum packages of pre-cooked roast beef inoculated with vegetative cells of *Clostridium perfringens* production of 2.4×10^5 spores / g was observed after 18 h at 37°C (Dework, 1972).

2.2.4. Inhibition of pathogens on pre-cooked, uncured roast beef

Modified atmospheric packaging (MAP), including vacuum packaging, has been employed primarily for the purpose of extending the shelf-life of pre-cooked, uncured meats, rather than as a means of controlling pathogenic growth (Farber, 1991). Shelf-life extension is due to an increase in the lag phase and a decreased growth rate as a result of the bacteriostatic effect of CO_2 (Farber, 1991). This effect is even more pronounced at lower temperatures due to increased dissolution of CO_2 at the meat surface (Carr and Marchello, 1986).

Hintlian and Hotchkiss (1987a) appear to have conducted the earliest studies on the growth of pathogens on pre-cooked, uncured roast beef stored under modified atmospheres. *Staphylococcus aureus*, *Salmonella typhimurium* and *Clostridium perfringens* were inhibited at a temperature of 12.8°C in an atmosphere containing 75% CO_2, 15% N_2 and 10% O_2. They suggested that elevated CO_2 level and reduced O_2 levels were responsible for inhibition of *Staphylococcus aureus* and *Salmonella typhimurium*, whereas the presence of O_2 was more likely responsible for inhibition of the anaerobic pathogen, *Clostridium perfringens* (Hintlian and Hotchkiss, 1987a). In a follow-up study, again on roast beef, the proliferation of pathogenic bacteria was monitored under a modified atmosphere of 75%CO_2 / 15%N_2 / 10%O_2 at 4.4°C (Hintlian and Hotchkiss, 1987b). While *Salmonella typhimurium* and *Clostridium perfringens* declined in numbers over the 42 day storage period, *Staphylococcus aureus* numbers remained unchanged. Since the storage temperature was below the minimum growth temperature for all three pathogens, growth was not expected. However, zero recovery of *Salmonella typhimurium* and *Clostridium perfringens* was obtained earlier in

storage than expected indicating the modified atmosphere had a bactericidal effect (Hintlian and Hotchkiss, 1987b).

Growth of *Listeria monocytogenes* has also been investigated on vacuum packaged and CO_2 packaged pre-cooked roast beef. Glass and Doyle (1989) reported very little growth of this species on vacuum packaged roast beef stored at 4.4°C compared to that observed on vacuum packaged sliced chicken and turkey. Hudson *et al.* (1994) reported growth of *Listeria monocytogenes* on vacuum packaged and CO_2 packaged roast beef slices stored at 3°C, although the lag phase was significantly longer under an atmosphere of 100% CO_2. *Listeria monocytogenes* was also observed to grow at -1.5°C in vacuum packages but declined in numbers under 100% CO_2 (Hudson *et al.*, 1994).

Michel *et al.* (1991) also investigated the survival and growth of *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli* and *Clostridium perfringens* on pre-cooked roast beef slices at refrigeration temperatures. In the presence of a natural competitive microflora, *Salmonella typhimurium*, *Listeria monocytogenes* and *Staphylococcus aureus* remained detectable on the slices after 70 d. Numbers of viable *Clostridium perfringens* and *Escherichia coli* cells declined over the study period with *Escherichia coli* numbers declining more rapidly during the first 14 d of storage (Michel *et al.*, 1991).

Organic acids and their salts, including sodium lactate and sodium propionate, have also been investigated for their ability to extend the shelf-life of pre-cooked roast beef (Papadopoulos *et al.*, 1991; Maca *et al.*, 1997). Information on the ability of these compounds to retard the growth of pathogens on cooked roast beef, however, is scarce.

Miller and Acuff (1994) recently investigated the effects of varying concentrations of sodium lactate on the growth of pathogens on cooked beef at 10°C. Sodium lactate concentrations of 3% and 4% were inhibitory towards the growth of *Salmonella typhimurium*, *Listeria monocytogenes* and *Escherichia coli* O157:H7. Growth differences were not observed with *Staphylococcus aureus* or *Clostridium perfringens*. Since the storage temperature used in this study was close to the minimum growth temperatures for these two species, the lack of inhibition by sodium lactate could be explained by difficulty in determining growth in the control and experimental trials (Miller and Acuff, 1994). Another method investigated as a means of destroying or reducing contaminants in pre-cooked roast beef is post process pasteurization. Cooksey *et al.* (1993) and Hardin *et al.* (1993) found that a reduction in listeriae populations could be achieved with this method, however, survivors were recovered by enrichment (Cooksey *et al.*, 1993; Hardin *et al.*, 1993).

Chapter 3

Preparation of Horseradish Distillate

3.1. Introduction

Steam distillation and solvent extraction are the two most common methods used to recover the essential oils of horseradish. Solvent extraction often involves the use of toxic solvents which must be separated from the final extract and disposed of accordingly. Interfering nonvolatile components may also be extracted in the process (MacLeod, 1976). Steam distillation, on the other hand, does not employ the use of toxic solvents to extract the essential oils of horseradish. Extracts prepared by the latter method would be more desirable for food applications, including use as a preservative for pre-cooked, uncured meat. Regardless of method used, the resulting extract will be primarily composed of allyl isothiocyanate (AIT) and β-phenethyl isothiocyanate (β-PEIT), the main volatile constituents of horseradish. Identification of these compounds, as well as other components present in the horseradish distillate, may be achieved by gas chromatography - mass spectroscopy (GC-MS). This is considered to be the best method for qualitative evaluation (MacLeod, 1976). The purpose of extracting the essential oils from horseradish roots by steam distillation was to provide sufficient horseradish distillate for further testing, including investigations into the antimicrobial efficiency of the distillate. The concentrations of AIT and β-PEIT present in the distillate were also determined.

3.2. Materials

Horseradish (*Amoracia lapathifolia*, Gilib.), grown at the Pacific Agriculture and AgriFood Research Centre (PARC), Summerland, BC, was harvested in the summer of 1996. The horseradish roots were rinsed of soil and debris, air dried and stored in 40 L - polyethylene containers at 2°C until further use. AIT (purity ≥ 98%) was obtained from Fluka Chemika-BioChemika (Edmonton, AB), while β-PEIT (purity ≥ 97%) was obtained from Sigma-Aldrich (Oakville, ON). Hexane (analytical grade) was obtained from Caledon Laboratories (Georgetown, ON).

3.3. Methods

3.3.1. Method of extraction

Horseradish root essential oils were obtained by the method of steam distillation outlined by Mazza (1984). Briefly, 750 g of horseradish root was grated with a food processor (Model A901D; Kenmore, New Hampshire, USA) and placed in a sealed 5 L - round bottom flask with 2 L of distilled water. After 30 min at room temperature the solution was brought to a boil and hydrodistillation was carried out for 15-20 min. The collected oil was placed at -20°C overnight to freeze out any water droplets. The aqueous-free distillate was subsequently stored at -30°C in a dark glass bottle and the head space of the bottle was replaced with nitrogen gas for increased stability (Sekiyama *et al.*, 1994b).

3.3.2. Qualitative determination of horseradish distillate composition

The composition of the horseradish distillate was determined by gas chromatography-mass spectroscopy (GC-MS; Model HP5890 and HP5970 mass selective detector, Hewlett Packard (Canada) Ltd., Richmond, BC). The chromatograph was fitted with a DB-210 capillary column (ID=0.32 mm, L=30 m) and was operated under the following conditions: initial oven temperature of 100°C (4 min), increasing oven temperature rate of 10°C/min (10 min), final oven temperature of 200°C (5 min), injector temperature of 250°C, detector temperature of 250°C and a carrier gas flow rate of 0.97 mL He/min (@ 100°C). The mass selective detector was adjusted to scan over 35-175 mass units at 3.8 scans/sec with an EM voltage of 1200 V and a threshold of 400. AIT and β-PEIT standards were diluted with hexane to concentrations of approximately 1 000 and 100 mg / L, respectively, while the horseradish distillate was diluted to a concentration of approximately 1 000 mg/L. An injection volume of 2 μL (split injection 15:1) was used in the analysis. All compound identifications were made using HP Chemstation G1034C Version C.02.00 that contained the Wiley library database of mass spectra.

3.3.3. Quantitative determination of horseradish distillate composition

The concentrations of AIT and β-PEIT present in the horseradish distillate were determined using a gas chromatograph (Model HP5890, Hewlett Packard (Canada) Ltd., Richmond, BC) equipped with a flame ionization detector and an electronic integrator (Model HP3390A, Hewlett Packard (Canada) Ltd., Richmond, BC). The operating conditions for the chromatograph were the same as previously stated (section 3.3.2) except that an injection

volume of 1 μL was used in the analysis. Standard curves of AIT and β-PEIT were prepared with all dilutions made in hexane. The horseradish distillate was also diluted in hexane to a concentration of approximately 2 000 mg/L. This analysis was performed three times over a one year period.

3.4. Results

3.4.1. Qualitative analysis of horseradish distillate

Twenty distillations were carried out over a four week period and the resulting distillates were pooled to form a composite of approximately 25 mL. All further experimentation involving horseradish distillate was conducted with this composite.

A chromatogram showing the composition of the extracted horseradish oil is shown in Figure 4. The compounds corresponding to each major peak are given in Table 1. Although the database used in mass spectrometry did not contain a spectrum for allyl thiocyanate, this compound, based on comparison with chromatograms and spectra found in the literature, was thought to correspond to peak 3 (Gilbert and Nursten, 1972; Grob and Matile, 1980; Mazza, 1984). Aside from the four major peaks listed in Table 1, a few minor peaks were also present in the chromatogram. Since these peaks did not match the spectra contained in the Wiley library database, definitive correlations could not be made.

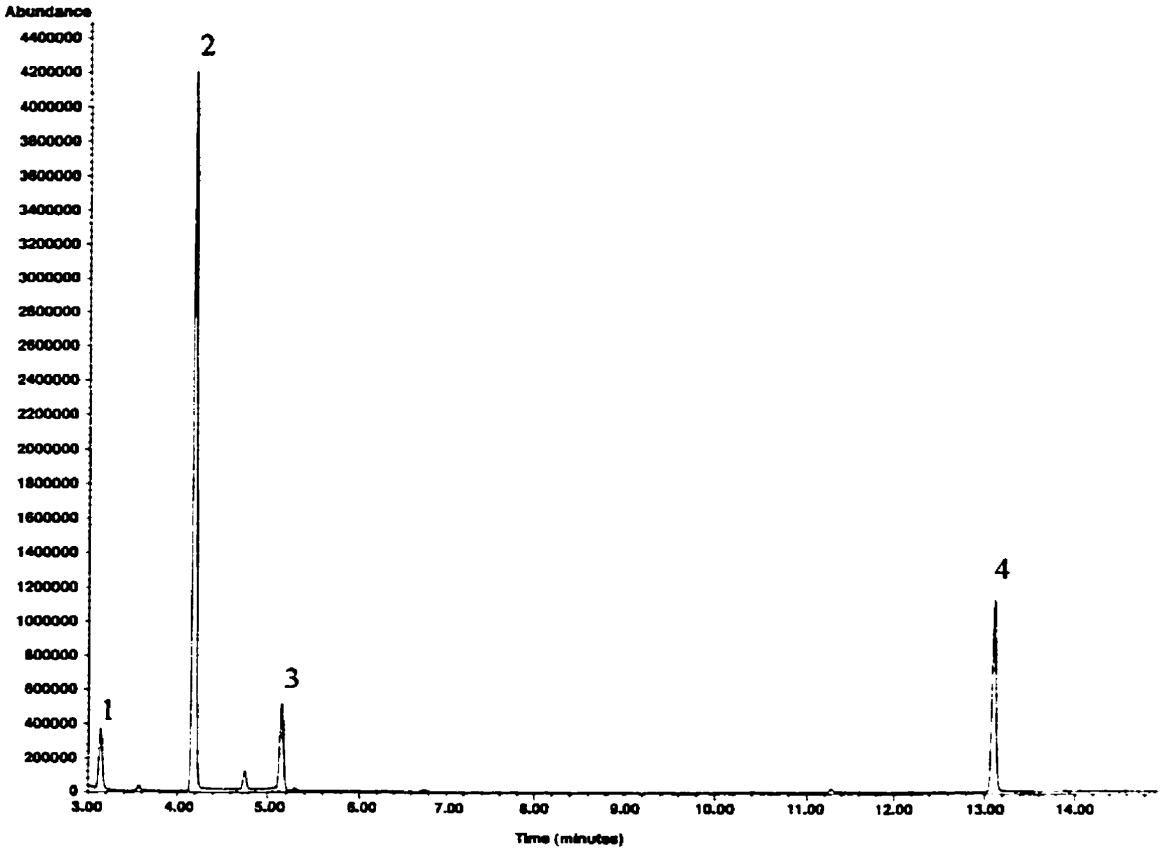

Figure 4. Gas chromatogram of horseradish distillate (components corresponding to numbered peaks are listed in Table 1.)

Table 1. Compounds identified in horseradish distillate[1].

Peak Number	Compound	Molecular Weight (Daltons)
1	acetonitrile	41
2	allyl isothiocyanate (AIT)	99
3	allyl thiocyanate	99
4	2-phenethyl isothiocyanate (β-PEIT)	163

[1]Identifications based on MS data.

3.4.2. Quantitative analysis of horseradish distillate

Standard concentration curves for AIT and β-PEIT are given in Appendix 1. Fresh standard curves for each compound were prepared each time a quantitative analysis was performed. The first quantitative analysis indicated that the distillate contained 87% AIT and 10% β-PEIT (average of two replicates). The second analysis was conducted two and one half months later and indicated that the distillate contained 92% AIT and 11% β-PEIT (average of four replicates). After 11 months at low temperature storage the distillate was found to contain 91% AIT and 11% β-PEIT (average of two replicates).

3.5. Discussion

The hydrodistillation method outlined by Mazza (1984) included a waiting period of 30 min after the addition of distilled water to the ground sample, and before commencement of the distillation. This allows the enzyme myrosinase, released by grinding of the tissue, sufficient time to react with the glucosinolates. Gil and MacLeod (1980a) reported that 20 min in distilled water were required for maximum conversion of sinigrin to AIT by action of myrosinase, while 30 min were required for maximum conversion of gluconasturtiin to β-PEIT.

As expected, AIT and β-PEIT were the two main components found in the horseradish distillate. Consistent with previous reports, AIT was found to be present in the distillate at a concentration approximately 10 times greater than that of β-PEIT (Gilbert and Nursten, 1972; Mazza, 1984). It also appears that these concentrations did not change over the extended period of low temperature storage. This is in agreement with Sekiyama *et al.* (1994b), who reported that cold storage and nitrogen replacement of head space gas maintains the stability of the distillate. In addition to the four major peaks identified, a few unidentified minor peaks were also observed on the chromatogram of the horseradish distillate. Gilbert and Nursten (1972) analysed several different types of horseradish and found five commonly present compounds, as well as other minor compounds. Mazza (1984) also detected several minor compounds in horseradish distillate. An initial peak corresponding to carbon disulphide was also reported by Mazza (1984). It has been theorized that the presence of carbon disulphide is due to hydrolysis of the isothiocyanates (Bailey *et*

al., 1961). Since our chromatograms began at three min, carbon disulphide could have been missed if present.

Although a quantitative determination of the distillate yield from horseradish root was not performed, a crude estimate of 2 mL / kg of root was made using a graduated cylinder. This is consistent with previously reported results of 1.32-1.85 g distillate / kg of root (Mazza, 1984). Mazza (1984) also reported the highest concentration of essential oil occurred in the primary roots, while the lowest concentration occurred in the tops (leaves) of the plant.

Chapter 4

Isolation and Identification of Bacteria Associated with Pre-cooked, Uncured Roast Beef

4.1. Introduction

The microflora of aerobically stored cooked roast beef is often dominated by *Pseudomonas* spp; however, this microflora, when stored under vacuum or high levels of CO_2, increasingly becomes dominated by lactic acid bacteria. Other microorganisms such as *Hafnia alvei*, *Serratia* spp, *Enterobacteriaceae* and *Brochothrix* spp. may also constitute part of the microflora (Anderson *et al.*, 1989; Penney *et al*,. 1993). The purpose of this work was to obtain spoilage microorganisms from both aerobically stored and vacuum packaged pre-cooked, uncured roast beef. Once characterized the isolates were used in subsequent studies on the antimicrobial efficiency of vaporized horseradish distillate.

4.2. Materials and Methods

4.2.1. Materials

Standard methods plate count agar (PCA) and peptone were purchased from Difco (Detroit, MI). Trypticase soy broth (TSB) and agar-agar for biological media were obtained from VWR Canlab (Mississauga, ON). MRS broth (MRSB; Oxoid), Anaerogen GasPacks and anaerobic indicator strips were obtained from Unipath, Inc. (Nepean, ON). Vacuum packaged roast beef slices (with spices; 26 d old) and freshly sliced deli roast beef were purchased from a local retail outlet and stored over night at 4°C.

4.2.2. Isolation of spoilage bacteria

Twenty-five grams of each roast beef product was massaged (separately) in a Stomacher (Model 400, Seward, London, UK) with 225 mL 0.1% peptone buffer (PB). Dilutions ranging from 10^{-2} to 10^{-7} in PB were inoculated onto agar plates by spread plating, in duplicate, on PCA and MRS agar (MRSA). PCA plates were incubated at 28°C for 24 h while the MRSA plates were incubated in an anaerobic jar (10% CO_2 generated by an Anaerogen GasPack) at 25°C for 48 h. Several colonies of varying morphology were removed from both the PCA and MRSA plates and purified by repeated streaking.

4.2.3. Taxonomy

The rapid KOH Gram stain method (Gregersen, 1978) was used to classify the unknown isolates as Gram negative or positive. Carbohydrate utilization was determined using BIOLOG GN and BIOLOG GP microplates (BIOLOG, Hayward, CA) and/or API 50 CHL carbohydrate strips (bioMèrieux Canada Inc., Montreal, PQ). Methods for detection of catalase, ability to hydrolyse arginine, ability to produce gas from glucose and type of carbohydrate metabolism (Hugh-Leifson carbohydrate assay) were derived from Gill and Greer (1993). Motility was observed by stab inoculating tubes containing trypticase soy agar supplemented with 0.6% yeast extract (TSA-YE) or tubes containing MRSA. In addition, motility was observed microscopically. Oxidase was determined using oxidase test strips (Difco, Detroit, MI). The configuration of lactate (D or DL) in Gram positive bacteria was determined using a D-lactate and L-lactate dehydrogenase assay (Boehringer Mannheim GmbH, Mannheim).

4.3. Results and Discussion

Bacterial counts in pre-cooked, uncured roast beef samples obtained from the local retail outlet are listed in Table 2. Total and lactic acid bacterial counts in roast beef slices from the deli counter were slightly lower than the vacuum packaged roast beef slices. Anderson *et al.* (1989) reported aerobic plate counts (APC) of 7.0 $\log_{10}$ colony forming units/g (cfu/g) for vacuum packaged roast beef slices stored for 28 d at 5°C, while Penney *et al.* (1993) reported APC of 7.4 $\log_{10}$ cfu/g for vacuum packaged roast beef slices stored for 10 d at 3°C. The bacterial counts for the vacuum packaged slices (labelled for 26 d) were slightly higher than those reported by Anderson *et al.* (1989) for a refrigerated storage period of 28 d. This may have resulted from temperature abuse during transportation and subsequent storage in the retail outlet. The storage length of the deli-purchased roast beef prior to slicing, on the other hand, is unknown and bacterial counts for this product could not be compared with those in the literature. It is interesting to note that the bacterial counts obtained on PCA are one log higher than those obtained on MRSA. This indicates that the microflora of the deli-purchased roast beef was not dominated by lactic acid bacteria to the same extent as the vacuum packaged roast beef slices.

Of fourteen pre-cooked roast beef isolates only one (isolated from the purge of vacuum packaged roast beef slices and labelled S12) was found to be Gram negative. According to the identification scheme of Vanderzant and Nickelson (1969), this Gram negative isolate belongs to the family Enterobacteriaceae since it is oxidase negative and is able to metabolize carbohydrates fermentatively (Hugh-Leifson carbohydrate assay).

Table 2. Bacterial loads in pre-cooked, uncured roast beef slices.

Roast beef product	Media	$\log_{10}$ cfu/g[1]
Fresh deli slices	PCA	7.39
	MRS agar	6.49
Vacuum packaged slices	PCA	8.43
	MRS agar	8.38
Purge from vacuum packaged slices	PCA	8.52
	MRS agar	8.35

[1] mean of two replicates.

An identification of *Serratia liquefaciens / grimesii* was obtained with the BIOLOG GN identification system (similarity index of 0.934 out of a possible 1.000). The ability to hydrolyse arginine was determined to differentiate between *Serratia liquefaciens* and *Serratia grimesii*. Using the test tube method and Nessler's reagent to determine hydrolysis of arginine (Gill and Greer 1993) only a weak positive result was obtained (top half of tube red, bottom half of tube orange). The arginine cupule of an expired API 20E carbohydrate strip (bioMèrieux Canada Inc., Montreal, PQ) also gave a positive result. According to Grimont and Grimont (1986), *Serratia liquefaciens* is unable to hydrolyse arginine. Therefore, based on the results of the arginine assay, and for the purpose of this study, isolate S12 will be referred to as *Serratia grimesii*. The biochemical reactions for this isolate, with the exception of the BIOLOG GN and API 20E results are listed in Table 3. The BIOLOG GN and API 20E carbohydrate utilization results may be found in Appendix 2a and 2b, respectively.

Table 3. Biochemical reactions observed for Gram negative isolate S12 - *Serratia grimesii*.

Microscopic morphology	coccoid/small rods; most often in pairs or singles
Hugh-Leifson carbohydrate utilization	fermenter
Catalase	+[1]
Motility	+
Methyl red	+
Lactose	-
Arginine	+
Nitrate reduction	+
Gas production from glucose	-
Oxidase	-

[1] '-', negative reaction; '+', positive reaction;

Only three Gram positive bacteria were identified since the other Gram positive isolates exhibited poor growth on both PCA and MRSA, and were difficult to maintain on agar slants at 4°C. API CHL50 carbohydrate strips and BIOLOG GP microplates were used to determine carbohydrate utilization. These assays indicated that one of the isolates belonged to the lactobacilli, but no definitive identifications could be made based on these observations alone. Further assays following the identification scheme for lactobacilli by Schillinger and Lücke (1987a) were conducted. The first Gram positive isolate (isolated from the deli-purchased roast beef and labelled S4) was catalase negative, did not produce gas from glucose and was a short rod (most often in pairs) that was able to grow at both 8°C and 15°C (Table 4). After testing positive for both D-lactate and L-lactate, and using the results from the API CHL50 carbohydrate strips and/or BIOLOG GP microplates, a positive identification of *Lactobacillus sake* was made. This identification was confirmed by comparing results with those reported by Hugas *et al.* (1993) and Schillinger and Lücke (1987b). Identification of

two other Gram positive isolates (isolated from the purge of vacuum packaged roast beef slices and labelled S10 and S13) was conducted using the same identification scheme. Both of these isolates were coccobacillary (in pairs or in chains of 8-9), catalase negative, produced gas from glucose, arginine negative and tested positive for D-lactate only (Table 4.03). According to Schillinger and Lücke (1987a) both isolates were leuconostocs but no further characterization was carried out. The BIOLOG GP and API CHL50 carbohydrate utilization results for these three lactic acid bacteria may be found in Appendix 2c and 2d, respectively.

Table 4. Biochemical reactions observed for three Gram positive isolates.

	Isolate S4 - *Lactobacillus sake*	Isolate S10 - *Leuconostoc* spp.	Isolate S13 - *Leuconostoc* spp.
Microscopic morphology	rods (3 times longer than wide), straight sided, commonly in pairs and oriented in a V-shaped pattern	coccobacillary, in pairs or short chains (8-9)	coccobacillary, in pairs or short chains (8-9)
Catalase	-[1]	-	-
Arginine	+	-	-
Gas production from glucose	-	+	+
Lactate configuration	DL	D	D
Growth at 8°C	+	+	+
Growth at 15°C	+	+	+
Growth at 37°C	ND	S	S
Growth at 45°C	-	-	-

[1] '-', negative reaction; '+', positive reaction; 'ND', not determined; 'S', slight.

Chapter 5

Agar Model System

5.1. Introduction

The antimicrobial effects of naturally occurring AIT have been documented throughout the century and various methods have been used to determine the effectiveness of this compound against bacterial growth and survival. AIT can be tested in the liquid or vapour phase. However, in terms of antimicrobial effectiveness, this compound has been reported to be more efficient in the vapour phase (Inouye *et al.*, 1983). Although early research suggested Gram positive bacteria are more resistant to AIT than Gram negative bacteria, this has not been supported by recent investigations (Inouye *et al.*, 1983; Sekiyama *et al.*, 1994b; Delaquis and Sholberg, 1997). Lactic acid bacteria, on the other hand, are consistently reported as exhibiting resistance to the antimicrobial effects of AIT (Llanos Palop *et al.*, 1995; Kyung and Fleming, 1997). In addition, pathogens also appear to be more resistant to AIT vapour than common Gram negative spoilage bacteria (Delaquis and Sholberg, 1997).

In the following study solidified agar inoculated with test microorganisms was placed in a sealed vessel and exposed to varying concentrations of gaseous horseradish distillate. Bacterial growth and survival were determined over a range of concentrations and temperatures. Results from this investigation should aid in further research on the use of essential oils from horseradish as a means of enhancing the safety and/or shelf-life of pre-cooked, uncured roast beef.

5.2. Materials

5.2.1. Media, chemicals and reagents

Trypticase soy broth (TSB) and agar-agar for biological media were purchased from VWR Canlab (Mississauga, ON) while MRS broth (MRSB; Oxoid), yeast extract (Oxoid), anaerogen gas packs (Oxoid) and anaerobic indicator strips (Oxoid) were purchased from Unipath, Inc. (Nepean, ON). Commercial grade canola oil was obtained from a local retailer. AIT (purity ≥ 98%) was obtained from Fluka Chemika-BioChemika (Edmonton, AB).

5.2.2. Bacterial cultures

Bacterial cultures tested included *Escherichia coli* O157:H7 (ATCC 43 985), *Listeria monocytogenes* (LCDC 81 861), *Salmonella typhimurium* (ATCC 14 028), *Staphylococcus aureus* (ATCC 25 923), *Serratia grimesii* (roast beef isolate S12) and *Lactobacillus sake* (roast beef isolate S4). Working cultures, with the exception of *Lactobacillus sake*, were maintained on slants of trypticase soy agar supplemented with 0.6% yeast extract (TSA-YE) at 4°C. *Lactobacillus sake* was maintained on MRS agar (MRSA) slants at 4°C. Stock cultures were stored at -70°C in 10% glycerol.

5.2.3. Model system

The model system consisted of a 2 L - Mason jar (Kerr, Los Angeles, CA) that housed a glass plate (20.5 cm x 7 cm) and a watch glass (diameter = 6.5 cm). The system was hermetically sealed with ceramic lined lids (Bernardin, Toronto, ON) that were individually modified with a silicone sealed rubber septum (Supelco, Bellefonte, PA) for sampling and

analysis of the head space vapours by gas chromatography (GC). For antimicrobial studies, 24 agar discs (1.77 cm^2) were aseptically removed from 20 mL solidified TSA-YE or MRSA plates with a sterile stainless steel cork borer and placed on the sterilized glass plate prior to inoculation. Each glass plate supported three strains randomly placed into test groups. The first test group was *Escherichia coli, Salmonella typhimurium* plus *Listeria monocytogenes.* While the second group was comprised of *Staphylococcus aureus, Serratia grimesii* and *Salmonella typhimurium*. Both groups were incubated at 35°C, while *Lactobacillus sake* was tested alone at 25°C. For the 12°C study, the bacteria were grouped as (A) *Escherichia coli, Salmonella typhimurium* and *Listeria monocytogenes* and (B) *Staphylococcus aureus, Serratia grimesii* and *Lactobacillus sake*. The watch glass, situated at the base of the Mason jar, was used as support for the horseradish:canola oil mixture. Figure 5 is an illustration of the model system housing inoculated agar discs.

5.3. Methods

5.3.1. Determination of antibacterial activity of vaporized horseradish distillate at elevated temperatures

The antibacterial activity of vaporized horseradish distillate was determined according to the method outlined by Delaquis and Sholberg (1997). A loopful of each working culture was added to a series of test tubes containing nine mL of TSB-YE, with the exception of *Lactobacillus sake.* A loopful of this culture was added to nine mL of MRSB. Cultures in TSB-YE were incubated at 35°C for 24 h, while *Lactobacillus sake* in MRSB was incubated at 25°C for the same length of time.

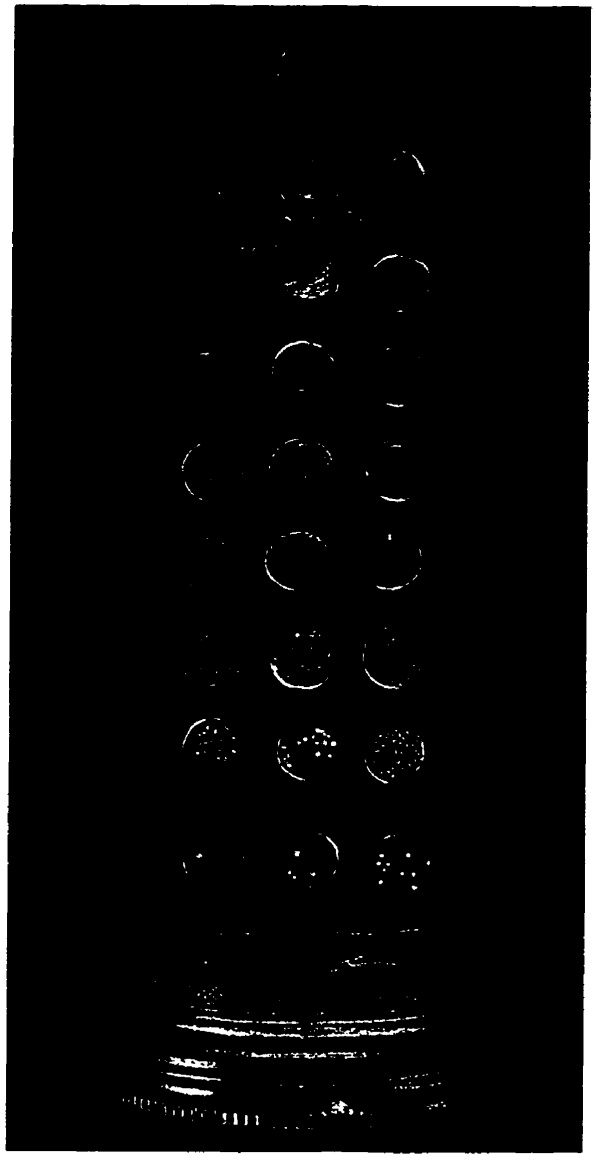

Figure 5. Model system used to assess the antimicrobial activity of gaseous horseradish distillate toward bacteria inoculated onto an agar surface. Shown are controls wherein each column of agar discs was inoculated with tenfold dilutions of individual bacterial cultures. The lids were fitted with a septum (not visible) to permit removal of gas samples.

Tenfold dilutions of each broth culture were then prepared in TSB-YE or MRSB, and 0.01 mL of each dilution was added to the surface of the agar discs (1.77 cm^2). The glass plates were inserted into 2 L - Mason jars prior to the addition of horseradish distillate. Horseradish distillate, diluted in commercial grade canola oil (1:10 v/v), was added to the system at a rate of 0, 500, 1 000 and 2 000 nL/L. The sealed jars were incubated at 35°C, with the exception of those containing *Lactobacillus sake*. These jars were incubated at 25°C.

After a 48 h incubation period the agar discs were removed from the model system and cfu were counted on those discs that exhibited the lowest number of visible cfu. Total inhibition (log cfu / cm^2) was calculated using the difference between the number of cfu on the experimental disc (B) and the inoculum density of the controls (without horseradish distillate) (A) (Equation 1). Agar discs without evidence of growth were placed on the surface of TSA-YE or MRSA plates to allow for possible recovery of injured cells. The recovery period was 72 h at 35°C for the pathogens and *Serratia grimesii,* while recovery of *Lactobacillus sake* was at 25°C in an anaerobic jar (10% CO_2 atmosphere generated with an Anaerogen pack (Oxoid, Nepean, ON)) for 72 h. The extent of irreversible inhibition was also calculated using Equation 1 with (B) corresponding to the number of visibly growing colonies present on the experimental agar discs after the recovery period.

$$Log\ cfu\ inhibited\ /\ cm^2 = Log\ \frac{A - B}{1.77\ cm^2}$$

Equation 1. Formula used to determine bacterial growth inhibition achieved with various concentrations of vaporized horseradish distillate. (A = the number of cfu at the highest dilution under controlled conditions multiplied by the dilution factor corresponding to that of B; B = the number of visible cfu observed at the highest dilution under experimental conditions).

5.3.2. Determination of antibacterial activity associated with vaporized horseradish distillate at 12 °C

The protocol for this study was similar to section 5.3.1 with modifications to the amount of distillate added to the system, to the temperature of incubation and to the length of incubation. Horseradish distillate was added to the system at a rate of 0, 2 000, 4 000 and 20 000 nL/L followed by incubation at 12°C for seven d to determine total inhibition. Conditions for recovery of injured or inhibited cells and determination of irreversible inhibition were the same as in section 5.3.1.

5.3.3. Analysis of vaporized AIT in the model system

Residual AIT concentrations in the head space of the model system were determined by gas chromatography (GC). In a preliminary investigation, chemical grade AIT was added to several Mason jars at a rate of 500, 1 000 and 2 000 nL/L, in the presence of dried air, dried nitrogen and in the presence of uninoculated TSA-YE discs. Incubation was at 37°C

and residual AIT concentrations were monitored each day over a five day period. Using a gas tight syringe, vapour samples (100 μL) were removed from the system via the rubber septum in the lid. A gas chromatograph (Model HP5700A; Hewlett Packard (Canada) Ltd., Richmond, BC) was fitted with a 60/80 mesh Carbowax 20M glass column (1/8" OD, 10' length) and operated under the following conditions: oven temperature of 100°C, injector temperature of 250°C, detector temperature of 250°C, and a carrier gas flow rate of 20 mL N_2 / min (100°C). Detection of AIT was conducted using a flame ionization detector that had a combined N_2-H_2 flow rate of 45 mL/min.

Residual AIT concentrations from horseradish distillate (initial concentrations of 500, 1 000 and 2 000 nL/L) were also monitored in the presence of dried nitrogen and uninoculated TSA-YE discs. For these trials the incubation temperature was 35°C and sampling was again conducted on days zero through five. In addition, residual AIT was monitored in experimental jars (initial inputs of 2 000, 4 000 and 20 000 nL/L) containing inoculated TSA-YE and MRSA discs and incubated at 12°C (section 5.3.2). Residual AIT determinations were conducted on days zero, one, two, four and seven. For each trial, the amount of residual AIT was expressed as percent AIT with day zero assigned a value of 100%.

5.3.4. Statistical analysis

Microbial and vapour data were analysed using SAS analytical software (SAS Institute Inc., Cary, NC). The general linear models procedure (PROC GLM) was used for the

analysis of variance while least-square means were used to determine significant differences at $P < 0.05$.

5.4. Results

5.4.1. Antibacterial activity of horseradish distillate at elevated temperatures

Figure 6 shows the total and irreversible inhibition profiles of bacteria achieved with volatile horseradish distillate (500, 1 000 and 2 000 nL/L) at 35°C, and the results of statistical analyses are given in Appendices 3 and 4. Similar trends were observed with the four pathogenic bacteria. Generally, an increase in total inhibition was observed with increased horseradish distillate concentration. Results obtained with *Staphylococcus aureus* deviated from this trend slightly since no significant differences were observed between total inhibition achieved with initial horseradish distillate concentrations of 1 000 and 2 000 nL/L.

The test strains varied in their ability to recover from exposure to the distillate. *Salmonella typhimurium* and *Escherichia coli*, the most resistant of the pathogenic bacteria, recovered almost completely from the inhibitory effects of vaporized horseradish distillate regardless of initial concentration. *Staphylococcus aureus* also exhibited resistance towards the lower vapour concentrations; however, an initial concentration of 2 000 nL/L caused significant irreversible inhibition. *Listeria monocytogenes*, the most sensitive pathogenic organism tested in the agar model system, was irreversibly inhibited at all vapour concentrations, and this effect increased with distillate concentration.

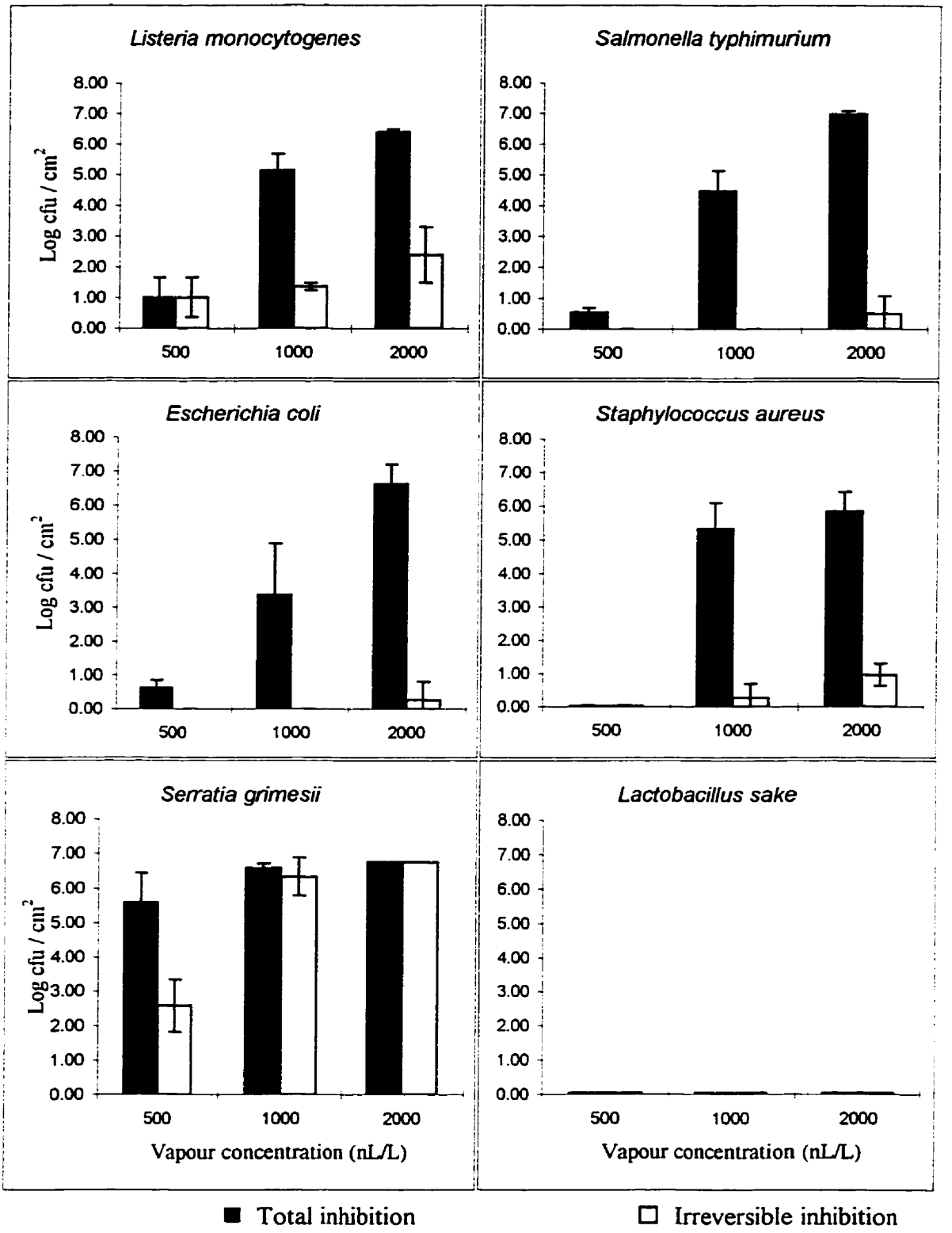

Figure 6. Total and irreversible inhibition of test microorganisms under 500, 1 000 and 2 000 nL horseradish distillate / L air on agar discs at 35°C (25°C for *L. sake* ; shown are means of four replicates ± standard deviation except with *S. grimesii* at 2 000 nL/L where only one replicate was conducted).

Serratia grimesii, a common spoilage bacterium in pre-cooked, uncured roast beef, was more susceptible to the effects of horseradish vapours than the pathogens. Significant inhibition was achieved with each initial concentration of horseradish distillate tested. In addition, irreversible inhibition increased with higher distillate concentration. The spoilage bacterium, *Lactobacillus sake*, was resistant to the horseradish distillate at all concentrations tested.

5.4.2. Antibacterial activity of horseradish distillate at 12 °C

Since limited irreversible inhibition was observed at 35°C and preliminary trials in the roast beef model system did not yield significant results (data not shown), the initial horseradish distillate concentrations were increased. Figure 7 illustrates the inhibition profiles (total and irreversible) of the test microorganisms with volatile horseradish distillate (2 000, 4 000 and 20 000 nL/L) at an abusive refrigeration temperature of 12°C, and results of statistical analyses are given in Appendices 4 and 5. The pathogenic bacteria were completely inhibited at the concentrations tested. With *Staphylococcus aureus*, inhibition at 20 000 nL/L was lower than at 2 000 or 4 000 nL/L, however this difference was not significant at P=0.01.

Differences were also observed in the extent of irreversible inhibition among the pathogenic bacteria. It was highest with 20 000 nL/L. Furthermore, inhibition achieved with 20 000 nL/L varied among genera. In terms of irreversible inhibition, *Staphylococcus aureus* was the most resistant pathogen while *Listeria monocytogenes*, *Salmonella typhimurium* and *Escherichia coli* were equally susceptible to the effects of the distillate.

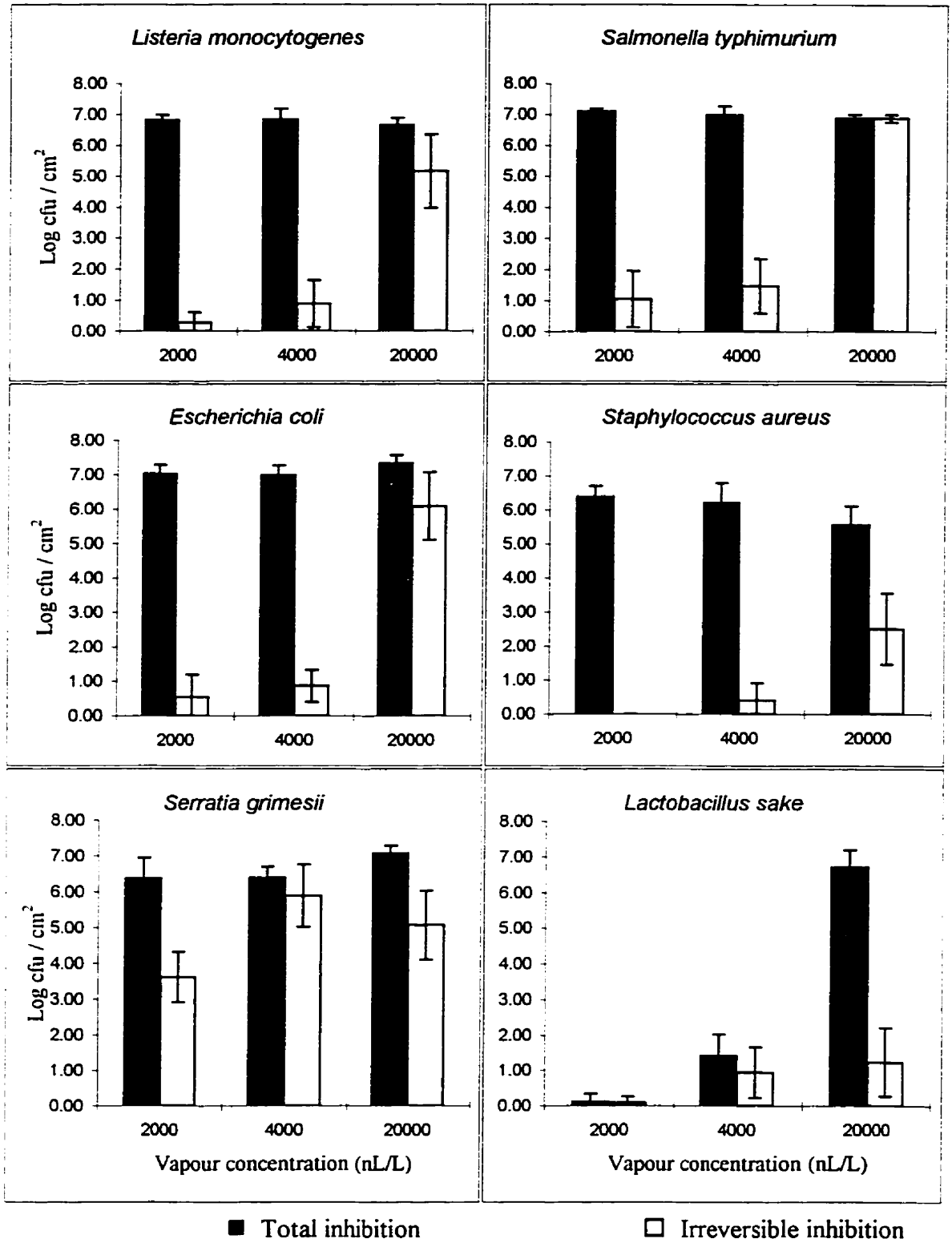

Figure 7. Total and irreversible inhibition of test microorganisms under 2 000, 4 000 and 20 000 nL horseradish distillate / L air on agar discs at 12°C (shown are means of four replicates ± standard deviation).

As reported earlier (section 5.4.1) *Serratia grimesii* was more sensitive to the horseradish distillate than the pathogens. At a concentration of 20 000 nL/L, total inhibition was significantly greater than that achieved with either 2 000 or 4 000 nL/L. A distillate concentration of 20 000 nL/L was also more lethal than 2 000 nL/L, although equivalent irreversible inhibition was obtained under 4 000 nL/L. While initial vapour concentrations of 2 000 and 4 000 nL/L caused significantly greater irreversible inhibition of *Serratia grimesii* than pathogenic bacteria, similar effects were achieved with 20 000 nL/L. Increasing concentrations of horseradish distillate slowed the growth of *Lactobacillus sake* and little growth occurred at the higher concentration. The extent of irreversible inhibition was limited, although significant differences were observed between 2 000 and 20 000 nL/L.

The experiments described above were carried out to determine whether the antimicrobial effects of horseradish distillate were affected by temperature. Statistical comparisons of data at a concentration of 2 000 nL/L revealed that temperature significantly affected inhibition of *Listeria monocytogenes*. Total inhibition was greater at 12°C while greater irreversible inhibition was observed at 35°C (Appendix 6a). Irreversible inhibition of *Staphylococcus aureus* and *Serratia grimesii* was also enhanced at 35°C, although total inhibition was unaffected by temperature (Appendix 6d-e). Inhibition of the remaining test strains was not significantly affected by a change in incubation temperature.

5.4.3. Fate of AIT in the model system

The fate of gaseous AIT was first evaluated in the model system flushed with dried air and nitrogen, and moist air generated by incorporation of agar discs. As shown in Figure 8, AIT concentrations decreased significantly over time (Appendix 7) in jars stored at 37°C, regardless of head space composition. Depletion of AIT was fastest in the head space above agar discs and approximately 20% of initial inputs remained after two d. More gradual depletion of AIT was observed in jars flushed with dried air and nitrogen, although there was considerable variability between measurements. Statistical comparison of mean residual AIT levels in the latter two treatments (Appendix 8) failed to reveal significant differences, likely as a result of this variability. Nevertheless, the shape of the curves indicates that losses were slowest when the model system was flushed with dried air prior to addition of AIT. AIT derived from horseradish distillate was also evaluated in nitrogen flushed jars with and without agar discs (Appendix 9). As shown in Figure 9, depletion of AIT at 35°C was again significantly faster in the head space above agar discs, and approximately 20% remained after two d (Appendix 10). Similar measurements were also performed at 12°C since some activity trials were conducted at this temperature, albeit with higher distillate concentrations (section 5.4.2). Figure 10 shows that a reduction in incubation temperature delayed depletion of AIT considerably. Residual concentrations with the lower input levels were approximately double those observed at 35°C. In addition, residual levels were significantly greater at the highest input of 20 000 nL/L (Appendix 11).

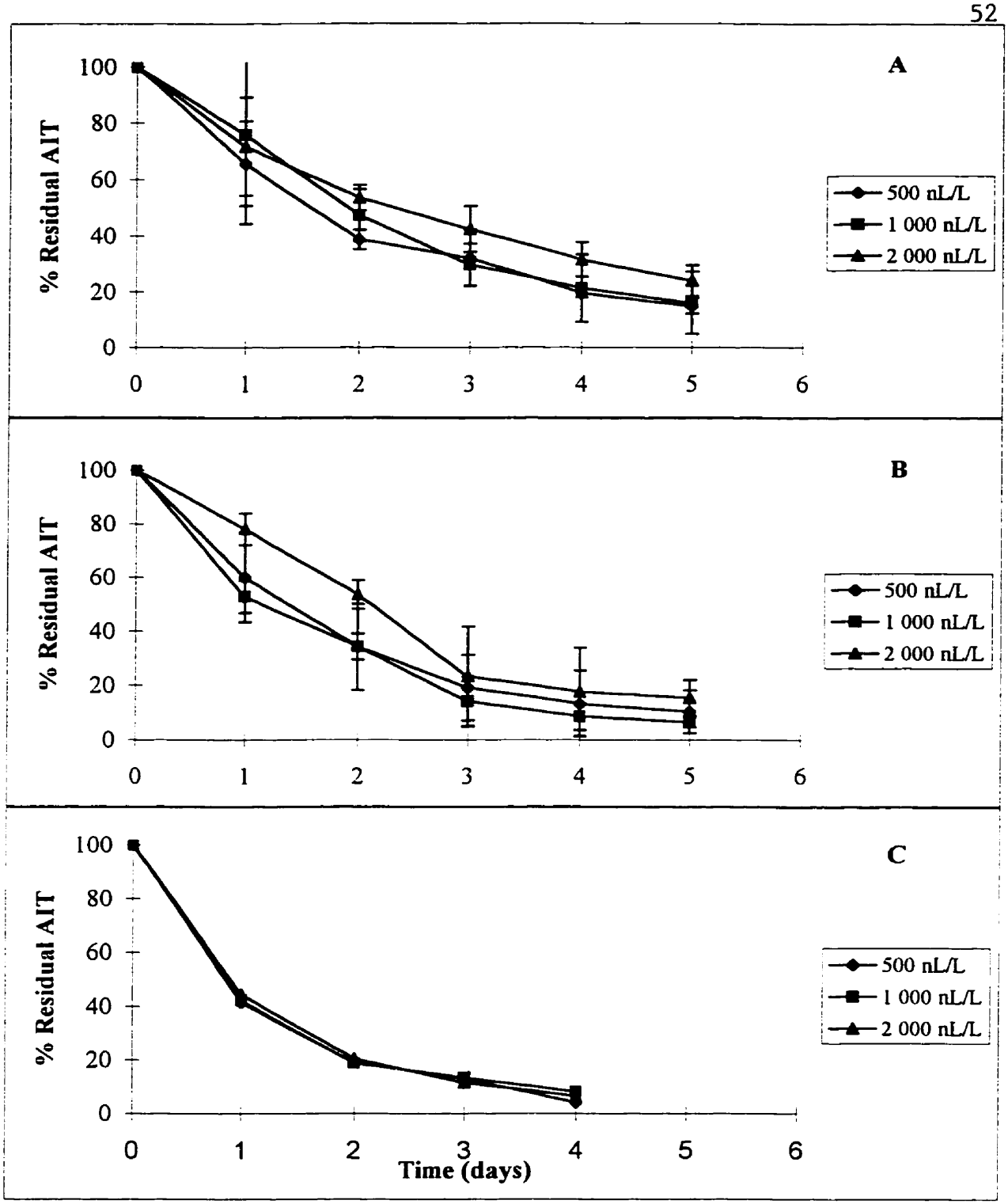

Figure 8. Depletion of chemical grade AIT in the model system over five d storage at 37°C (A- flushed with dried air, B- flushed with dried nitrogen, C- in the presence of 21 TSA-YE discs) (shown are the means of two replicates ± standard deviation except with TSA-YE trial where only one replicate was conducted).

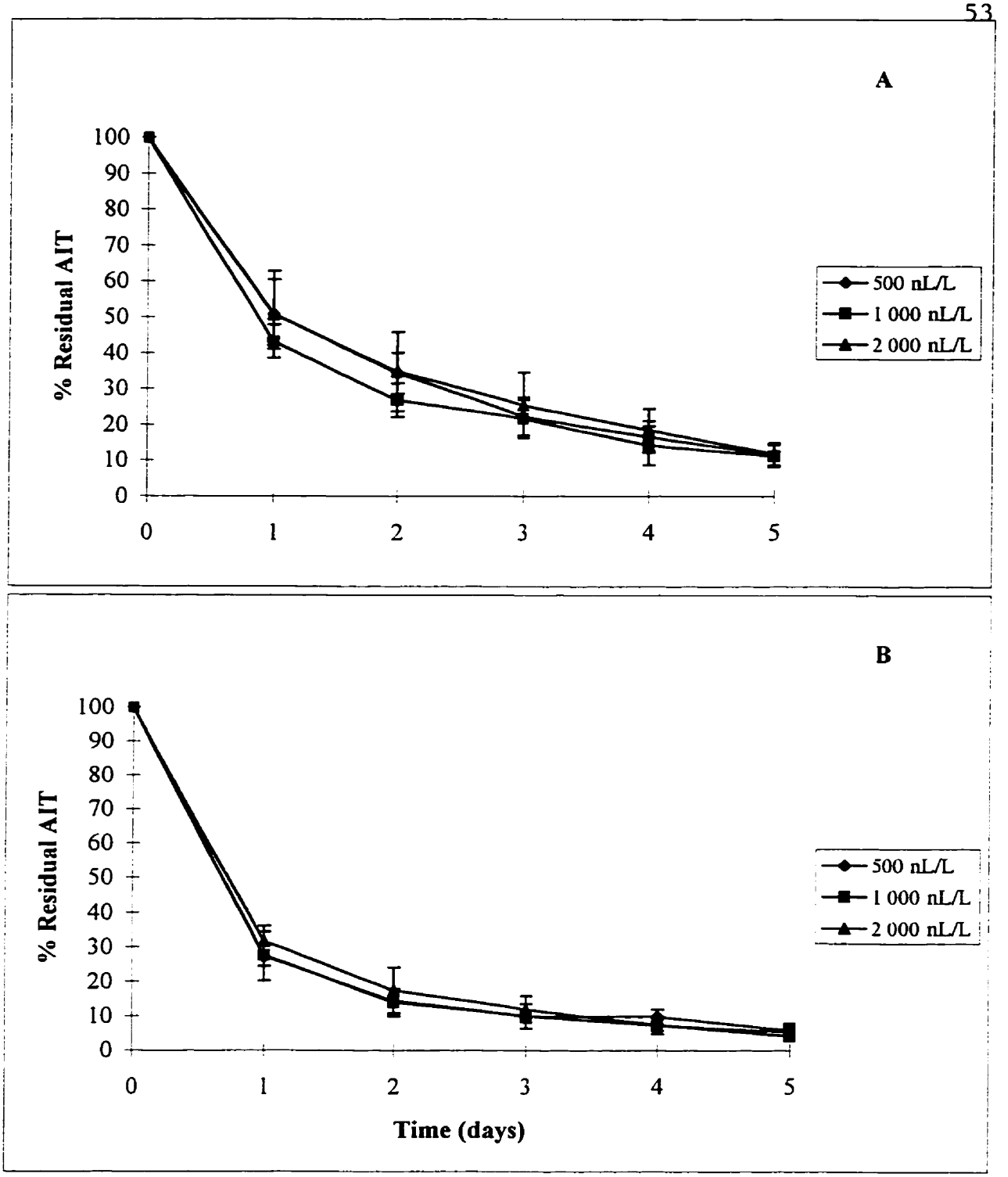

Figure 9. Depletion of AIT from horseradish distillate in the model system over five d at 35°C (A- model system flushed with dried nitrogen, B- in the presence of uninoculated TSA-YE discs; shown are means of four replicates ± standard deviation).

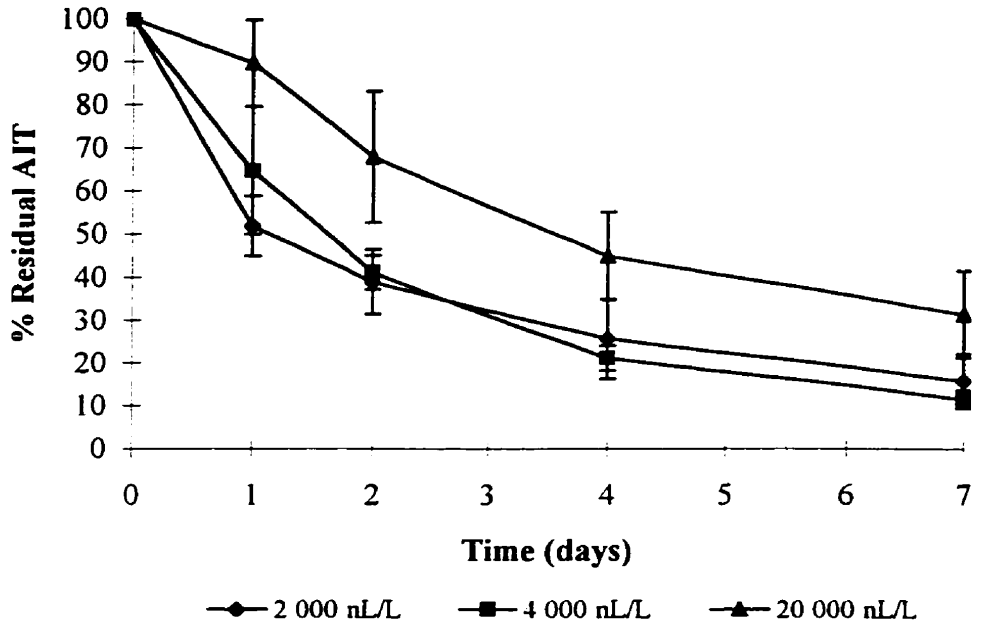

Figure 10. Depletion of AIT from horseradish distillate in an inoculated agar model system over seven d of storage at 12°C (shown are means of four replicates ± standard deviation).

Overall, studies carried out in the model system suggest that the presence of oxygen does not influence the fate of AIT in the gas phase. Depletion of AIT was accelerated above a wet substratum however, particularly at higher temperatures.

5.5. Discussion

Differences in the extent of inhibition observed between the test genera exposed to vaporized horseradish distillate indicate that the antimicrobial effect of horseradish distillate is genus specific. Similar observations with vaporized mustard extract and chemical grade AIT have been reported by Foter and Golick (1938), Inouye *et al.* (1983), Isshiki *et al.* (1992), Sekiyama *et al.* (1994b), Brabban and Edwards (1995) and Delaquis and Sholberg (1997). Inouye *et al.* (1983) and Isshiki *et al.* (1992) also reported that Gram negative bacteria were more susceptible to vaporized AIT than Gram positive bacteria. There was no evidence of a relationship between sensitivity to isothiocyanates and the Gram-staining reaction in our experiments. Interestingly, all the pathogens tested exhibited a similar range of sensitivity. Delaquis and Sholberg (1997) reported a similar observation for *Listeria monocytogenes*, *Salmonella typhimurium* and *Escherichia coli* O157:H7 using purified AIT. Sekiyama *et al.* (1994b) also observed that *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus* were equally affected by an AIT-rich extract from mustard seed.

Lactobacillus sake was consistently more resistant to the distillate than any of the test species, regardless of incubation temperature and initial concentration. Resistance to AIT by lactic acid bacteria has been previously reported. Kyung and Fleming (1997) found that concentrations of AIT required to inhibit growth of *Lactobacillus plantarum* and

Lactobacillus brevis were 1.5 times greater than those required for growth inhibition of *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimurium*. While investigating the ability of *Lactobacillus agilis* strain R16 to degrade the glucosinolate, sinigrin, Llanos Palop *et al.* (1995) found this bacterium was initially inhibited by AIT (2.5 mM) but was able to resume growth after 72 h at 30°C. Recent studies in our laboratory have also shown that six additional lactic acid bacteria isolated from cooked roast beef exhibit extreme resistance towards vaporized horseradish distillate (Delaquis, unpublished).

Interestingly, changes in incubation temperature did not appear to significantly affect the antimicrobial effectiveness of AIT against *Salmonella typhimurium*, *Escherichia coli* and *Lactobacillus sake*. This was in contradiction with the observations of Foter and Golick (1938) who reported that antimicrobial effects associated with vapours from crushed horseradish were more pronounced at 37.5°C and were diminished by lowering incubation temperatures. The ability of *Listeria monocytogenes* to grow at low refrigeration temperatures may be an important factor. This microorganism may reach the log phase earlier than bacteria that do not grow well at low temperature and thus, be more susceptible to the vapours.

Residual AIT concentrations were observed to decline over the storage period in each trial. Both dried air and nitrogen were introduced in the preliminary trials as means of retarding AIT depletion since interaction with water molecules is reported to decompose AIT (section 2.1.3.3). However, information on the impact of temperature and oxygen on vaporized AIT is negligible. Although oxygen (air) did not appear to have an impact in AIT

depletion, the results indicate that degradation is accelerated in the presence of agar discs. In aqueous media spontaneous decomposition and nucleophilic attack of water and hydroxide ions are believed to account for the disappearance of AIT (Kawakishi and Namiki, 1969; Llanos Palop *et al.*, 1995; Ohta *et al.*, 1995a).

Depletion of AIT in experimental systems has been reported previously. Foter and Golick (1938) observed that the inhibitory properties of horseradish vapours were rapidly exhausted over six h at 37.5°C. Although they did not measure the residual AIT concentrations during the experimental period, the decrease in antimicrobial activity could have been caused by depletion of AIT. Development of resistance to AIT is unlikely based on current literature. Sekiyama *et al.* (1994a) also reported a depletion of AIT to 17% of the initial concentration over 24 h at 30°C but no explanation for this occurrence was provided. In a follow-up study, Sekiyama *et al.* (1996) observed AIT depletion over seven d at 27°C to 1/10th of the initial AIT concentration. They reported 15-20% of AIT was absorbed by the agar media, while leakage and interaction with the plastic container were likely responsible for the additional depletion. Reactions with medium components cannot be discounted since AIT is known to react with free amino groups in proteins and peptides (Kroll *et al.*, 1994; Kroll and Rawel, 1996). Furthermore, dissolution of AIT in a moisture layer on the glass surface could account for some loss from the vapour phase. Conversely, Ohta *et al.* (1995b) reported that polysaccharides and dextrins are able to suppress AIT decomposition in the presence of water. As polysaccharides make up a large proportion of the components present in the agar discs, they may have retarded the depletion of AIT. Absorption onto the container surfaces or leakage may have also contributed to the depletion of residual AIT over time in

our model system. Sekiyama *et al.* (1995) reported that AIT is absorbed by plastics but interactions with glass have not been previously described.

At the higher distillate input level (20 000 nL/L), residual concentrations on a percentage basis were always significantly greater. This suggests that the model system contains a finite number of adsorption sites or reactive components that were saturated at the higher input. Therefore, maintenance of sufficient AIT concentrations in the head space above a food product may require consideration of losses due to such phenomena.

Chapter 6

Roast Beef Model System

6.1. Introduction

The availability of pre-cooked, uncured meat products has expanded over the past several years primarily due to modified atmosphere packaging and improvements to refrigerated distribution. Although the shelf-life may be extended, concerns regarding the safety of these products have not been fully addressed (Farber, 1991). Pathogenic bacteria, including *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* and *Staphylococcus aureus*, are reported to survive and/or grow on pre-cooked roast beef at refrigeration temperatures. The purpose of the following study was to determine the effects of horseradish distillate in the vapour phase against pathogenic and spoilage microorganisms inoculated onto the surface of roast beef, and a microflora associated with post-process contaminated roast beef. Sensory changes to roast beef exposed to vaporized horseradish distillate were also determined. In addition, volatile compound profiles of refrigerated pre-cooked roast beef stored under horseradish distillate were determined by gas chromatography-mass spectrometry.

6.2. Materials and Methods

6.2.1. Materials, media and reagents

Several cook-in bag restructured beef roasts (ca. 2 kg/roast) were obtained from a local meat processor and cooled to -1.5°C prior to slicing. Microbiological analyses were

performed with trypticase soy broth (TSB; VWR Canlab, Mississauga, ON), agar-agar for biological media (VWR Canlab), MRS broth (MRSB; Oxoid, Unipath, Nepean, ON), yeast extract (Oxoid, Unipath), violet red bile glucose agar (VRBG; Oxoid, Unipath), pseudomonas agar base (Oxoid, Unipath) and pseudomonas C-F-C selective supplement (Oxoid, Unipath). Anaerogen gas packs (Oxoid) and anaerobic indicator strips (Oxoid) were also obtained from Unipath. Peptone buffer was purchased from Difco (Detroit, MI), while allyl isothiocyanate (AIT) was purchased from Fluka Chemika-Biochemika (Edmonton, AB). Reagents included: ammonium molybdate (Fisher Scientific Ltd., Nepean, ON), ammonium nitrate (Baker Chemical Co., Phillipsburg, NJ), ammonium thiocyanate (Baker Chemical Co.), citric acid (Mallinckrodt Inc., Chesterfield, MO), ferrous ammonium sulfate (Baker Chemical Co.), hydrochloric acid (Caledon Inc., Georgetown, ON), nitric acid (Fisher Scientific Ltd.), phenolphthalein (Baker Chemical Co.), potassium permanganate (Merck and Co., Ltd., Montreal, PQ), silver nitrate (Nichols Chemical Co., Toronto, ON), dibasic sodium phosphate-heptahydrate (Mallinckrodt, Inc.), monobasic sodium phosphate (Fisher Scientific Ltd.), sodium chloride (Merck and Co., Ltd.), sodium hydroxide (Caledon Inc.) and sulfuric acid (Caledon Inc.). Horseradish distillate was prepared by steam distillation and commercial grade canola oil was obtained from a local retailer.

6.2.2. Preparation and proximate analysis of roast beef

A cook-in-bag, restructured roast (ca. 2 kg) was aseptically removed from its packaging in a laminar flow hood and placed on a sterile piece of aluminum foil. Prior to transferring the roast to a clean and sanitized meat slicer (Model 410, Hobart, Don Mills, ON;

width setting = 20) each side of the roast was flamed with 95% ethanol. The blade and working surfaces were also doused with 95% ethanol and ignited. The resulting roast beef slices (0.5 cm thickness) were placed into sterile sample bags (Canlab, ON) and stored at -30°C until further use.

The moisture content of pre-cooked, uncured roast beef was determined using the vacuum oven method (AOAC, 1975). Ash content was determined using the muffle furnace method described by Kramlich *et al.* (1973). Protein was calculated from nitrogen values obtained with a nitrogen analyser (Model FP-528; Leco Instruments Ltd., Mississauga, ON) and fat content was calculated by difference. Salt content was determined as percent sodium chloride (%NaCl) using the Volhard method as described by Kramlich *et al.* (1973). Briefly, this involved wet ashing five grams of a pre-ground roast beef sample with silver nitrate and nitric acid, followed by titration of excess silver nitrate with potassium thiocyanate. Finally, the phosphate content was determined as percent phosphorus pentoxide ($\%P_2O_5$) using the titration method described by Koniecko (1985). This method involved forming a precipitate from a dry ashed sample of pre-ground roast beef using hydrochloric acid, ammonium nitrate and a citro-molybdate solution. The resulting precipitate was then titrated with sodium hydroxide (0.1N) and back titrated with sulphuric acid (0.5N).

6.2.3. Preparation of bacterial cultures

Test microorganisms included *Escherichia coli* O157:H7 (ATCC 43 985), *Listeria monocytogenes* (LCDC 81 861), *Salmonella typhimurium* (ATCC 14 028), *Staphylococcus aureus* (ATCC 25 923), *Serratia grimesii* (roast beef isolate S12) and *Lactobacillus sake*

(roast beef isolate S4). Bacterial cultures were grown in 30 mL of TSB-YE for 24 h at 35°C. *Lactobacillus sake* was grown in 30 mL of MRSB for 24 h at 25°C. Twenty-five millilitres (25 mL) of each culture was sedimented by centrifugation (Sorvall RC-5 automatic superspeed refrigerated centrifuge, Du Pont, Newtown, CT; 10 min, 3000 x *g*), washed twice and resuspended in 0.05 M phosphate buffer solution (pH 7.2; PBS). Bacterial cultures were further diluted with PBS until an optical density (OD) at 600 nm corresponding to approximately 5 $\log_{10}$ cfu/mL was obtained. The desired $OD_{600\ nm}$ for each culture was determined from standard curves for $OD_{600\ nm}$ vs bacterial count (log cfu/mL) (Appendix 12). Standard curves for each culture were prepared from 24 h broth cultures (grown in either TSB-YE or MRSB) that were centrifuged, washed, diluted twice and resuspended in PBS as above. Optical densities were recorded for various culture dilutions in PBS at 600 nm and were further correlated to cfu by plating the dilutions, in duplicate, on TSA-YE. Incubation was at 35°C for 24 h. Enumeration of *Lactobacillus sake* was conducted by plating dilutions, in duplicate, on MRSA, followed by anaerobic incubation at 25°C for 48 h.

6.2.4. Determination of antimicrobial effectiveness of vaporized horseradish distillate in a roast beef model system at 12 °C

Thawed roast beef slices were aseptically cut into four cm^2 pieces and placed on sterile glass plates (20.5 cm x 7 cm) to obtain six roast beef pieces per plate. Four of the roast beef pieces were inoculated, in duplicate, with 0.04 mL of the test microorganisms, while the two remaining roast beef pieces served as controls. Two genera of bacteria were tested per Mason jar and were randomly grouped as (A) *Escherichia coli* and *Staphylococcus aureus*,

(B) *Salmonella typhimurium* and *Listeria monocytogenes* and © *Serratia grimesii* and *Lactobacillus sake*. The glass plates supporting the inoculated roast beef pieces were inserted into oven-dried 2L-Mason jars and 0, 80 or 400 µL of a 1:10 dilution of horseradish distillate to canola oil were added to individual jars to yield test concentrations of 0, 4 000 and 20 000 nL/L, assuming complete vaporization of the distillate. The jars were immediately sealed with ceramic lined lids (Bernardin, Toronto, ON) that were fitted with rubber septa (Supelco, Bellefonte, PA) and placed in an incubator at 12°C.

Bacterial cell densities (log cfu/cm^2) were determined after zero, one, two, four and seven d of storage. Each roast beef piece was massaged with 100 mL of PBS in a Stomacher (Model 400, Canlab, ON; 120 sec., normal speed). Decimal dilutions in PBS were spread plated in duplicate on TSA-YE (MRSA for *Lactobacillus sake*). TSA-YE plates were incubated at 35°C for 24 h, while MRSA plates were incubated anaerobically at 25°C for 48 h to enumerate *Lactobacillus sake*.

6.2.5. Analysis of residual AIT at 12 °C

Gas chromatography (GC) was used to analyse the residual AIT concentrations present in the head space of the roast beef model system. Sampling and GC conditions were as previously described in section 5.3.3. Head space analysis for residual AIT was conducted parallel to the microbiological analysis on days zero, one, two, four and seven of storage at 12°C by withdrawal of samples through septa in the lids.

6.2.6. Shelf-life of roast beef exposed to volatile horseradish distillate at 4 °C

Thawed roast beef slices were contaminated by placing them, for two min (each side) on a non-sanitized, stainless steel, cafeteria food preparation counter, and on a mirrored serving platter that held sandwich meats at room temperature for one h. Contaminated slices were cut into several pieces (4 cm^2), placed on sterilized glass plates and were inserted into 2L-Mason jars (see section 6.2.4). Twelve jars were flushed with nitrogen gas for five min via a septum in the lid prior to incubation. A needle was inserted into a second septum in the lid to accommodate flushing of the jars. Four hundred microlitres (400 µL) of a 1:10 dilution of horseradish distillate to canola oil were added to the watch glass situated at the base of each test jar prior to incubation. The distillate was added with a syringe through one of the septa used for gas flushing. Control jars containing air or nitrogen received no distillate. All jars were incubated at 4°C.

Bacterial cell densities (log cfu/cm^2) were determined after 0, 1, 3, 7, 14, 21 and 28 d in storage at 4°C. Duplicate roast beef pieces were individually massaged with 100 mL PB in a Stomacher. Decimal dilutions in PB were spread plated in duplicate on PCA, MRSA, VRBG and pseudomonas base agar supplemented with pseudomonas C-F-C selective supplement (PCFC). PCA plates were incubated at 30°C (48 h) for the enumeration of total aerobic bacteria. PCFC plates were incubated at 25°C (48 h) for the enumeration of pseudomonads. VRBG plates were incubated at 35°C (24 h) for the enumeration of enteric bacteria, while MRSA plates were incubated anaerobically at 25°C (72 h) for the enumeration of lactic acid bacteria.

Nitrogen, oxygen and carbon dioxide levels in the gas flushed jars were monitored by GC. Prior to microbial analysis, 100 µL gas samples were removed with a gas-tight syringe via a septum in the lid. A Shimadzu gas chromatograph (Model GC-14; Shimadzu Corp., Kyoto, Japan) and a Shimadzu chromatopac (Model C-R5A; Shimadzu Corp., Kyoto, Japan) were used to analyse the gas samples. The gas chromatograph was fitted with two columns, one packed with 80/100 mesh Molecular Sieve 5A (2.44 m), the second packed with 80/100 Porapac Q (1.83 m).

Surface colour changes were also monitored on each sampling day. A Minolta chroma meter (Model Cr-300; Minolta Canada Inc., Mississauga, ON) was used to record L*, a*, and b* measurements. Three measurements were made on each of the remaining roast beef pieces not used in microbiological analysis. Measurements were not conducted on pieces with visible evidence of microbial growth.

6.2.7. Sensory evaluation of roast beef exposed to volatile horseradish distillate at 4 °C

6.2.7.1. Sample preparation

Thawed roast beef slices were cut into four cm^2 pieces and placed on several sterile glass plates (12 roast beef pieces per plate). Each plate was inserted into a sterile glass 2L-Mason jar and 0, 80 or 400 µL of a 1:10 dilution of horseradish distillate to canola oil was added to the base of the jars. All jars were sealed with ceramic lined lids and stored at 4°C. Roast beef samples for the morning evaluation were prepared 1-1.5 h before the evaluation and panellists were presented with samples in 30 mL plastic sample containers with fitted lids. Preparation of the afternoon samples was also done at this time. Each 30 mL sample

container was modified by puncturing a hole (diameter = 6.4 mm) in the lids. Roast beef samples in the modified containers were returned to the 4°C cooler until one h before the afternoon evaluation. Roast beef samples for colour analysis were kept in transparent petri dishes (60 x 15 mm) and were removed from the cooler (4°C) approximately one h before each session.

6.2.7.2. Sensory analysis

Twelve judges, four men and eight women, all employees of PARC and ranging in age from 21-45 years, volunteered to participate in the study. On two planning sessions, preliminary descriptive terms and tentative reference standards were selected. The panel round-table discussion and orientation session was conducted the day before the evaluation. During this session the reference standards and definitions were provided and discussed (Table 5).

Table 5. Definitions of sensory attributes associated with roast beef exposed to varying concentrations of vaporized horseradish distillate.

Processed meat aroma	the magnitude of a typical cooked meat product aroma, representative of cold cuts and deli meats.
Horseradish aroma	the magnitude of a typical vegetal aroma, such as watercress or celery; the character associated with 0.0001 mg horseradish oil / L canola oil.
Horseradish irritation aroma	the magnitude of burning, tingling and pungent sensations experienced in the nose.
Processed meat flavour	the magnitude of cooked meat product flavour, representative of cold cuts and deli meats.
Horseradish flavour	the magnitude of vegetal taste, such as watercress or celery.
Horseradish irritation flavour	the magnitude of burning, tingling and pungent sensations experienced in the mouth.

Standards were prepared at 0.005, 0.001, 0.0005 and 0.0001 mg horseradish distillate / L canola oil. The general consensus was that the horseradish aroma was detectable at a concentration of 0.0001 mg/L, but pungency was not detectable until 0.001 mg/L. Some difficulty was expressed by panellists in distinguishing the horseradish aroma from the pungency aroma at the higher concentrations. Freshly thawed roast beef and roast beef exposed to 20 000 nL horseradish distillate / L air (24 h) were provided as examples of low horseradish/horseradish irritation flavour and high horseradish/horseradish irritation flavour, respectively. Roast beef stored under air (24 h) and roast beef exposed to 20 000 nL/L were provided as examples of low pink colour and high pink colour, respectively.

The attribute intensities for the roast beef samples were evaluated on an unstructured 10 cm line (Appendix 13). To prevent end-effects (O'Mahony, 1982) the low and high reference standards were anchored at 1.0 and 9.0 cm, respectively. When evaluating pink colour, the Munsell colour samples, 2.5 YR 5/4 and 7.5 YR 5/2 (Munsell Book of Colour, New Windsor, NY), were used as the low and high reference points, respectively. Physical references were not provided for aroma or flavour during the evaluation, but standards diluted in canola oil were available to the panellists prior to the evaluations so that they could familiarize themselves with low and high levels of horseradish/irritation aroma. In addition, L*, a* and b* measurements were taken immediately after each evaluation session using a Minolta chroma meter.

6.2.8. Analysis of volatile compound profiles in roast beef stored at 4 °C

Thawed roast beef slices were cut into four cm^2 pieces, placed on glass plates (six pieces/plate) and inserted into 2L-Mason jars as described in sections 6.2.4 and 6.2.6. The control jars were immediately sealed and 400 μL of diluted horseradish distillate (1:10 dilution made in canola oil) was added to the base of the test jars to yield 20 000 nL horseradish distillate / L air. All jars were incubated at 4°C.

Sampling was conducted on days 0, 3, 7 and 15. Three roast beef pieces were manually diced and placed into a sealed glass vessel equipped with a water jacket held at 30°C. The sample was purged (Model LSC 2000; Tekmar Company, Cincinnati, OH) for 30 min (flow rate of 100 mL He/min) and the trapped volatiles were analysed by GC-MS. The chromatograph (Model HP5890, Hewlett Packard, Richmond, BC) was fitted with a Supelcowax 10 capillary column (ID=0.25 mm, L=60 m) and was operated under the following conditions: initial oven temperature of 35°C (10 min), increasing oven temperature rate of 3°C/min (45 min), final oven temperature of 170°C (0 min), detector temperature of 280°C and a carrier gas (He) pressure of 30 psi. The mass selective detector (Model HP5970, Hewlett Packard, Richmond, BC) was adjusted to scan over 33-250 mass units with an EM voltage of 1800 V and a threshold of 500.

6.2.9. Statistical analysis

Microbial, chemical and sensory data were analysed using the general linear models procedure (PROC GLM) of SAS (SAS institute, Cary, NY) and significant differences were determined by least square means and/or Duncan's multiple range test ($P < 0.05$).

6.3. Results

6.3.1. Proximate analysis of roast beef

The moisture content of pre-cooked, uncured roast beef used in the antimicrobial, sensory and volatile profiling studies was 75.22 % ● 0.60 % (wwb). Ash content was 3.05% ± 0.01%, protein content was 17.00% ± 0.38% and fat content was 4.73%. Salt content was 2.36 % ± 0.05 % NaCl and phosphate content was 0.42 % ± 0.04 % P_2O_5. Equations used for all determinations are listed in Appendix 14.

6.3.2. Antimicrobial effectiveness of horseradish distillate at 12 °C

Figure 11 shows the inhibitory effect of vaporized horseradish distillate toward bacteria tested at the surface of pre-cooked, uncured roast beef, and the results of statistical analyses are shown in Appendices 15 and 16. *Listeria monocytogenes* was the most resistant of the four pathogenic bacteria tested. Growth was unaffected by 2 000 nL/L, while 4 000 nL/L resulted in growth inhibition until day four. By day seven cfu of *Listeria monocytogenes* under 4 000 nL/L were similar to those under air. The shape of the curve indicates that the distillate induced an extension of the lag phase. This effect was enhanced under 20 000 nL/L and an increase of 1.1 log cfu/cm^2 was observed after seven d. *Staphylococcus aureus* and *Escherichia coli* were more susceptible to the distillate and each test concentration significantly inhibited growth over seven d. Addition of 20 000 nL/L also resulted in reductions of 1.1 and 0.9 log cfu/cm^2 over seven d for *Staphylococcus aureus* and *Escherichia coli*, respectively.

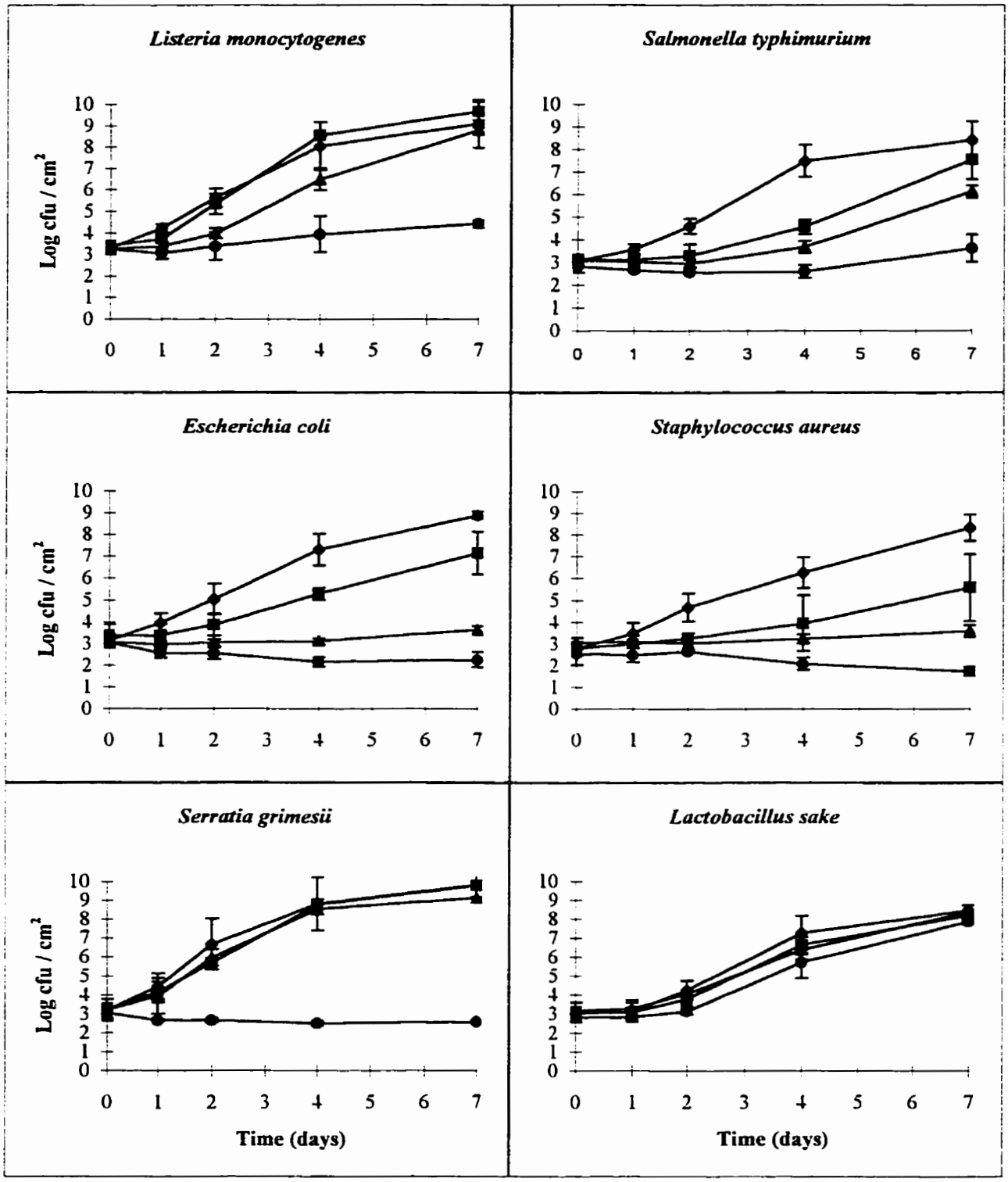

Figure 11. Effect of vaporized horseradish distillate on the growth of bacteria on pre-cooked, uncured roast beef at 12°C (◆ control, ■ 2 000 nL/L, ▲ 4 000 nL/L, ● 20 000 nL/L; shown are means of two replicates ± standard deviation; exceptions - three replicates with *E. coli* and *L. monocytogenes* @ 4 000 nL/L and four with *S. typhimurium* @ 4 000 nL/L).

Although *Salmonella typhimurium* was inhibited by all distillate test concentrations, the shapes of the curves for this bacterium were different than for the other microorganisms. Following an extension of the lag phase rapid growth occurred after two d under 2 000 and 4 000 nL/L, and after four d under 20 000 nL/L. *Salmonella typhimurium* was more tolerant of higher residual levels of AIT following two d or more exposure than were *Staphylococcus aureus* and *Escherichia coli*.

Serratia grimesii and *Lactobacillus sake* were more resistant to horseradish distillate than the pathogenic bacteria. *Serratia grimesii* was completely inhibited throughout the experimental period under a vapour concentration of 20 000 nL/L, however, no significant differences in growth were observed with 2 000 and 4 000 nL/L. *Lactobacillus sake* was the most resistant strain tested and an extension of the lag phase required 20 000 nL/L.

6.3.3. Analysis of residual AIT

Depletion of residual AIT in the roast beef model system is shown in Figure 12 and the results of statistical analyses are given in Appendix 17. Residual AIT for an initial input of 20 000 nL/L fell to 56.29% after 24 h at 12°C, while that of 4 000 nL/L and 2 000 nL/L were depleted to 20.28% and 6.88%, respectively. After seven d in storage these concentrations had dropped to 21.21%, 2.36% and 1.11%, respectively.

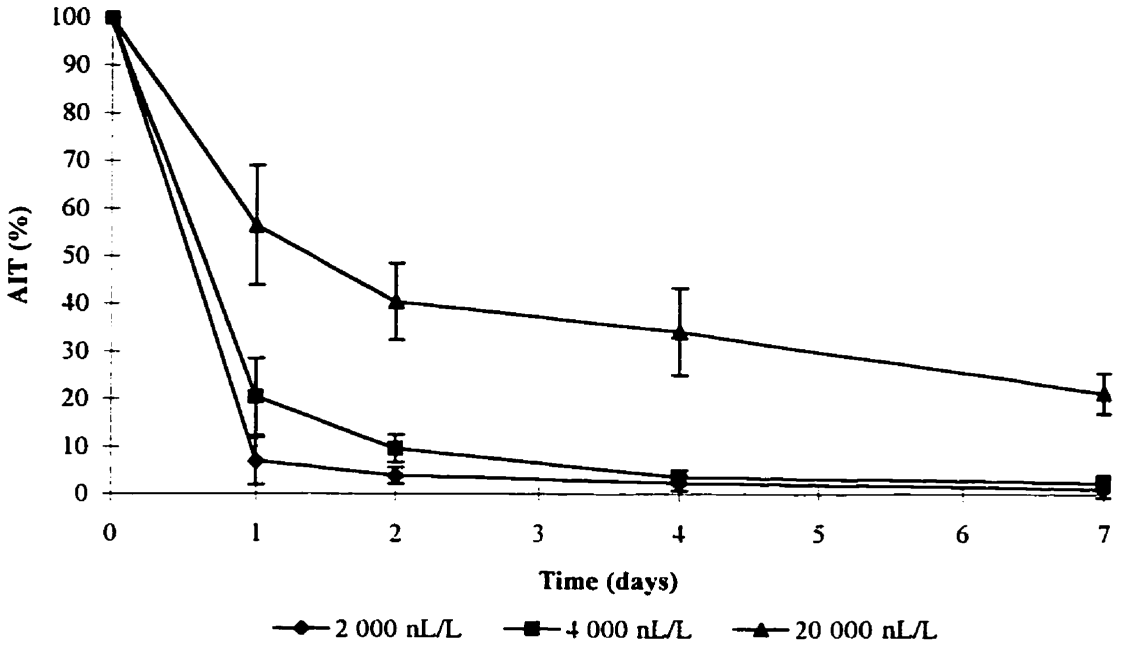

Figure 12. Depletion of residual AIT in a roast beef model system over seven d at 12°C (shown are means of six replicates ± standard deviation).

6.3.4. Shelf-life of pre-cooked, uncured roast beef exposed to volatile horseradish distillate at 4 °C

Figures 13 - 16 show bacterial numbers on contaminated roast beef stored under different atmospheric concentrations of horseradish distillate (0 nL/L-air (control), 0 nL/L-nitrogen (control-nitrogen), 20 000 nL/L-air, 20 000 nL/L-nitrogen). All replicates are shown to illustrate the variability observed in these studies. Differences between replicates made statistical comparisons between treatments impossible. Nevertheless, bacterial numbers under control and control-nitrogen test conditions appeared similar, as were those under 20 000 nL/L and 20 000 nL/L-nitrogen. Therefore, statistical comparisons between controls and test conditions could be performed if nitrogen was omitted as a factor (Appendix 18).

Total aerobic counts were significantly affected by horseradish distillate and growth suppression was clearly visible with replicates A and C (Figure 13). However, inhibition in replicate B was less dramatic. Pseudomonads were the most susceptible group of bacteria and were completely inhibited throughout the experimental period. In addition, bacteriostatic/bactericidal activity was observed with replicates A and C (Figure 14). Enterics were also suppressed by 20 000 nL/L (Figure 15). Again, results in replicate B slightly differed from replicates A and C with inhibition delayed until later on in storage. Lactic acid bacteria were only slightly inhibited by 20 000 nL/L in two of the replicates while in at least one (replicate B) there was evidence of unusual resistance to the distillate (Figure 16).

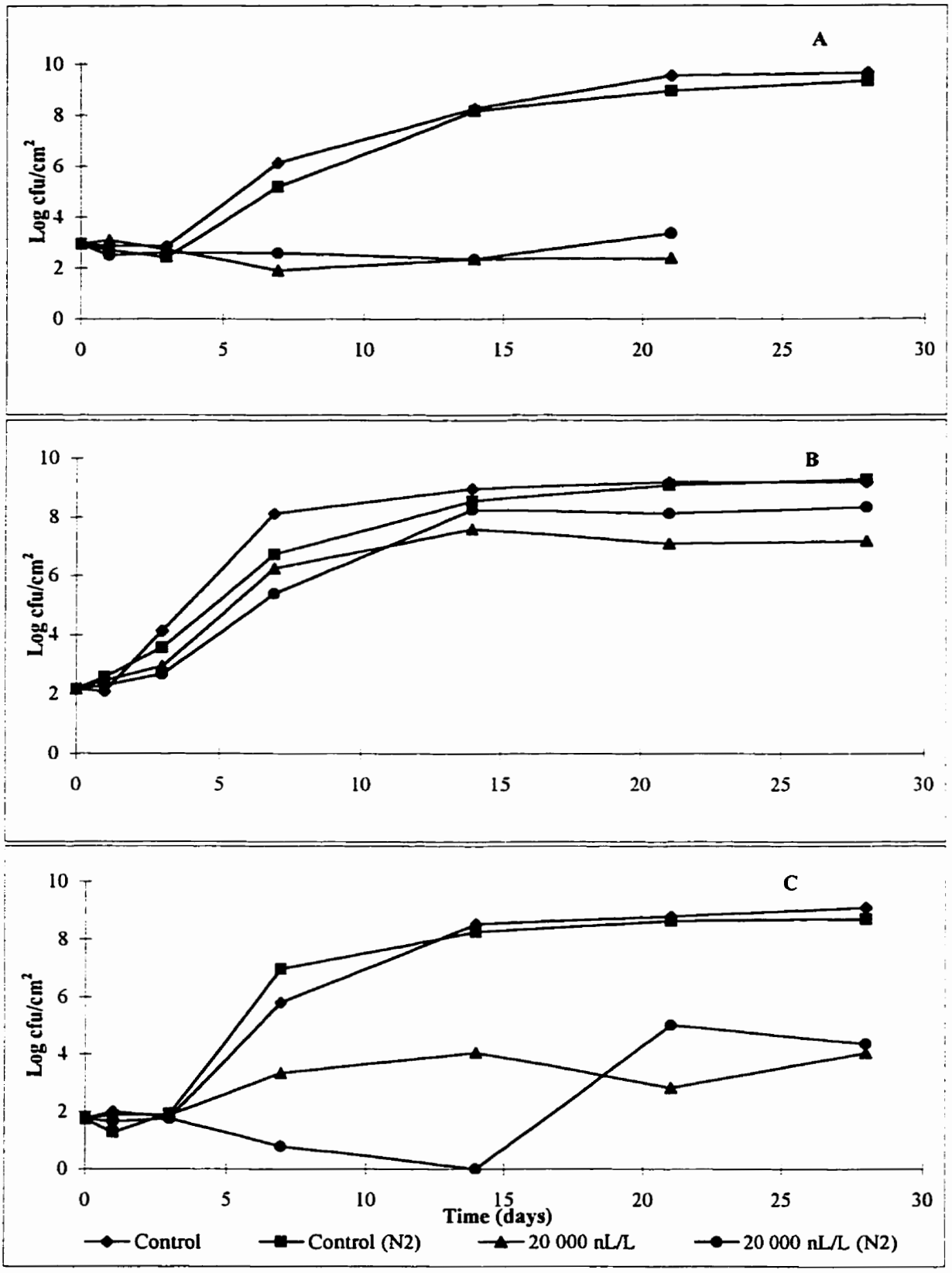

Figure 13. Effect of vaporized horseradish distillate on total aerobic counts on roast beef (PCA media - 48 h at 30°C; A - replicate 1, B - replicate 2, C - replicate 3).

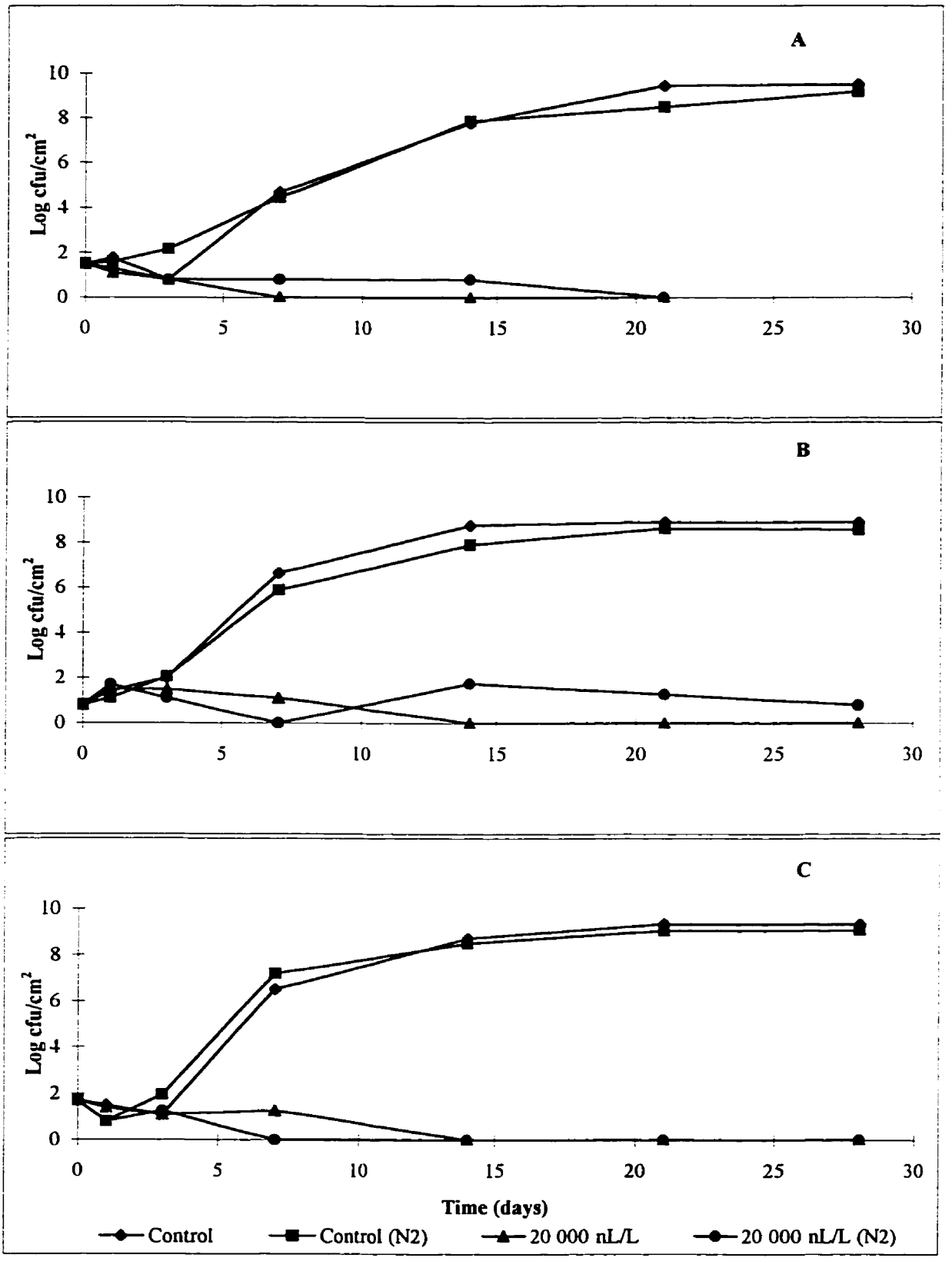

Figure 14. Effect of vaporized horseradish distillate on pseudomonads on roast beef (PCFC media - 48 h at 30°C; A - replicate 1, B - replicate 2, C - replicate 3).

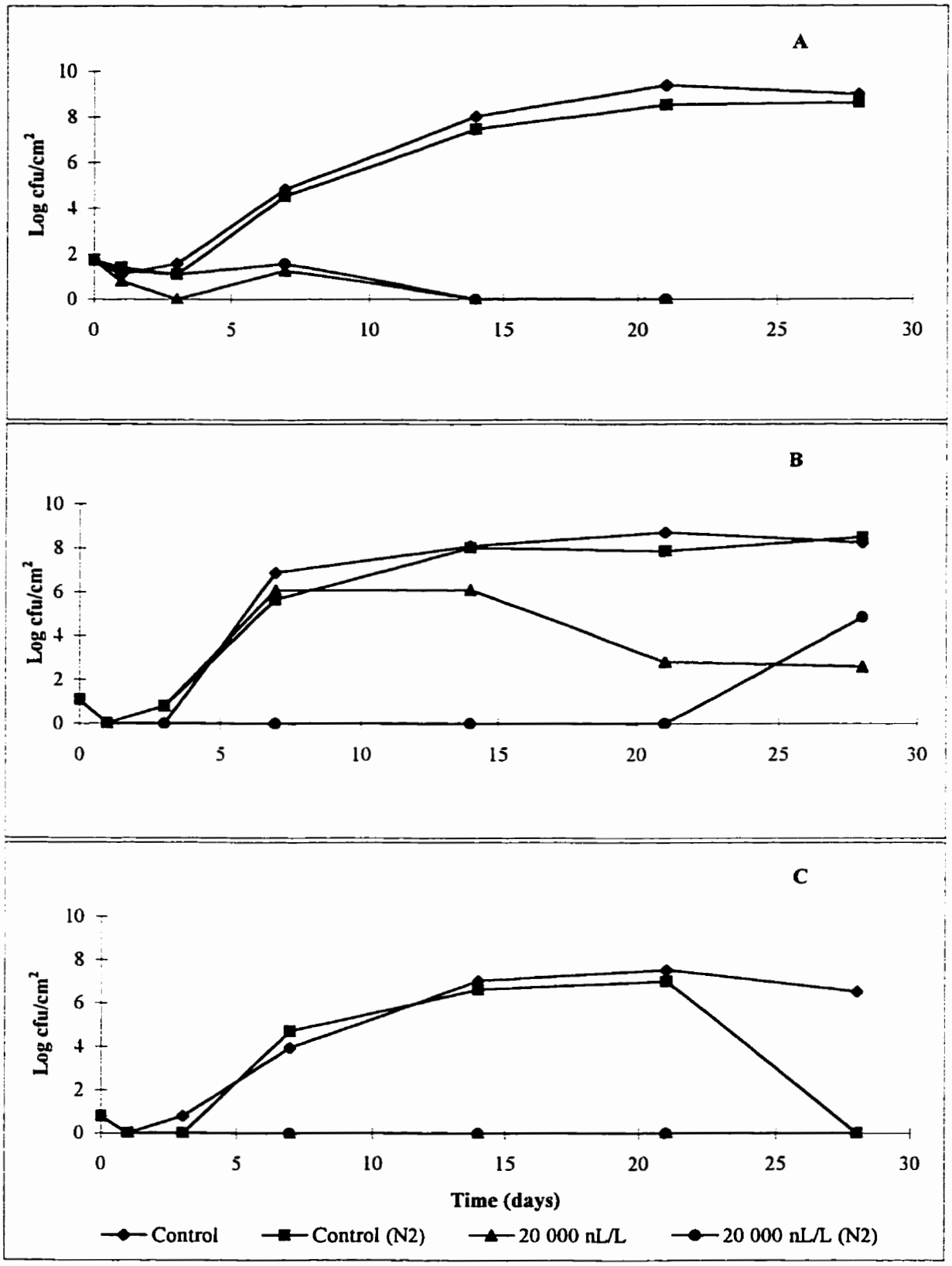

Figure 15. Effect of vaporized horseradish distillate on enteric bacteria on roast beef (VRBG media - 24 h at 35°C; A - replicate 1, B - replicate 2, C - replicate 3).

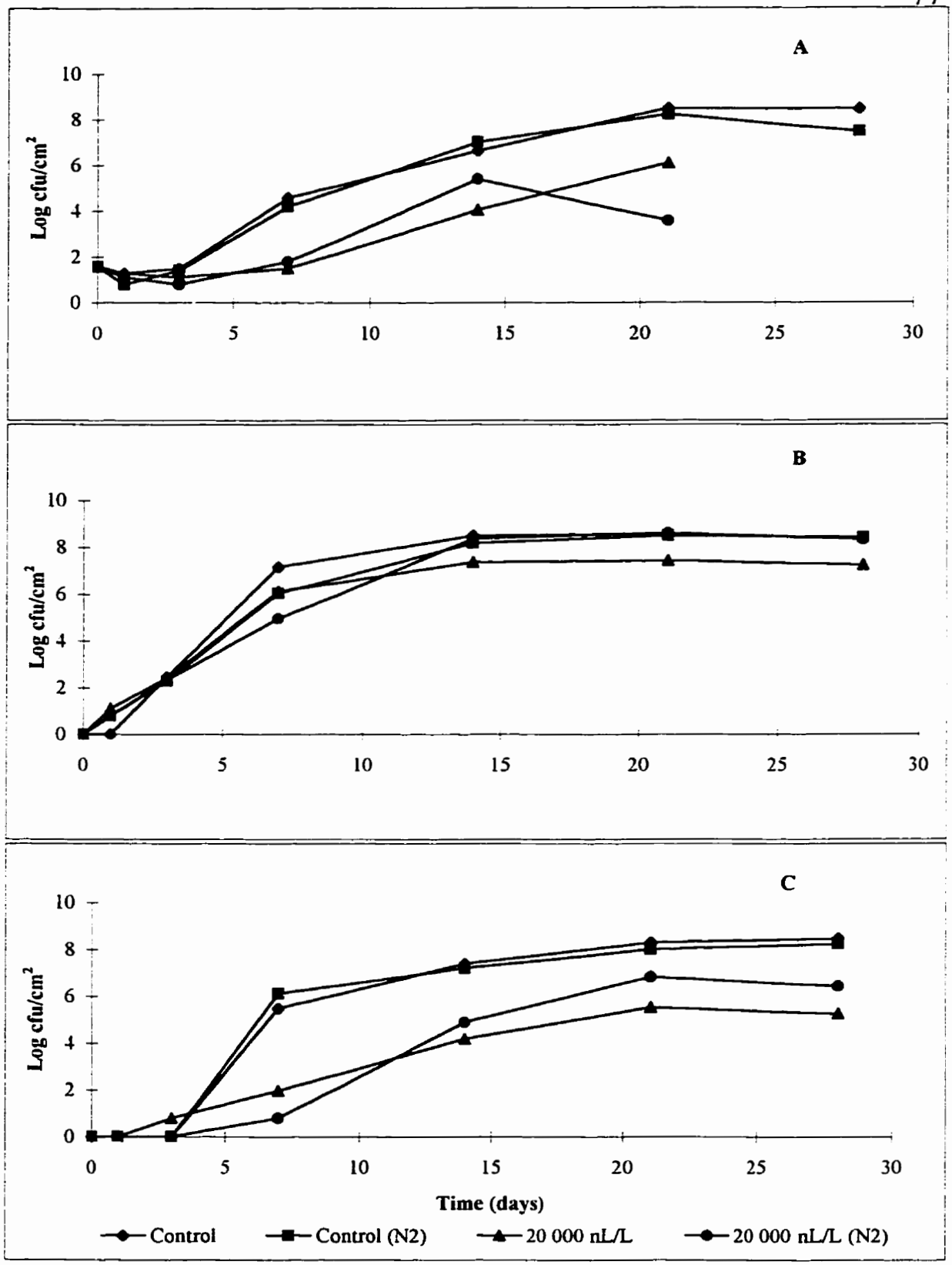

Figure 16. Effect of vaporized horseradish distillate on lactic acid bacteria on roast beef (MRS media - 72 h at 25°C; A - replicate 1, B - replicate 2, C - replicate 3).

The gas composition in the nitrogen-flushed jars is presented in Table 6. Significant differences in the amounts of nitrogen, oxygen and carbon dioxide were found between control and experimental treatments (Appendix 19). Differences between replicates for nitrogen and oxygen were also significant. Over the length of storage at 4°C, nitrogen levels increased until day 14 and then decreased in the presence of vaporized horseradish distillate. Oxygen levels increased until day 7 then decreased in the presence of air, however in the presence of vaporized horseradish distillate oxygen levels after day 3 increased over time. Carbon dioxide levels were not detectable until day 14 and did not differ significantly over time within each treatment.

Changes in surface colour were also monitored by measurement of L*, a* and b* values as shown in Table 7. L* (measure of luminance) and b* (measure of yellow/blue colour) values did not significantly change over time, nor did exposure to horseradish distillate and/or nitrogen gas affect these values (Appendix 20). Significant differences in a* values (measure of green/red colour) were observed in roast beef samples stored under horseradish distillate (regardless of presence/absence of nitrogen gas) over time. The observed increases in a* values for samples stored under horseradish distillate indicates increases in the amount of red colour. These colour differences are illustrated in Figure 17.

Table 6. Percent nitrogen, oxygen and carbon dioxide present in the head space of nitrogen flushed mason jars containing pre-cooked, uncured roast beef pieces with and without vaporized horseradish distillate at 4°C[1].

Time (days)	Control (%N_2)	20 000 nL/L (%N_2)
1	96.23 ± 1.29	92.08 ± 1.47
3	94.13 ± 3.19	93.77 ± 3.24
7	93.34 ±3.50	93.51 ± 2.77
14	92.28 ± 3.05	93.70 ± 3.64
21	93.06 ± 3.64	91.16 ± 3.61
28	93.44 ± 3.53	88.41 ± 0.06
	Control (%O_2)	20 000 nL/L (%O_2)
1	3.77 ± 1.29	7.92 ± 1.47
3	5.87 ± 3.19	6.23 ± 3.24
7	6.65 ± 3.50	6.49 ± 2.77
14	6.11 ± 3.11	6.11 ± 3.35
21	3.43 ± 2.91	8.60 ± 3.33
28	1.68 ± 1.52	11.13 ± 0.26
	Control (%CO_2)	20 000 nL/L (%CO_2)
14	1.61 ± 1.69	0.19 ± 0.33
21	3.51 ± 1.63	0.25 ± 0.37
28	4.69 ± 1.46	0.31 ± 0.35

[1]mean values of three replicates ● standard deviation.

Table 7. L*, a* and b* values for pre-cooked, uncured roast beef samples stored at 4°C under varying concentrations of vaporized horseradish distillate and with or without the addition of nitrogen gas.

Time (days)	Control	Nitrogen Control	20 000 nL/L	Nitrogen 20 000 nL/L
	L* values			
0	49.44 ± 2.65	49.45 ± 2.65	49.45 ± 2.65	49.45 ± 2.65
1	48.92 ± 0.41	48.01 ● 0.18	49.86 ± 1.33	47.70 ● 0.97
3	48.08 ± 1.90	49.31 ± 0.86	50.02 ± 1.05	48.28 ± 1.48
7	49.23 ± 0.92	48.03 ± 1.49	50.36 ● 0.78	49.37 ± 1.43
14	ND[2]	ND	50.97 ± 1.41	49.52 ± 1.08
21	ND	ND	51.54 ● 1.16	48.40 ± 1.63
	a* values			
0	8.32 ± 0.40	8.32 ± 0.40	8.32 ± 0.40	8.32 ± 0.40
1	8.38 ± 0.29	8.56 ± 0.28	10.61 ± 0.27	10.31 ± 0.29
3	8.85 ± 0.26	8.64 ± 0.16	11.01 ± 0.45	10.95 ± 0.18
7	8.80 ± 0.24	8.59 ± 0.35	11.13 ± 0.13	11.00 ± 0.30
14	ND	ND	10.67 ± 0.55	10.77 ± 1.07
21	ND	ND	10.58 ± 0.02	10.62 ± 0.18
	b* values			
0	7.12 ± 0.30	7.12 ± 0.30	7.12 ± 0.30	7.12 ± 0.30
1	7.25 ± 1.14	7.54 ± 0.26	7.50 ± 0.26	6.84 ± 0.92
3	7.74 ± 1.24	8.16 ± 0.32	7.69 ± 0.41	7.37 ± 0.53
7	7.38 ± 0.15	6.79 ± 0.82	7.79 ± 0.60	7.06 ± 0.28
14	ND	ND	8.14 ± 0.05	7.62 ± 0.29
21	ND	ND	8.82 ± 0.02	7.77 ± 0.33

[1]mean values of three replicates ± standard deviation.
[2]ND-not determined

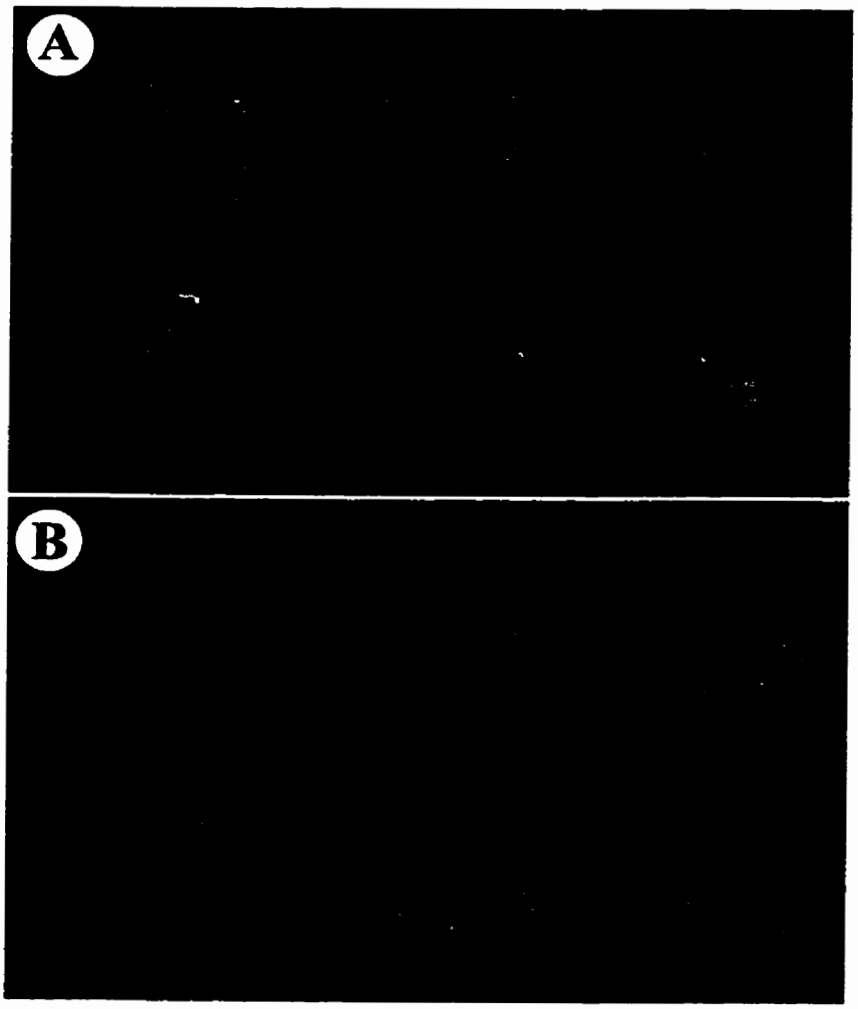

Figure 17. Roast beef pieces exposed to 0 (A) and 20 000 nL horseradish distillate / L air (B) for 72 hours at 4°C.

6.3.5. Sensory analysis

The sensory evaluation revealed significant aroma, flavour and colour differences in pre-cooked, uncured roast beef exposed to volatile horseradish distillate (Table 8; Appendix 21). Since significant differences were not observed between the morning and afternoon evaluations, means were averaged across sessions and plotted on cobweb diagrams as shown in Figure 18.

Table 8. Mean ratings[1] and standard deviations for sensory attributes of roast beef pieces exposed to varying concentrations of vaporized horseradish distillate for two storage periods (storage temperature of 4°C).

	3 days storage			15 days storage		
	Control	4 000 nL/L	20 000 nL/L	Control	4 000 nL/L	20 000 nL/L
Beef aroma	6.8 ± 1.5^{a}	4.9 ± 1.8^{b}	3.0 ± 1.3^{c}	6.7 ± 2.9^{a}	5.7 ± 2.2^{b}	3.4 ± 1.6^{c}
Horseradish aroma	1.5 ± 1.4^{a}	3.7 ± 2.0^{c}	6.5 ± 1.4^{e}	1.2 ± 1.1^{a}	2.6 ± 2.2^{b}	5.8 ± 1.9^{d}
Horseradish irritation aroma	1.1 ± 0.9^{a}	2.4 ± 1.5^{b}	5.2 ± 2.1^{d}	0.9 ± 0.8^{a}	1.8 ± 1.5^{b}	4.4 ± 2.0^{c}
Beef flavour	7.3 ± 1.5^{e}	5.3 ± 1.4^{c}	2.8 ± 1.2^{a}	N/A[2]	6.4 ± 2.1^{b}	3.9 ± 1.9^{d}
Horseradish flavour	1.6 ± 1.6^{a}	3.3 ± 2.3^{b}	7.3 ± 1.5^{d}	N/A	2.7 ± 2.4^{b}	5.8 ± 2.5^{c}
Horseradish irritation flavour	1.1 ± 0.9^{a}	2.3 ± 1.7^{b}	7.3 ± 1.4^{d}	N/A	1.8 ± 1.5^{ab}	5.1 ± 2.5^{c}
Pink colour	3.1 ± 1.1^{b}	5.3 ± 1.7^{c}	7.3 ± 1.4^{d}	2.1 ± 1.2^{a}	5.0 ± 1.5^{c}	5.5 ± 1.6^{c}

[1] average of 48 values (12 panellists x 2 sessions (am/pm) x 2 replicates); except on day 15, 4 000 nL/L (mean of 33 values).

[a-d] values within the same row with the same superscript are not significantly different ($P > 0.05$).

[2] N/A - attribute not assessed.

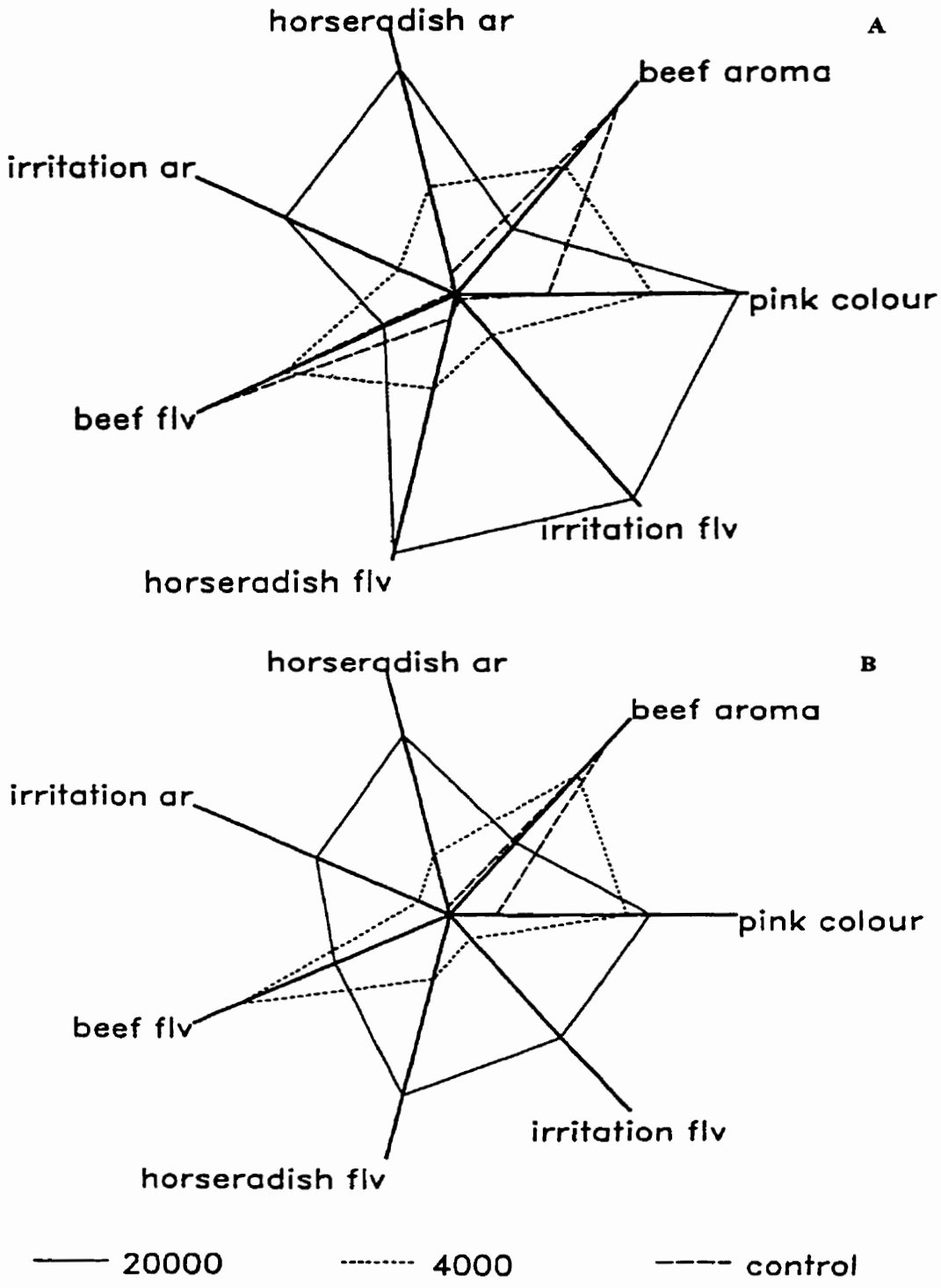

Figure 18. Cobweb diagrams illustrating intensities of roast beef attributes with respect to level of vaporized horseradish distillate (nL/L) and length of exposure at 4°C (A - 3 days; B - 15 days) (Flavours not assessed for control - day 15).

Beef aroma scores were highest when roast beef samples were stored in an atmosphere devoid of horseradish distillate, and scores decreased with increasing distillate concentrations, regardless of storage length. Conversely, scores increased for horseradish aroma with increased distillate concentration. Significant differences in this attribute were also observed between the two storage periods. Roast beef stored under 4 000 and 20 000 nL/L for three d scored higher than roast beef exposed to equivalent distillate concentrations for 15 d. A similar trend was observed with horseradish irritation aroma.

Beef flavour decreased with increased horseradish distillate concentrations. In addition, roast beef stored for 15 d at 4°C had more beef flavour than roast beef stored for three d. Attributes for both horseradish flavour and horseradish irritation flavour increased with increased horseradish distillate concentrations. For each of these attributes, roast beef exposed to 20 000 nL/L and stored for three d scored significantly higher than roast beef stored at an equivalent horseradish distillate concentration for 15 d.

Pink colour was the only colour attribute assessed by the sensory panel. The typical colour of pre-cooked, uncured roast beef degraded rapidly in control samples. In all cases these samples were scored lower than those stored under horseradish distillate (Table 8). After three d of storage, roast beef stored under 20 000 nL/L scored significantly higher in pink colour than roast beef stored under 20 000 nL/L for 15 d.

Figure 19 is a graphical representation of results obtained by Principle Component Analysis. Principle component 1 (factor 1) explained 94.9% of the variability in the data, while factor 2 explained an additional 2.8% resulting in a total of 97.7% variability described.

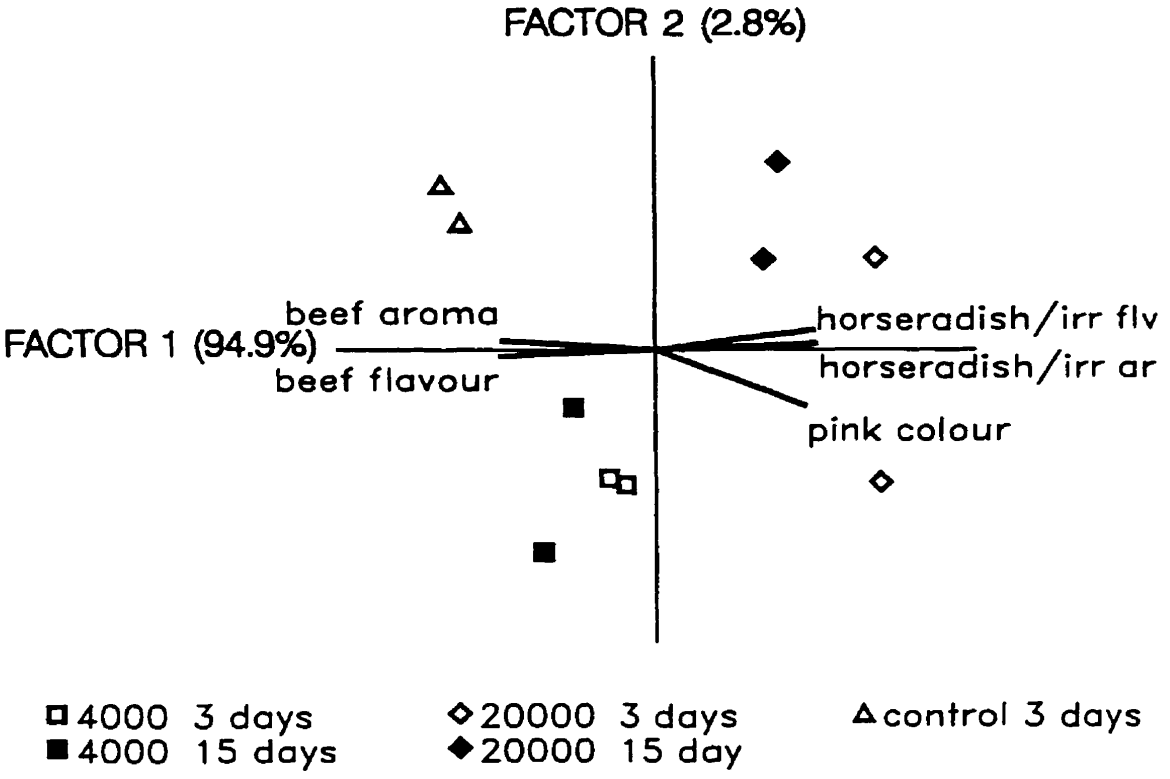

Figure 19. Principle Component Analysis diagram illustrating differences in roast beef attributes with respect to level of vaporized horseradish distillate (nL/L) and length of exposure at 4°C.

The 180° orientation of these two vectors suggested that beef flavour and aroma are inversely related to horseradish and horseradish irritation flavours and aromas, as shown by the correlation matrix (Table 9). Differences in horseradish distillate treatments are clearly separated. The control samples were located to the far left of the figure characterized by the presence of beef aroma/flavours and the absence of pink colour. In contrast, the roast beef samples exposed to 20 000 nL/L were located to the far right. They could be characterized by the presence of horseradish aroma/flavours and pink colour. Those exposed to intermediate concentrations were more centrally located, representing intermediate intensities of beef and horseradish. Also evident is the relative location of the three and 15 day samples. At both test concentrations the three day samples had more horseradish character and less beef character than the 15 day samples. This suggests that the horseradish compounds dissipate from the sample. It could be further speculated that pink colour is an indicator of the intensity of horseradish aroma/flavours. Although colour and aroma/flavour are not normal related, they appear concomitantly together in this study.

Table 9. Correlation matrix among the descriptive attributes (storage temperature of 4°C; df = 3)

Attribute	1	2	3	4	5	6	7
1. Pink colour	1.00						
2. Beef aroma	-0.91*	1.00					
3. Horseradish aroma	0.91*	-1.00***	1.00				
4. Horseradish irritation aroma	0.89*	-0.99***	0.99***	1.00			
5. Beef flavour	-0.92*	0.99**	-0.99***	-0.99**	1.00		
6. Horseradish flavour	0.90*	-0.98**	0.99**	1.00***	-0.99**	1.00	
7. Horseradish irritation flavour	0.87	-0.94*	0.96**	0.99**	-0.97**	0.99***	1.00

*, **, ***, significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Colour differences at the surface of roast beef exposed to three levels of horseradish distillate (0, 4 000 and 20 000 nL/L) and stored at 4°C for three d and 15 d were also determined using a Minolta chroma meter (Table 10; Appendix 22). L* values were not significantly different for the six treatments, although differences were observed with the a* values. Roast beef under 20 000 nL/L and stored at 4°C for three d had the highest a* value of six treatments. Control roast beef stored for 15 d at 4°C had the lowest a* value. The only significant difference observed with the b* values was between control roast beef stored for 15 d and roast beef under 20 000 nL/L stored for 3 d.

L*, a* and b* values were again monitored after the same roast beef samples were exposed to air for approximately 1.75 h (length of sensory evaluation) (Table 11; Appendix 23). In parallel with results obtained for fresh samples no significant differences were observed in L* values and a* values were higher for roast beef stored under horseradish distillate. Slight differences in b* values between roast beef exposed to 4 000 nL/L for 15 d and roast beef exposed to 20 000 nL/L for three d were also observed.

Table 10. Initial L*, a* and b* values[1] for pre-cooked, uncured roast beef samples stored at 4°C under varying concentrations of vaporized horseradish distillate for two time periods.

	Control	4 000 nL/L	20 000 nL/L	Control	4 000 nL/L	20 000 nL/L
	3 days storage			15 days storage		
L*	49.6 ± 0.9[a]	49.6 ± 0.7[a]	49.8 ± 1.3[a]	50.1 ± 1.3[a]	50.4 ± 0.9[a]	50.6 ± 1.0[a]
a*	8.6 ± 0.2[a]	9.5 ± 0.3[b]	10.7 ± 0.6[c]	7.7 ± 0.5[d]	9.2 ± 0.4[ab]	9.2 ± 0.2[ab]
b*	7.8 ± 0.4[ab]	7.7 ± 0.4[ab]	7.2 ± 0.5[a]	7.9 ± 0.5[b]	7.7 ± 0.5[ab]	7.5 ± 0.3[ab]

[1] mean of four values (2 sessions (am/pm) x 2 replicates) ● standard deviation; values obtained 15 min after removal from refrigerated storage with chroma meter.
[a-d] means within same row with different superscripts are significantly different ($P < 0.05$).

Table 11. L*, a* and b* values[1] for pre-cooked, uncured roast beef samples exposed to air at an ambient temperature for 1.75 h after being stored at 4°C under varying concentrations of vaporized horseradish distillate for two time periods.

	Control	4 000 nL/L	20 000 nL/L	Control	4 000 nL/L	20 000 nL/L
	3 days storage			15 days storage		
L*	49.5 ± 0.9[a]	49.4 ± 1.0[a]	49.8 ± 1.3[a]	50.1 ± 1.1[a]	50.6 ± 1.1[a]	50.6 ± 0.8[a]
a*	8.1 ± 0.3[b]	9.1 ± 0.3[c]	9.7 ± 0.4[d]	7.0 ± 0.6[a]	8.6 ± 0.2[bc]	8.6 ± 0.2[bc]
b*	7.4 ± 0.4[ab]	7.5 ± 0.5[ab]	7.0 ± 0.5[a]	7.8 ● 0.2[b]	7.6 ± 0.3[b]	7.3 ● 0.2[ab]

[1] mean of four values (2 sessions (am/pm) x 2 replicates) ● standard deviation; values obtained 1.75 h after removal from refrigerated storage with chroma meter.
[a-d] means within same row with different superscripts are significantly different ($P < 0.05$).

6.3.6. Volatile profiles of pre-cooked, uncured roast beef at 4 °C

Gas chromatograms of volatile compounds from roast beef samples stored under air or horseradish distillate for 0, 3, 7 and 15 d at 4°C are shown in Figures 20 and 21. The compounds and retention times corresponding to the labelled peaks on the chromatograms are listed in Tables 12 and 13.

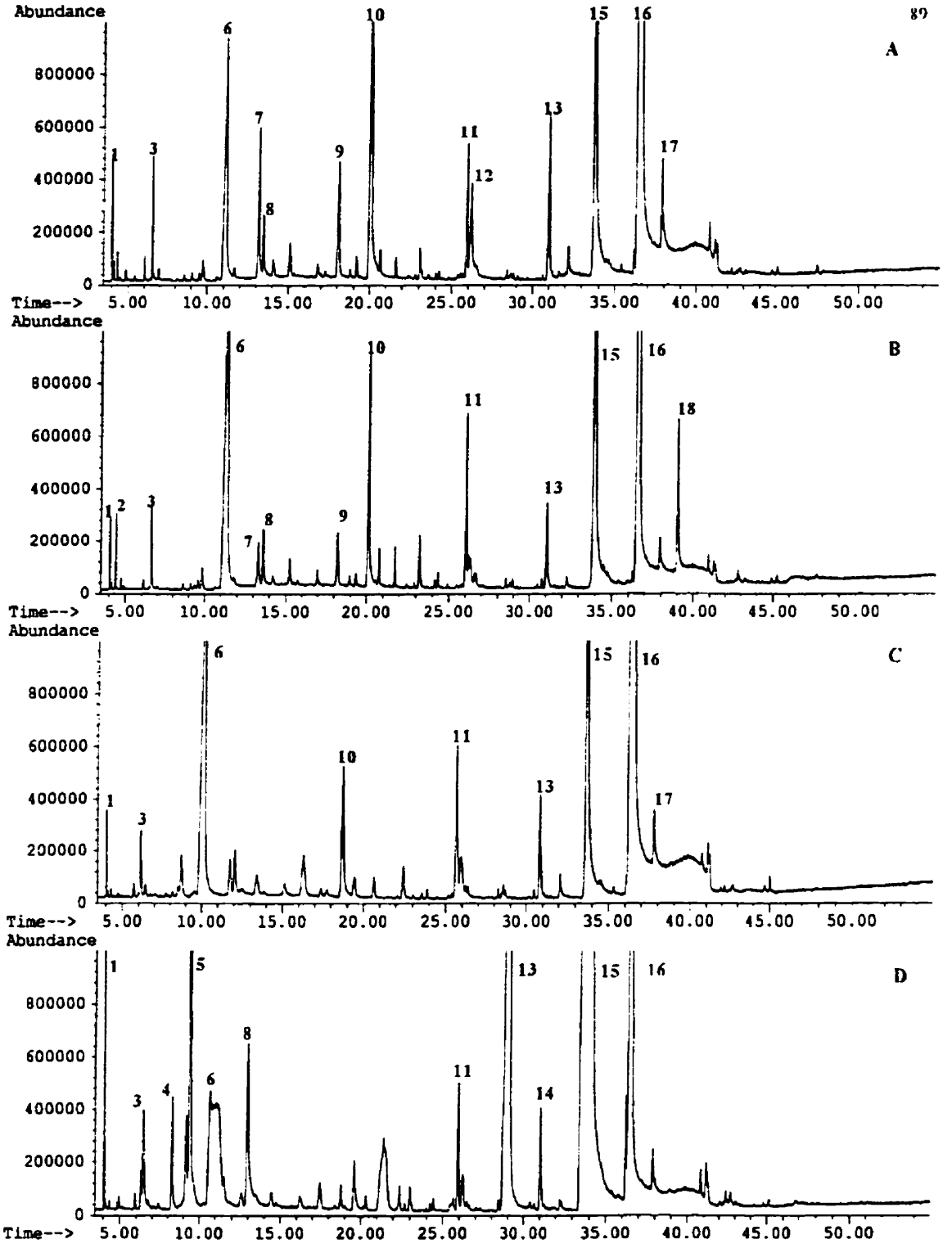

Figure 20. Gas chromatograms of volatile compounds in the head space above pre-cooked, uncured roast beef stored at 4°C without horseradish distillate (A - 0 days; B - 3 days; C - 7 days; D - 15 days; compounds corresponding to numbered peaks are listed in Table 12).

Table 12. Compounds corresponding to labelled peaks in chromatograms from control samples of pre-cooked, uncured roast beef.

Peak number	Retention time (min)	Compound
1	4.0	carbon dioxide
2	4.4	hexane
3	6.4	2-propanone
4	8.3	acetic acid
5	9.4	3-methyl butanal
6	10.9	ethanol
7	13.2	pentanal
8	13.4	2,3-butanedione
9	18.2	camphene
10	19.6	hexanal
11	26.0	di-limonene
12	26.1	heptanal
13	31.0	1-pentanol
14	29.1	3-methyl-1-butanol
15	33.9	3-hydroxy butanone
16	36.5	allyl isothiocyanate
17	37.8	nonanal
18	39.0	2-butoxy ethanol

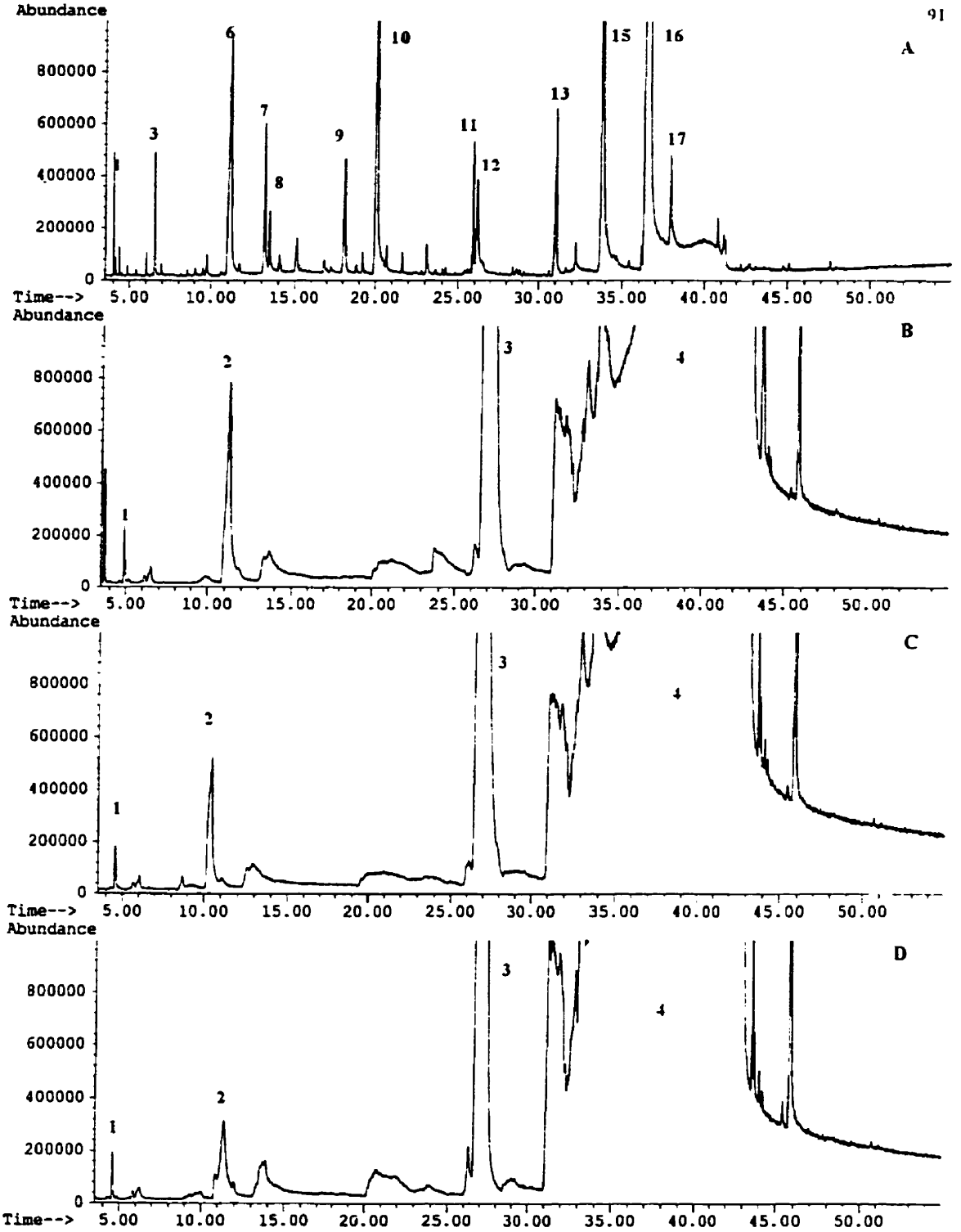

Figure 21. Gas chromatograms of volatile compounds above the head space of pre-cooked, uncured roast beef exposed to 20 000 nL horseradish distillate/L air and stored at 4 °C (A - 0 days; B - 3 days; C - 7 days; D - 15 days; compounds corresponding to numbered peaks are listed in Table 13 with the exception of A (0 days of storage) which are listed in Table 12).

Table 13. Compounds corresponding to labelled peaks in chromatograms from pre-cooked, uncured roast beef exposed to 20 000 nL horseradish distillate / L air.

Peak number	Retention time (min)	Compound
1	4.6	carbon disulphide
2	10.9	ethanol
3	26.9	3-butene nitrile
4	39.8	allyl isothiocyanate

The head space above roast beef stored in air contained a complex mixture of volatile compounds that included several alcohols, aldehydes and ketones. An AIT peak (RT=36.5) was also observed in control profiles indicating that a residue was retained either on the column, in the trap or on the glassware used for purging the sample. Since the column and trap were routinely flushed with helium carrier gas at high temperatures, it is more likely that the residue was retained on the glassware and linings of the screwcap lids. Volatile profiles of roast beef exposed to horseradish distillate vapours were considerably less complex. Compounds such as pentanal, nonanal, hexanal, 2-propanone and 2,3-butanedione could not be detected. The broad AIT peak blocked or overlapped compounds with higher retention times, including 3-hydroxybutanone (RT=33.7), 4-isothiocyanatobutene (RT=43.0) and di-2 propenyl disulphide (RT=43.7). A compound with a molecular weight of 99 was also observed (RT=45.8) and was tentatively identified as allyl thiocyanate. In addition, 3-butene nitrile and carbon disulphide were present in the volatile profiles of roast beef exposed to gaseous horseradish distillate.

6.4. Discussion

Differences between literature values for moisture, ash, protein, fat, salt and phosphate contents of pre-cooked, uncured roast beef and those found in this study were minimal. Glass and Doyle (1989) reported roast beef moisture, protein and fat contents of 64.2%-68.7%, 21.8%-25.1% and 4.2%-11.8%, respectively. Carter *et al.* (1992) reported moisture, ash, protein and fat contents of roast beef as 70.53%, 2.78%, 20.56% and 6.17%, respectively. Salt contents, as percent sodium chloride, are reported to range from 0.5%-2.05% (Glass and Doyle, 1989; Carter *et al.*, 1992; Miller and Acuff, 1994). Literature values for phosphate content are also comparable to those found in this study (0.30-0.38%; Carter *et al.*, 1992; Miller and Acuff, 1994) implying that the roast beef used in the antimicrobial, sensory and volatile profiling studies was representative of a standard commercial product.

The inhibitory effects of vaporized horseradish distillate on pre-cooked, uncured roast beef were lower than expected. Bacterial growth (with the exception of *Lactobacillus sake*) on bacteriological media at 12°C was inhibited by 2 000 nL/L after seven d of storage, while 4 000 to 20 000 nL/L were required for inhibition of all microorganisms on pre-cooked, uncured roast beef at the same temperature. Lower concentrations of residual AIT in the roast beef model system may partially account for the decrease in antimicrobial activity over the storage period. Decreases in antimicrobial activity have been previously observed when plant extracts tested in bacteriological media were applied to foods (Shelef *et al.*, 1984; Ismaiel and Pierson, 1990). Shelef *et al.* (1984) reported that microbial resistance to volatile components in sage oil increased in products with low water contents and high proportions of protein and fat. Furthermore, they hypothesized that the lipophilic antimicrobial

compounds may lose activity by becoming solubilized in the lipid fraction of the food product. This protective effective by fat was also suggested by Ismaiel and Peirson (1990). While the roast beef used in this study had some fat marbling, randomization of the samples minimized effects of small differences in sample marbling. The high protein content of roast beef (17.00%) should also be considered a factor since isothiocyanate interactions with proteins may have depleted the AIT available for bacterial inhibition. Furthermore, Hao *et al.* (1998) suggested that the porous surface of cooked beef may reduce bacterial contact with antimicrobial agents by providing a refuge for the microorganisms. The poor solubility of AIT in aqueous media may prevent diffusion into deep crevices.

In addition to an overall decrease in antibacterial activity in the roast beef model system, the resistance of each bacterium towards horseradish distillate differed from observations made in the agar model system. *Listeria monocytogenes*, the most sensitive pathogenic bacterium in the agar model system, exhibited greater resistance towards the inhibitory effects of vaporized horseradish distillate on pre-cooked, uncured roast beef than *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium*. Resistance of *Listeria monocytogenes* towards other plant extracts on cooked beef has been previously reported (Hao *et al.*, 1998).

Since bacteriostatic and bactericidal effects were observed against test microorganisms individually inoculated on to roast beef, the effectiveness of vaporized horseradish distillate against a mixed microflora associated with post-process contaminated roast beef was also assessed. Although the distillate was generally effective in suppressing the growth of contaminants, some differences were observed between replicate experiments. These can be

attributed in part to the lack of control over inoculum types and levels. Statistical comparison of data from the replicate experiments was not possible due to the variation between replicates. However, when nitrogen was omitted as a factor, each individual replicate could be analyzed. Although total aerobic counts were lower in samples stored under vaporized horseradish distillate, the higher counts observed with replicate B suggest that part of the contaminating microflora was resistant to the antimicrobial effect. Enterics appear to contribute to the higher numbers during the first 14 d, while lactic acid bacteria may make up the majority of the microflora at later stages. In addition, enteric counts increased on day 28 in replicate B (nitrogen flushed system with 20 000 nL/L). The enteric bacterium, *Hafnia alvei*, has been associated with roast beef stored for extended periods under modified atmospheric conditions (Anderson *et al.*, 1989). It is plausible that this or related species were initially inhibited by vaporized horseradish distillate but resumed growth later, perhaps as a result of AIT depletion. Resistance to vaporized horseradish distillate was also observed with lactic acid bacteria. Although extension of the lag phase occurred with each replicate, greater resistance was observed in replicate B. Differing levels of resistance towards AIT for lactic acid bacteria have been previously reported (Kyung and Fleming, 1997), suggesting that the type(s) of lactic acid bacteria in replicate B differ from those in replicates A and C.

Replacement of the head space gases with nitrogen did not significantly contribute to growth suppression of any of the bacterial groups examined. The residual oxygen in the nitrogen flushed jars supported the growth of Pseudomonas spp. to high densities. Differences in the concentrations of individual gases in the head space of control and test samples may be attributed to dissimilar respiration patterns, perhaps induced by alterations

to the microflora by the distillate and/or leakage. Since incorporation of the distillate generally reduced the total microbial load on roast beef, less carbon dioxide from microbial respiration was released into the jars. As a result, higher concentrations of oxygen were detected throughout the storage period, likely from slow leakage through the lids. Conversely, oxygen levels declined in control samples due to respiration by the higher microbial biomass.

Exposure to vaporized horseradish distillate also affected the surface colour of roast beef as determined by physical measurements. Samples stored with distillate maintained higher red colour as indicated by significantly higher a* values. This result was confirmed by sensory evaluation (section 6.3.5). The preservation of red colour could be related to the antioxidant properties of horseradish extracts, which have been previously documented by Yamaguchi *et al.*(1984). Generally, the 2-thiobarbituric acid test (TBA) is used to determine lipid oxidation in meats and meat products (Koneicko, 1985b). Preliminary TBA analyses were conducted in this study (data not shown). Chemical changes to a TBA-pigment complex could not be detected however, likely due to insufficient concentrations of malonaldehyde in the meat samples. Nevertheless, it appears that the application of horseradish distillate to pre-cooked, uncured roast beef prevents colour changes generally associated with oxidative reactions involving myoglobin. Furthermore, Dymicky *et al.* (1975) reported that denatured myoglobin can react with nitrogen containing substances to produce pink hemochromes. It is plausible that AIT (a nitrogen containing substance) is able to chelate with iron in the heme portion of the protein and produce a pink colour that remains stable upon storage.

Results from the sensory analysis of roast beef slices exposed to volatile horseradish distillate contradict statements by Isshiki *et al.* (1992). These authors claim that various food products such as fresh beef, cured pork, sliced tuna etc. exposed to volatiles from naturally derived AIT possessed only a slight AIT odour after removal of the packaging. This odour was reported to quickly dissipate once the food packages were left open for a short period of time. In contrast, results presented in section 6.3.5 indicate that exposure to vaporized horseradish distillate does alter the sensory properties of pre-cooked, uncured roast beef. Aroma, flavour and colour changes remained significant after the treated product was exposed to an atmosphere free of horseradish vapours for up to four h at refrigeration temperatures. This suggests that the volatile compounds in horseradish are solubilized in roast beef over time. Futhermore, higher scores for horseradish/irritation aromas and horseradish/irritation flavours were obtained with roast beef that had been exposed to horseradish distillate for 3 d compared to 15 d. This observation suggests that the isothiocyanates responsible for aroma and flavour changes are themselves altered through reactions with food components, as outlined previously. Use of horseradish distillates in the preservation of pre-cooked roast beef therefore induces sensory changes in the product as a result of reactions with food components.

Volatile compounds in the head space above roast beef stored with and without horseradish distillate were partially characterized in an effort to determine whether the isothiocyanates inhibit oxidation reactions in pre-cooked meat. While investigating volatiles associated with warmed over flavour in reheated pre-cooked beef, St. Angelo *et al.* (1987) reported that the major components associated with oxidizing cooked beef include pentanal,

3-hydroxy-2-butanone, hexanal, heptanal, nonanal, ethanol, 2-propanone, hexane, acetic acid, 3-methyl butanal, 2,3-butanedione, 1-pentanol and 3-methyl-1butanol. These compounds were detected in stored control samples in the present study. Incorporation of horseradish distillate reduced or prevented formation of several key volatiles. Carbonyls such as hexanal and nonanal are formed via oxidation of unsaturated meat lipids and are normally associated with oxidizing fat in cooked meats (Ramarathnam *et al.*, 1991). Neither compound was detected in pre-cooked roast beef stored under horseradish distillate. The remaining volatiles detected in control samples are often reported in cooked meat undergoing oxidation (St. Angelo *et al.*, 1987; Ramarathnam *et al.*, 1991; Kerler and Grosch, 1996). These results suggest that horseradish distillate contains powerful antioxidant(s) that block reactions responsible for oxygen induced degradation of meat lipids. AIT has been reported to have antioxidant properties (Mazza, 1998) and is a likely source of this activity. Metal chelation by AIT could retard the initiation step of autoxidation resulting in suppression of undesirable aromas and flavours, and enhance the formation of pink colour. However, the exact mechanism(s) responsible is unknown.

Chapter 7

General Discussion

Although extracts of cruciferous plants and purified isothiocyanates have long been known to have antimicrobial activity, there is divergence in the data reported in the scientific literature. In addition, few studies have been carried out in food products and little is known about interactions between food components and the main constituent of the essential oil of horseradish, AIT. Differences in methodology, test microorganisms and origin of the antimicrobial (ie: chemically produced or naturally occurring) make it difficult to compare results of this study with literature values. Early investigators reported results as absence/presence of growth while more recent measurements are reported as minimum inhibitory concentration or minimum inhibitory dose (MIC/MID). Aside from these differences, a general trend of increased inhibition with increased isothiocyanate/distillate concentration in the gas phase has been reported (Isshiki *et al.*, 1991; Sekiyama *et al.*, 1994a; Delaquis and Sholberg, 1997).

Interestingly, results obtained in the agar model system at 12°C did not parallel those observed in the roast beef model system. Although inhibition increased with distillate concentration in each system, complete inhibition required higher inputs in the roast beef model system. The difference was ascribed to differing substrate composition and its influence on the fate of AIT. In addition, ecological considerations related to the surface microenvironment may have led to the differences.

Regardless of substrate, significant inhibition was achieved at refrigeration temperatures under a horseradish distillate concentration of 20 000 nL/L. Mechanisms responsible for the antimicrobial activity of horseradish distillate and/or isothiocyanates are not fully understood. Most authors have speculated that interactions with proteins are likely significant. Zsolnai (1966) theorized that isothiocyanates exert their activity by inhibiting intracellular sulphydryl enzymes. At least one such enzyme, cytochrome c oxidase has been shown to lose activity in yeast exposed to AIT (Kojima and Ogawa, 1971). The latter observation is of interest since the most resistant bacterium tested or detected in this study belonged to the lactic acid bacteria group. These bacteria do not possess cytochrome c. Inhibition of a single enzyme by AIT is doubtful, however, given the highly reactive nature of this compound. Increasing bacteriostatic and bactericidal activity at higher concentrations indicated that additional effects are likely. Recently, Matsuoka *et al.* (1997) reported on the antimicrobial activity of an isothiocyanate breakdown product associated with radish. These authors suggested that damage to RNA, DNA, proteins, peptidoglycan and lipid synthesis are responsible for inhibition.

Each microorganism tested in this study responded differently to vaporized horseradish distillate. While the absence of cytochrome c in lactic acid bacteria may partially explain the resistance of lactic acid bacteria, there appears to be no explanation for differences in susceptibility between pathogenic bacteria and *Serratia grimesii*, or for differences in susceptibility between the pathogens tested. In the course of an investigation with several microorganisms isolated from environmental sources for their potential to detoxify rapemeal, Brabban and Edwards (1994) found that four strains of *Bacillus*, two strains of *Streptomyces*,

one strain of *Staphylococcus* and one unidentified fungus were able to use sinigrin as a carbon source. Regardless of whether glucose or sinigrin was present as the carbon source these microorganisms exhibited similar growth yields. Similarly, Nugon-Baudon *et al.* (1990) isolated a *Lactobacillus* strain from chickens that exhibited myrosinase-like activity and was able to degrade sinigrin. Tani *et al.* (1974) and Llanos Palop *et al.* (1995) also identified microorganisms that exhibited ability to degrade sinigrin. In all cases, these microorganisms must possess mechanisms to avoid the toxic effects of AIT, the breakdown product of sinigrin. Differences between species related to their ability to avoid the effects of AIT could therefore account for the range in susceptibility to the compound.

It is also useful to speculate on the influence of growth rates on the effectiveness of AIT. Pseudomonads are often reported to be very susceptible to the antimicrobial effects of AIT, particularly at lower temperatures. Since most species are psychrotrophic, elevated growth rates under these conditions would be common in this genus (Jay, 1992). Therefore, a link between higher respiration rates associated with faster growth rates and the extent of inhibition by AIT may exist. Delaquis and Sholberg (1997) presented data obtained with mesophilic pathogens exposed to AIT at temperatures below those permitting growth which is in support of this hypothesis. The susceptibility of pseudomonads to AIT was also confirmed on roast beef in the present study.

The results of experiments carried out in both the agar and roast beef model systems suggest that maintenance of elevated AIT concentrations is required to ensure inhibition of microorganisms in prolonged storage. For this reason, the fate of AIT was examined under a variety of conditions. Overall, AIT concentrations decreased over time in both the agar and

roast beef model systems and the reactions responsible for AIT depletion appeared to be complex. Oxygen did not affect the fate of AIT and slightly faster depletion occurred in the presence of agar discs. Loss of AIT due to reactions involving water have been previously reported (Llanos Palop *et al.*, 1995). The phenomenon was attributed to a spontaneous chemical conversion of AIT, although products of such a reaction were not detected. Sekiyama *et al.* (1996) also observed depletion of residual AIT in a model system. They reported that 15-20% of the initial input was absorbed by the agar medium, while leakage and interaction with the container were most likely responsible for further depletion over time. Hence, dissolution into the substrate or reactions with components thereof can account for some losses. In addition, absorption by several types of plastics has been reported (Sekiyama *et al.*, 1995). The reactivity of AIT with glass is unknown, however, and the influence of this factor on depletion of AIT in the present model system could not be assessed. Finally, reactions with substrate components, particularly proteins, doubtless led to some losses. These observations suggest that the use of vaporized AIT as a preservative for packaged foods requires careful consideration of the nature of the packaging system and potential interactions with food components.

In terms of consumer acceptability, considerable flavour changes occur when roast beef is exposed to 20 000 nL/L. Oral and nasal irritation at this concentration was high. Since this product has been altered from a sensory point of view it would have to be labelled as seasoned or flavoured product. In contrast, at 4 000 nL/L the flavour and aroma changes were less marked, and the product could possibly be marketed without further labelling (perhaps as preserved with natural horseradish oil).

The finding that horseradish distillate possesses antioxidant activity was unexpected. Colour degradation and undesirable organoleptic changes are major obstacles to the large scale production and distribution of pre-cooked, uncured roast beef. The results of this study indicate that such alterations could be minimized by incorporation of either horseradish distillate or AIT in the packaged product. However, these findings will need to be confirmed in more rigorous investigations on the chemistry of colour stability and fat oxidation in the presence of AIT. Improved retention of cooked meat colour in the presence of AIT probably derives from reactions with myoglobin, while reduced fat oxidation is likely due to interference with oxygen catalyzed reactions. The latter is an important reaction in the flavour chemistry of cooked meats, since the reactants can influence aroma nuances in these products (Ramarathnam *et al.*, 1991). Hence, horseradish distillate does induce changes in meat aroma and flavour, as illustrated by the results of sensory evaluation.

This work has demonstrated that horseradish distillate and/or AIT could be effective antimicrobial agents for pre-cooked, uncured roast beef stored in impermeable containers. Most microorganisms tested were sensitive to the effect, although it is clear that some have unusual resistance to AIT. Fortunately, all were species of lactic acid bacteria, a group of microorganisms that contains no known pathogenic species. Indeed, their presence in meat products is considered to offer a measure of protection against food-borne pathogens, due to their ability to successfully compete with such species. The isothiocyanates are not inert compounds, however, and do react with meat components. This results in sensory changes which would require modification of labelling.

Chapter 8

Summary and Recommendations

Results obtained in the agar model system revealed that 2 000 nL horseradish distillate / L air was sufficient to inhibit *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* and *Staphylococcus aureus* at 35°C and 12°C. *Serratia grimesii* was slightly more sensitive and was inhibited by 1 000 and 2 000 nL/L at 35°C and 12°C, respectively. *Lactobacillus sake* exhibited resistance to vaporized horseradish distillate at 25°C, although significant inhibition was achieved with 20 000 nL/L at 12°C. Bactericidal effects were also observed against each test microorganism, however, these were genus specific and concentration dependent. Higher distillate concentrations (up to 20 000 nL/L) were required for inhibition of bacteria in the roast beef model system, and *Serratia grimesii* and *Lactobacillus sake* exhibited greater resistance than the pathogens. An extension of the lag phase was observed with each test bacterium in the presence of horseradish distillate. Bactericidal effects against *Escherichia coli, Staphylococcus aureus* and *Serratia grimesii* on roast beef were also observed under 20 000 nL/L. Bacteriostatic effects were also observed at 4°C against aerobic bacteria, pseudomonads and enterics on roast beef. Lactic acid bacteria again exhibited resistance.

Depletion of residual AIT concentrations in the head space of both systems partially accounted for recovery of the test bacteria. Resistance to vaporized horseradish distillate on roast beef may be attributed to greater depletion of AIT due to substrate composition. Hydrated agar is largely composed of polysaccharides, which are reported to suppress AIT

decomposition. Meat, on the other hand contains more protein and lipids. Interaction with proteins or solubilization of AIT in the lipid fraction may decrease the amount of AIT available for inhibition.

In addition to the bacteriostatic effects achieved on roast beef changes to aroma, flavour and colour were observed under vaporized horseradish distillate. Roast beef stored for three d under 20 000 nL/L had the highest sensory scores for horseradish/irritation aroma, horseradish/irritation flavour and pink colour. In parallel to these results, a* measurements taken with a Minolta chroma meter were higher for roast beef stored for three d under 20 000 nL/L indicating greater retention of red colour. Analysis of volatile compounds in stored roast beef also showed that vaporized horseradish distillate suppressed the formation of compounds associated with pre-cooked roast beef. The antioxidant properties of horseradish may account for these findings.

In summary, inhibition of bacteria by vaporized horseradish distillate is specific among genera and resistance may be related to the ability of bacteria to degrade sinigrin as a carbon source and metabolize the reaction products. Since aroma and flavour changes remain significant under low levels of vaporized horseradish distillate, some difficulty may be encountered in marketing treated pre-cooked roast beef unless directed to speciality niches. However, beneficial aspects such as anticarcinogenicity, improvements to colour and the fact that the antimicrobial agent was of natural origin may be of value for marketing purposes. Also, resistance exhibited by lactics could prove beneficial since this group of microorganisms are known to inhibit growth of undesirable bacteria, including pathogens. Recommendations for further research based on results from this study include:

1. Investigations into the mode of action of isothiocyanates (ie: horseradish distillate) against bacterial growth. Investigations should focus on interactions with key metabolic enzymes, notably those involved in oxidative metabolism.

2. Investigations into substrate composition and its role in AIT depletion. Supplementing agar with various levels of proteins and lipids may reveal the fate of AIT in various substrates.

3. Investigations into the nature of the antioxidant properties associated with AIT/horseradish distillate. Perhaps a relationship between the antimicrobial and antioxidant properties exists.

4. Investigations into the antimicrobial activity of AIT/horseradish distillate in conjunction with other antimicrobial agents (ie: monolaurin). Use of AIT in conjunction with another antimicrobial agent may result in suppression of AIT depletion, or result in an additive or synergistic antimicrobial effect.

5. Investigations on the use of horseradish distillate in packaged pre-cooked, uncured meat products, including MAP storage. In order to use vaporized horseradish distillate as an antimicrobial agent, the rapid depletion of AIT must be addressed. Development of a packaging system that allows a timed-release of vapours would be advantageous.

6. Further investigations on the retention of aromas and flavours as perceived by human observers. Once the microbiological and chemical properties of roast beef exposed to vaporized horseradish distillate have been sufficiently investigated, consumer acceptability will have to be determined.

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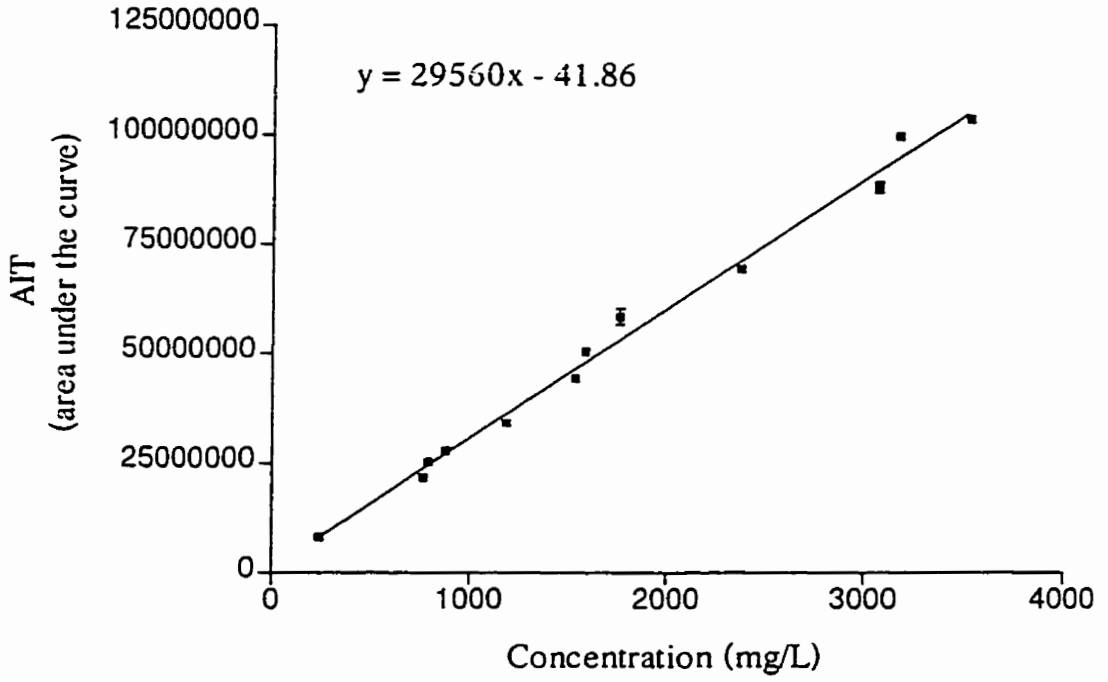
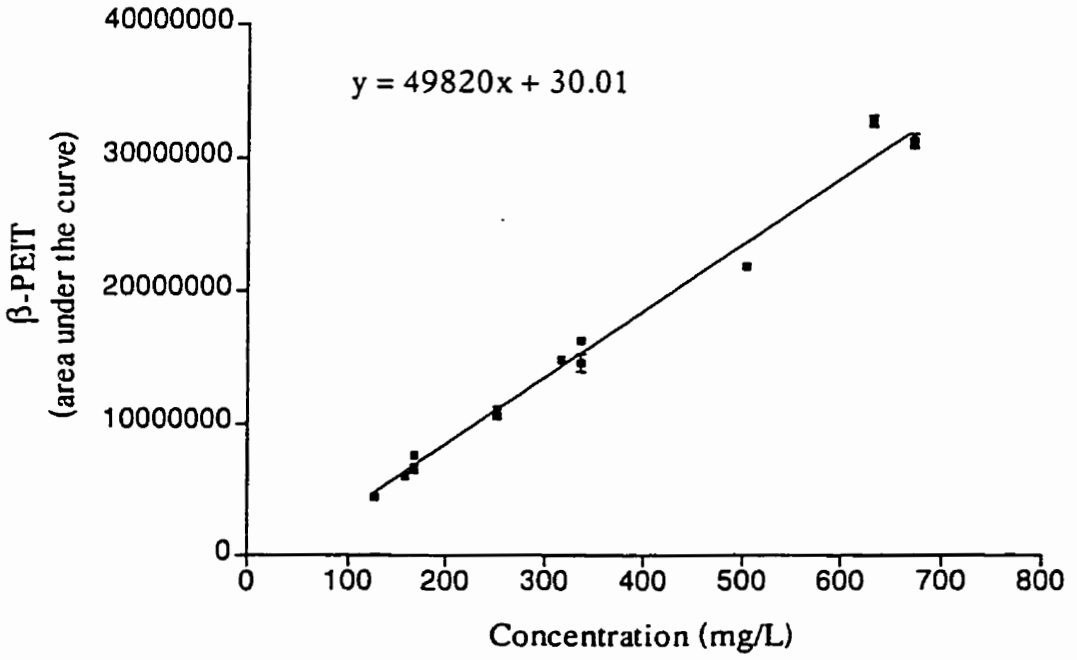
Appendices

Appendix 1. Standard curves used in the quantitative analysis of components present in horseradish distillate (A - AIT; B - β-PEIT).

A



B



Appendix 2a. Carbohydrate utilization by Gram negative roast beef isolate S12 - *Serratia grimesii* determined using a BIOLOG GN MicroPlate.

α-cyclodextrin	+[1]	turanose	+	alaninamide	+
dextrin	+	xylitol	+	D-alanine	+
glycogen	+	methyl pyruvate	+	L-alanine	+
tween 40	+	mono-methyl succinate	+	L-alanyl-glycine	+
tween 80	+	acetic acid	+	L-asparagine	+
N-acetyl-D-galactosamine	+	cis-aconitic acid	+	L-aspartic acid	+
N-acetyl-D-glucosamine	+	citric acid	+	L-glutamic acid	+
adonitol	+	formic acid	+	glycyl-L-aspartic acid	+
L-arabinose	+	D-galactonic acid lactone	+	glycyl-L-glutamic acid	+
D-arabitol	+	D-galacturonic acid	+	L-histidine	+
cellobiose	+	D-gluconic acid	+	hydroxy L-proline	+
i-erythritol	-	D-glucosaminic acid	+	L-leucine	-
D-fructose	+	D-glucuronic acid	+	L-ornithine	+
L-frucose	+	α-hydroxybutyric acid	+	L-phenylalanine	+
D-galactose	+	β-hydroxybutyric acid	+	L-proline	+
gentiobiose	+	γ-hydroxybutyric acid	-	L-pyroglutamic acid	-
α-D-glucose	+	p-hydroxy phenylacetic acid	+	D-serine	+
m-inositol	+	itaconic acid	-	L-serine	+
α-D-lactose	+	α-keto butyric acid	-	L-threonine	+
lactulose	+	α-keto glutaric acid	+	D, L-carnitine	-
maltose	+	α-keto valeric acid	-	γ-amino butyric acid	+
D-mannitol	+	D, L-lactic acid	+	urocanic acid	+
D-mannose	+	malonic acid	-	inosine	+
D-melibiose	+	propionic acid	-	uridine	+
β-methyl-D-glucoside	+	quinic acid	-	thymidine	+
D-psicose	+	D-saccharic acid	-	phenyl ethylamine	-
D-raffinose	+	sebacic acid	-	putrescine	+
L-rhamnose	+	succinic acid	+	2-amino ethanol	-
D-sorbitol	+	bromo succinic acid	+	2, 3 butanediol	-
sucrose	+	succinamic acid	+	glycerol	+
D-trehalose	+	glucuronamide	-	glucose-6-phosphate	+
D, L-α-glycerol phosphate	+	glucose-1-phosphate	+		

[1] '+', positive reaction; '-', negative reaction.

Appendix 2b. Carbohydrate utilization by Gram negative roast beef isolate - *Serratia grimesii* determined using API 20E carbohydrate plates.

ONPG	+	mannitol	+
arginine	+	inositol	+
lysine	+	sorbitol	+
ornithine	+	rhamnose	-
citrate	+	sucrose	+
H_2S	-	melibiose	-
urea	-	amygdalin	+
tryptophane	-	L-arabinose	+
tryptophane (formation of indole)	-	gelatin (liquefaction)	+
VP (sodium pyruvate/ creatine)	S	glucose	+

[1] '-', negative reaction; '+', positive reaction; 'S', slight

Appendix 2c. Carbohydrate utilization by Gram positive roast beef isolate S4 - *Lactobacillus sake* determined using a BIOLOG GP MicroPlate.

α-cyclodextrin	-[1]	α-methyl D-glucoside	-	propionic acid	-
β-cyclodextrin	-	β-methyl D-glucoside	-	pyruvic acid	-
dextrin	-	α-methyl D-mannoside	-	succinamic acid	-
glycogen	-	palatinose	-	succinic acid	-
inulin	-	D-psicose	-	N-acetyl L-glutamic acid	-
mannan	-	D-raffinose	-	alaninamide	-
tween 40	-	L-rhamnose	-	D-alanine	-
tween 80	-	D-ribose	+	L-alanine	-
N-acetyl-D-glucosamine	+	salicin	-	L-alanyl-glycine	-
N-acetyl-D-mannosamine	+	sedoheptulosan	-	L-asparagine	-
amygdalin	-	D-sorbitol	-	L-glutamic acid	-
L-arabinose	+	stachyose	-	glycyl-L-glutamic acid	-
D-arabitol	-	sucrose	+	L-pyroglutamic acid	-
arbutin	-	D-tagatose	-	L-serine	-
cellobiose	+	D-trehalose	-	putrescine	-
D-fructose	+	turanose	-	2, 3-butanediol	-
L-frucose	-	xylitol	-	glycerol	+
D-galactose	+	D-xylose	-	adenosine	+
D-galacturonic acid	-	acetic acid	-	2'-deoxy adenosine	-
gentiobiose	-	α-hydroxybutyric acid	-	inosine	+
D-gluconic acid	+	β-hydroxybutyric acid	-	thymidine	+
m-inositol	-	γ-hydroxybutyric acid	-	uridine	+
α-D-lactose	S	p-hydroxyphenyl acetic acid	-	adenosine-5'-monophosphate	-
lactulose	-	α-keto glutaric acid	-	thymidine-5'-monophosphate	-
maltose	+	α-keto valeric acid	-	uridine-5'-monophosphate	-
maltotriose	-	lactamide	-	fructose-6-phosphate	-
D-mannitol	-	D-lactic acid methyl ester	-	glucose-1-phosphate	-
D-mannose	+	L-lactic acid	S	glucose-6-phosphate	-
D-melezitose	-	D-malic acid	-	D, L-α-glycerol phosphate	-
D-melibiose	+	L-malic acid	-	α-D-glucose	+
α-methyl D-galactoside	-	methyl pyruvate	-	β-methyl-D-galactoside	-
3-methyl glucose	-	mono-methyl succinate	-		

[1] '+', positive reaction; '-', negative reaction; 'S', slight

Appendix 2d. Carbohydrate utilization by three Gram positive isolates determined using API CHL50 carbohydrate plates.

	Isolate S4 *L. sake*	Isolate S10 *Leuconostoc* spp.	Isolate S13 *Leuconostoc* spp.		Isolate S4 *L. sake*	Isolate S10 *Leuconostoc* spp.	Isolate S13 *Leuconostoc* spp.
Glycerol	-[1]	-	-	α Methyl-D-mannoside	-	-	-
Erthritol	-	-	-	α Methyl-D-glucoside	-	+	+
D-Arabinose	-	-	-	N Acetyl glucosamine	+	+	S
L-Arabinose	+	+	-	Amygdaline	S	-	-
Ribose	+	+	+	Arbutine	-	-	-
D-Xylose	-	-	-	Esculine	+	+	+
Adonitol	-	-	-	Salicine	+	+	-
β Methyl-xyloside	-	-	-	Cellobiose	+	S	S
Galactose	+	+	+	Maltose	+	+	-
D-Glucose	+	+	+	Lactose	+	-	-
D-Fructose	+	+	+	Melibiose	+	+	-
D-Mannose	+	+	S	Saccharose	+	+	+
L-Sorbose	-	-	-	Trehalose	+	+	S
Rhamnose	+	+	-	Inuline	-	-	-
Dulcitol	-	-	-	Melezitose	-	-	-
Inositol	-	-	-	D-Raffinose	-	-	-
Mannitol	-	-	-	Amidon	-	-	-
Sorbitol	-	-	-	Glycogene	-	-	-
D-Fucose	-	-	-	Xylitol	-	-	-
L-Fucose	-	-	-	β Gentiobiose	+	S	S
D-Arabitol	-	-	-	D-Turanose	-	+	+
L-Arabitol	-	-	-	D-Lyxose	-	-	-
Gluconate	S	S	S	D-Tagatose	-	-	-
2 ceto-gluconate	-	-	-	5 ceto-gluconate	-	-	-

[1] '-', negative reaction; '+', positive reaction; 'S', slight

Appendix 3a. Analysis of variance of total and irreversible inhibition of *Listeria monocytogenes* on TSA-YE at 35°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	2	63.6723	31.8361	135.10	0.0001*
Error	9	2.1209	0.2357		
Corrected Total	11	65.7932			
	R-square 0.9678	C.V. 11.6089	Root MSE 0.4854	Total inhibition Mean 4.1817	

1 treatment - 500 nL/L, 1 000 nL/L, 2 000 nL/L.
*, significantly different at $p < 0.05$.

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	4.1402	2.0701	5.07	0.0335*
Error	9	3.6751	0.4083		
Corrected Total	11	7.8153			
	R-square 0.5298	C.V. 40.3166	Root MSE 0.6390	Irreversible inhibition Mean 1.5850	

*, significantly different at $p < 0.05$.

Appendix 3b. Analysis of variance of total and irreversible inhibition of *Salmonella typhimurium* on TSA-YE at 35°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	84.3988	42.1994	272.42	0.0001*
Error	9	1.3942	0.1549		
Corrected Total	11	85.7930			
	R-square 0.9838	C.V. 9.8601	Root MSE 0.3936	Total inhibition Mean 3.9917	

*, significantly different at $p < 0.05$.

Appendix 3b. Continued.

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	0.6534	0.3267	3.00	0.1004
Error	9	0.9803	0.1089		
Corrected Total	11	1.6337			
	R-square 0.3400	C.V. 200.0204	Root MSE 0.3300	Irreversible inhibition Mean 0.1650	

Appendix 3c. Analysis of variance of total and irreversible inhibition of *Escherichia coli* on TSA-YE at 35°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	72.0401	36.0201	41.00	0.0001*
Error	9	7.9065	0.8785		
Corrected Total	11	79.9466			
	R-square 0.9011	C.V. 26.4893	Root MSE 0.9373	Total inhibition Mean 3.5383	

*, significantly different at $p < 0.05$.

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	0.1980	0.0990	1.00	0.4053
Error	9	0.8911	0.0990		
Corrected Total	11	1.0891			
	R-square 0.1818	C.V. 346.4102	Root MSE 0.3147	Irreversible inhibition Mean 0.0908	

Appendix 3d. Analysis of variance of total and irreversible inhibition of *Staphylococcus aureus* on TSA-YE at 35°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	83.0104	41.5052	133.56	0.0001*
Error	9	2.7969	0.3108		
Corrected Total	11	85.8073			
	R-square	C.V.	Root MSE	Total inhibition Mean	
	0.9674	14.9822	0.5575	3.7208	

*, significantly different at $p < 0.05$.

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	1.9145	0.9573	10.11	0.0050*
Error	9	0.8520	0.0947		
Corrected Total	11	2.7665			
	R-square	C.V.	Root MSE	Irreversible inhibition Mean	
	0.6920	73.9900	0.3077	0.4158	

*, significantly different at $p < 0.05$.

Appendix 3e. Analysis of variance of total and irreversible inhibition of *Serratia grimesii* on TSA-YE at 35°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	2.3961	1.1980	3.21	0.1126
Error	9	2.2374	0.3729		
Corrected Total	11	4.6334			
	R-square	C.V.	Root MSE	Total inhibition Mean	
	0.5171	9.9185	0.6106	6.1567	

Appendix 3e. Continued.

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	32.8392	16.4196	37.13	0.0004*
Error	9	2.6535	0.4422		
Corrected Total	11	35.4927			
	R-square 0.9252	C.V. 14.1159	Root MSE 0.6650	Irreversible inhibition Mean 4.7111	

*, significantly different at $p < 0.05$.

Appendix 4. Total and irreversible inhibition achieved with various concentrations of vaporized horseradish distillate on agar discs stored at 35°C (A; 25°C for *L. sake*) and 12°C(B)[1].

A.	Total inhibition (Log cfu / cm²)			Irreversible inhibition (Log cfu / cm²)		
Vapour concentration	500 nL/L	1 000 nL/L	2 000 nL/L	500 nL/L	1 000 nL/L	2 000 nL/L
L. monocytogenes	1.01 ± 0.64[2a]	5.15 ± 0.54[b]	6.40 ± 0.09[c]	1.01 ± 0.64[a]	1.36 ± 0.11[a]	2.39 ± 0.90[b]
S. typhimurium	0.53 ± 0.15[a]	4.47 ± 0.66[b]	6.97 ± 0.10[c]	0[a]	0[a]	0.50 ± 0.57[a]
E. coli	0.62 ± 0.24[a]	3.38 ± 1.50[b]	6.62 ± 0.58[c]	0[a]	0[a]	0.27 ± 0.55[a]
S. aureus	0.01 ± 0.03[a]	5.33 ± 0.76[b]	5.83 ± 0.59[b]	0.01 ± 0.03[a]	0.28 ± 0.41[a]	0.96 ± 0.34[b]
S. grimesii	5.58 ± 0.85[a]	6.58 ± 0.13[a]	6.75[3a]	2.58 ± 0.76[a]	6.33 ± 0.55[b]	6.75[3b]
L. sake	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
B.						
Vapour concentration	2 000 nL/L	4 000 nL/L	20 000 nL/L	2 000 nL/L	4 000 nL/L	20 000 nL/L
L. monocytogenes	6.83 ± 0.15[a]	6.84 ± 0.35[a]	6.65 ± 0.23[a]	0.28 ± 0.33[a]	0.88 ± 0.76[a]	5.15 ± 1.19[b]
S. typhimurium	7.10 ± 0.09[a]	6.99 ± 0.28[a]	6.88 ± 0.12[a]	1.05 ± 0.91[a]	1.47 ± 0.87[a]	6.88 ± 0.12[b]
E. coli	7.03 ± 0.25[a]	6.99 ± 0.28[a]	7.34 ± 0.24[a]	0.55 ± 0.65[a]	0.87 ± 0.46[a]	0.24 ± 0.98[b]
S. aureus	6.39 ± 0.31[a]	6.21 ± 0.58[ab]	5.57 ± 0.56[b]	0[a]	0.41 ± 0.50[a]	2.51 ± 1.05[b]
S. grimesii	6.37 ± 0.58[a]	6.39 ± 0.31[a]	7.07 ± 0.23[b]	3.62 ± 0.70[a]	5.89 ± 0.87[b]	5.07 ± 0.96[b]
L. sake	0.11 ± 0.23[a]	1.41 ± 0.61[b]	6.73 ± 0.47[c]	0.09 ± 0.18[a]	0.95 ± 0.72[ab]	1.24 ± 0.97[b]

[1] total inhibition at 35°C/25°C determined after 48 hours and irreversible inhibition determined after 72 hours; total inhibition at 12°C determined after seven days and irreversible inhibition determined after 72 hours.

[2] mean cell densities of 4 replicates ± standard deviation.

[3] value represents only one replicate.

[a-c] means within the same row and type of inhibition with different superscripts are significantly different ($P < 0.05$).

Appendix 5a. Analysis of variance of total and irreversible inhibition of *Listeria monocytogenes* on TSA-YE at 12°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	2	0.0893	0.0446	0.68	0.5308
Error	9	0.5907	0.0656		
Corrected Total	11	0.6800			
	R-square	C.V.	Root MSE	Total inhibition Mean	
	0.1313	3.7833	0.2562	6.7717	

1 treatment - 2 000 nL/L, 4 000 nL/L, 20 000 nL/L.

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	56.4496	28.2248	40.06	0.0001*
Error	9	6.3409	0.7045		
Corrected Total	11	62.7904			
	R-square	C.V.	Root MSE	Irreversible inhibition Mean	
	0.8990	39.9225	0.8394	2.103	

*, significantly different at $p < 0.05$.

Appendix 5b. Analysis of variance of total and irreversible inhibition of *Salmonella typhimurium* on TSA-YE at 12°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	0.0946	0.04733	1.43	0.2880
Error	9	0.2970	0.0330		
Corrected Total	11	0.3916			
	R-square	C.V.	Root MSE	Total inhibition Mean	
	0.2417	2.5997	0.1817	6.9875	

Appendix 5b. Continued.

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	84.5779	42.2889	78.99	0.0001*
Error	9	4.8186	0.5354		
Corrected Total	11	89.3965			
	R-square 0.9461	C.V. 23.3712	Root MSE 0.7317	Irreversible inhibition Mean 3.1308	

*, significantly different at $p < 0.05$.

Appendix 5c. Analysis of variance of total and irreversible inhibition of *Escherichia coli* on TSA-YE at 12°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	0.2831	0.1416	2.19	0.1682
Error	9	0.5824	0.0647		
Corrected Total	11	0.8655			
	R-square 0.3271	C.V. 3.5731	Root MSE 0.2544	Total inhibition Mean 7.119	

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	77.3899	38.6949	72.64	0.0001*
Error	9	4.7943	0.5327		
Corrected Total	11	82.1842			
	R-square 0.9417	C.V. 29.2140	Root MSE 0.7299	Irreversible inhibition Mean 2.4983	

Appendix 5d. Analysis of variance of total and irreversible inhibition of *Staphylococcus aureus* on TSA-YE at 12°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	1.5023	0.7512	3.05	0.0974
Error	9	2.2168	0.2463		
Corrected Total	11	3.7191			
	R-square 0.4039	C.V. 8.1976	Root MSE 0.4963	Total inhibition Mean 6.0542	

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	14.4541	7.2271	16.06	0.0011*
Error	9	4.0500	0.4500		
Corrected Total	11	18.5041			
	R-square 0.7811	C.V. 69.0972	Root MSE 0.6708	Irreversible inhibition Mean 0.9708	

*, significantly different at $p < 0.05$.

Appendix 5e. Analysis of variance of total and irreversible inhibition of *Serratia grimesii* on TSA-YE at 12°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	1.2521	0.6260	3.90	0.0603
Error	9	1.4451	0.1606		
Corrected Total	11	2.6972			
	R-square 0.4642	C.V. 6.0637	Root MSE 0.4007	Total inhibition Mean 6.6083	

Appendix 5e. Continued.

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	10.5621	5.2810	7.30	0.0131*
Error	9	6.5151	0.7239		
Corrected Total	11	17.0772			
	R-square 0.6185	C.V. 17.5127	Root MSE 0.8508	Irreversible inhibition Mean 4.8583	

*, significant at $p < 0.05$.

Appendix 5f. Analysis of variance of total and irreversible inhibition of *Lactobacillus sake* on MRSA at 12°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	98.1838	49.0919	227.22	0.0001*
Error	9	1.9445	0.2161		
Corrected Total	11	100.1282			
	R-square 0.9806	C.V. 16.9023	Root MSE 0.4648	Total inhibition Mean 2.75	

*, significant at $p < 0.05$.

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	2.8675	1.4337	2.88	0.1080
Error	9	4.4814	0.4979		
Corrected Total	11	7.3488			
	R-square 0.3902	C.V. 93.1541	Root MSE 0.7056	Irreversible inhibition Mean 0.7575	

Appendix 6a. Analysis of variance of total and irreversible inhibition of *Listeria monocytogenes* on TSA-YE exposed to 2 000 nL horseradish distillate / L air at 12°C and 35°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Temperature	1	0.3698	0.3698	24.17	0.0027*
Error	6	0.0918	0.0153		
Corrected Total	7	0.4616			
	R-square	C.V.	Root MSE	Total inhibition Mean	
	0.8011	1.8713	0.1237	6.61	

*, significant at $p < 0.05$.

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Temperature	1	8.9253	8.9253	19.57	0.0045*
Error	6	2.7363	0.4560		
Corrected Total	7	11.6616			
	R-square	C.V.	Root MSE	Irreversible inhibition Mean	
	0.7654	50.6326	0.6753	1.3338	

*, significant at $p < 0.05$.

Appendix 6b. Analysis of variance of total and irreversible inhibition of *Salmonella typhimurium* on TSA-YE exposed to 2 000 nL horseradish distillate / L air at 12°C and 35°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Temperature	1	0.0300	0.0300	3.34	0.1175
Error	6	0.0534	0.0090		
Corrected Total	7	0.0840			
	R-square 0.3573	C.V. 1.3484	Root MSE 0.09485	Total inhibition Mean 7.0338	

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Temperature	1	0.6105	0.6105	1.06	0.3433
Error	6	3.4614	0.5769		
Corrected Total	7	4.0719			
	R-square 0.1499	C.V. 98.4812	Root MSE 0.7595	Irreversible inhibition Mean 0.7713	

Appendix 6c. Analysis of variance of total and irreversible inhibition of *Escherichia coli* on TSA-YE exposed to 2 000 nL horseradish distillate / L air at 12°C and 35°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Temperature	1	0.3445	0.3445	1.75	0.2343
Error	6	1.1824	0.1971		
Corrected Total	7	1.5268			
	R-square 0.2256	C.V. 6.5042	Root MSE 0.4439	Total inhibition Mean 6.825	

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Temperature	1	0.1485	0.1485	0.41	0.5437
Error	6	2.1514	0.3586		
Corrected Total	7	2.2999			
	R-square 0.0646	C.V. 146.4956	Root MSE 0.5988	Irreversible inhibition Mean 0.4088	

Appendix 6d. Analysis of variance of total and irreversible inhibition of *Staphylococcus aureus* on TSA-YE exposed to 2 000 nL horseradish distillate / L air at 12°C and 35°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Temperature	1	0.6385	0.6385	2.86	0.1416
Error	6	1.3379	0.2230		
Corrected Total	7	1.9764			
	R-square	C.V.	Root MSE	Total inhibition Mean	
	0.3230	7.7317	0.4722	6.1075	

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Temperature	1	1.8432	1.8432	32.26	0.0013*
Error	6	0.3428	0.0571		
Corrected Total	7	2.1860			
	R-square	C.V.	Root MSE	Irreversible inhibition Mean	
	0.8432	49.7970	0.2391	0.4800	

*, significantly different at $p < 0.05$.

Appendix 6e. Analysis of variance of total and irreversible inhibition of *Serratia grimesii* on TSA-YE exposed to 2 000 nL horseradish distillate / L air at 12°C and 25°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Temperature	1	0.1155	0.1155	0.35	0.5976
Error	6	1.0008	0.3336		
Corrected Total	7	1.1163			
	R-square 0.1035	C.V. 8.9603	Root MSE 0.5776	Total inhibition Mean 6.4460	

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Temperature	1	7.8375	7.8375	15.99	0.0280*
Error	6	1.4708	0.4903		
Corrected Total	7	9.3083			
	R-square 0.8420	C.V. 16.4906	Root MSE 0.7002	Irreversible inhibition Mean 4.2460	

*, significantly different at $p < 0.05$.

Appendix 6f Analysis of variance of total and irreversible inhibition of *Lactobacillus sake* on MRSA exposed to 2 000 nL horseradish distillate / L air at 12°C and 25°C.

Total inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Temperature	1	0.0253	0.0253	1.00	0.3559
Error	6	0.1519	0.0253		
Corrected Total	7	0.1772			
	R-square 0.1429	C.V. 282.8427	Root MSE 0.1591	Total inhibition Mean 0.05625	

Irreversible inhibition.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Temperature	1	0.0153	0.0153	1.00	0.3559
Error	6	0.0919	0.0153		
Corrected Total	7	0.1072			
	R-square 0.1429	C.V. 282.8427	Root MSE 0.1237	Irreversible inhibition Mean 0.0438	

Appendix 7a. Analysis of variance of chemical AIT remaining in a model system flushed with dried air and stored at 37°C.

Day 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	2	101.9220	50.9610	0.64	0.6093
Replicate	1	1354.2033	1354.2033	17.04	0.0540
Error	2	158.9226	79.4613		
Corrected Total	5	1615.0479			
	R-square 0.9016	C.V. 12.5610	Root MSE 8.9141	AIT Mean 70.9667	

[1] treatment - 500 nL/L, 1 000 nL/L and 2 000 nL/L.

Day 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	224.6377	112.3189	2.35	0.2986
Replicate	1	22.8931	22.8931	0.48	0.5605
Error	2	95.6504	47.8252		
Corrected Total	5	343.1812			
	R-square 0.7213	C.V. 14.8690	Root MSE 6.9156	AIT Mean 46.51	

Day 3.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	183.1851	91.5926	0.98	0.5057
Replicate	1	38.2538	38.2538	0.41	0.5883
Error	2	187.4107	93.7054		
Corrected Total	5	408.8496			
	R-square 0.5416	C.V. 27.9249	Root MSE 9.6801	AIT Mean 34.6650	

Appendix 7a. Continued.

Day 4.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	168.6769	84.3385	1.82	0.3543
Replicate	1	93.8522	93.8522	2.03	0.2904
Error	2	92.5561	46.2781		
Corrected Total	5	355.0852			
	R-square 0.7393	C.V. 28.2567	Root MSE 6.8028	AIT Mean 24.0750	

Day 5.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	97.1803	48.5902	1.00	0.5012
Replicate	1	63.7004	63.7004	1.30	0.3717
Error	2	97.6476	48.8238		
Corrected Total	5	258.5283			
	R-square 0.6223	C.V. 38.2976	Root MSE 6.9874	AIT Mean 18.2450	

Appendix 7b. Analysis of variance of chemical AIT remaining in a model system flushed with dried nitrogen and stored at 37°C.

Day 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	2	671.6369	335.8185	8.54	0.1048
Replicate	1	270.1446	270.1446	6.87	0.1199
Error	2	78.6432	39.3216		
Corrected Total	5	1020.4247			
	R-square 0.9229	C.V. 9.8539	Root MSE 6.2707	AIT Mean 63.6367	

[1] treatment - 500 nL/L, 1 000 nL/L and 2 000 nL/L.

Day 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	501.9469	250.9735	2.36	0.2974
Replicate	1	90.4040	90.4040	0.85	0.4536
Error	2	212.4386	106.2193		
Corrected Total	5	804.7896			
	R-square 0.7360	C.V. 25.3755	Root MSE 10.3063	AIT Mean 40.6150	

Day 3.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	84.0492	42.0246	1.87	0.3489
Replicate	1	520.0566	520.0566	23.09	0.0407*
Error	2	45.0372	22.5186		
Corrected Total	5	649.1430			
	R-square 0.9306	C.V. 25.1344	Root MSE 4.7454	AIT Mean 18.8800	

*, significantly different at $p < 0.05$.

Appendix 7b. Continued.

Day 4.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	82.7684	41.3842	1.25	0.4442
Replicate	1	370.6776	370.6776	11.21	0.0788
Error	2	66.1563	33.0782		
Corrected Total	5	519.6023			

R-square	C.V.	Root MSE	AIT Mean
0.8727	43.7810	5.7514	13.1367

Day 5.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	81.4800	40.7400	3.97	0.2012
Replicate	1	86.3363	86.3363	8.42	0.1011
Error	2	20.5189	10.2595		
Corrected Total	5	188.3352			

R-square	C.V.	Root MSE	AIT Mean
0.8911	29.8235	3.2030	10.7400

Appendix 8. Analysis of variance of chemical grade AIT (initial concentration - 2 000 nL/L) exposed to various atmospheres at 37°C.

Day 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	2	775.6954	387.8477	2.29	0.3040
Error	2	338.7495	169.3747		
Corrected Total	4	1114.4449			
	R-square 0.6960	C.V. 18.9278	Root MSE 13.0144		AIT Mean 68.7580

[1] treatment - flushed with dried air, flushed with dried nitrogen and in the presence of 21 uninoculated TSA-YE discs.

Day 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	881.2634	440.6317	17.79	0.0532
Error	2	49.5497	24.7749		
Corrected Total	4	930.8131			
	R-square 0.9468	C.V. 10.6056	Root MSE 4.9774		AIT Mean 46.9320

Day 3.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	727.1283	363.5641	1.80	0.3573
Error	2	404.2970	202.1485		
Corrected Total	4	1131.4253			
	R-square 0.6427	C.V. 49.7791	Root MSE 14.2179		AIT Mean 28.5620

Appendix 8. Continued.

Day 4.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	455.7446	227.8723	1.51	0.3986
Error	2	302.0594	151.0297		
Corrected Total	4	757.8040			
	R-square 0.6014	C.V. 58.6887	Root MSE 12.2894		AIT Mean 20.9400

Day 5.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[2]	1	71.5716	71.5716	1.97	0.2956
Error	2	72.6856	36.3428		
Corrected Total	3	144.2572			
	R-square 0.4961	C.V. 30.6482	Root MSE 6.0285		AIT Mean 19.6700

[2] treatment - flushed with dried air, flushed with dried nitrogen.

Appendix 9a. Analysis of variance of residual AIT from horseradish distillate in a model system flushed with dried nitrogen and stored at 35°C.

Day 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	2	140.1667	70.0833	1.43	0.3110
Replicate	3	506.2500	168.7500	3.44	0.0925
Error	6	294.5000	49.0833		
Corrected Total	11	940.9167			
	R-square 0.6870	C.V. 14.5704	Root MSE 7.0059	AIT Mean 48.0833	

[1] treatment - 500 nL/L, 1 000 nL/L and 2 000 nL/L.

Day 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	165.5000	82.7500	2.30	0.1813
Replicate	3	330.6667	110.2222	3.06	0.1129
Error	6	215.8333	35.9722		
Corrected Total	11	712.0000			
	R-square 0.6969	C.V. 18.7428	Root MSE 5.9977	AIT Mean 32.0000	

Day 3.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	27.1667	13.5833	0.89	0.4584
Replicate	3	315.0000	105.0000	6.89	0.0227*
Error	6	91.5000	15.2500		
Corrected Total	11	433.6667			
	R-square 0.4890	C.V. 16.8567	Root MSE 3.9051	AIT Mean 23.1667	

*, significantly different at $p < 0.05$.

Appendix 9a. Continued.

Day 4.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	36.1667	18.0833	2.64	0.1508
Replicate	3	205.5833	68.5278	9.99	0.0095*
Error	6	41.1667	6.8611		
Corrected Total	11	282.9167			
	R-square 0.8545	C.V. 15.9556	Root MSE 2.6194	AIT Mean 16.4167	

*, significantly different at $p < 0.05$.

Day 5.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	1.1667	0.5833	0.34	0.7219
Replicate	3	79.3333	26.4444	15.61	0.0031*
Error	6	10.1667	1.6944		
Corrected Total	11	90.6667			
	R-square 0.8879	C.V. 11.5750	Root MSE 1.3017	AIT Mean 11.6667	

*, significant different at $p < 0.05$.

Appendix 9b. Analysis of variance of residual AIT from horseradish in a model system containing 21 TSA-YE discs and stored at 35°C.

Day 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	2	46.5000	23.2500	0.48	0.6390
Replicate	3	40.9167	13.6389	0.28	0.8360
Error	6	288.8333	48.1389		
Corrected Total	11	376.2500			
	R-square 0.2323	C.V. 24.1329	Root MSE 6.9382	AIT Mean 28.7500	

[1] treatment - 500 nL/L, 1 000 nL/L and 2 000 nL/L.

Day 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	72.0000	36.0000	1.92	0.2271
Replicate	3	61.5833	20.5278	1.09	0.4214
Error	6	112.6667	18.7778		
Corrected Total	11	246.2500			
	R-square 0.5425	C.V. 30.4094	Root MSE 4.3333	AIT Mean 14.2500	

Day 3.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	18.6667	9.3333	0.90	0.4540
Replicate	3	44.0000	14.6667	1.42	0.3264
Error	6	62.0000	10.3333		
Corrected Total	11	124.6667			
	R-square 0.5027	C.V. 31.1086	Root MSE 3.215	AIT Mean 10.3333	

Appendix 9b. Continued.

Day 4.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	21.5000	10.7500	0.62	0.5705
Replicate	3	260.2500	86.7500	4.98	0.0456*
Error	6	104.5000	17.4167		
Corrected Total	11	386.2500			
	R-square 0.7295	C.V. 53.8494	Root MSE 4.1733	AIT Mean 7.7500	

*, significantly different at $p < 0.05$.

Day 5.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	9.5000	4.7500	0.49	0.6336
Replicate	3	118.9167	39.6389	4.11	0.0665
Error	6	57.8333	9.6389		
Corrected Total	11	186.2500			
	R-square 0.6895	C.V. 65.3612	Root MSE 3.1047	AIT Mean 4.7500	

Appendix 10. Analysis of variance of AIT from horseradish distillate (initial concentration - 2 000 nL/L) exposed to various atmospheres at 35°C.

Day 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	1	722.0000	722.0000	8.95	0.0243*
Error	6	484.0000	80.6667		
Corrected Total	7	1206.0000			

R-square	C.V.	Root MSE	AIT Mean
0.5987	21.9060	8.9815	41.0000

[1] treatment - flushed with dried nitrogen and in the presence of 21 uninoculated TSA-YE discs.
*, significantly different at $p < 0.05$.

Day 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	1	612.5000	612.5000	7.27	0.0358*
Error	6	505.5000	84.2500		
Corrected Total	7	1118.0000			

R-square	C.V.	Root MSE	AIT Mean
0.5479	35.3030	9.1788	26.0000

*, significantly different at $p < 0.05$.

Day 3.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	1	351.1250	351.1250	7.15	0.0369*
Error	3	294.7500	49.1250		
Corrected Total	7	645.8750			

R-square	C.V.	Root MSE	AIT Mean
0.5436	37.6318	7.0089	18.6250

*, significantly different at $p < 0.05$.

Appendix 10. Continued.

Day 4.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	1	242.0000	242.0000	10.23	0.0187*
Error	6	142.0000	23.6667		
Corrected Total	7	384.0000			
	R-square 0.6302	C.V. 37.4218	Root MSE 4.8648	AIT Mean 13.0000	

*, significantly different at $p < 0.05$.

Day 5.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	1	120.1250	120.1250	20.74	0.0039*
Error	6	34.7500	5.7917		
Corrected Total	7	154.8750			
	R-square 0.7756	C.V. 29.6196	Root MSE 2.4066	AIT Mean 8.1250	

.*, significantly different at $p < 0.05$.

Appendix 11. Analysis of variance of residual AIT from horseradish in a model system containing 24 inoculated TSA-YE/MRSA discs and stored at 12°C.

Day 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	2	2599.5121	1299.7561	13.01	0.0017*
Error	10	999.3044	99.9304		
Corrected Total	12	3598.8166			
	R-square 0.7223	C.V. 15.0937	Root MSE 9.9965	AIT Mean 66.2300	

[1] treatment - 2 000 nL/L, 4 000 nL/L and 20 000 nL/L.
*, significantly different at $p < 0.05$.

Day 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	2576.6138	1288.3069	9.69	0.0031*
Error	12	1594.6205	132.8850		
Corrected Total	14	4171.2344			
	R-square 0.6177	C.V. 22.8432	Root MSE 11.5276	AIT Mean 50.4640	

*, significantly different at $p < 0.05$.

Day 4.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	1269.4445	634.7223	8.74	0.0054*
Error	11	799.2922	72.6630		
Corrected Total	13	2068.7367			
	R-square 0.6136	C.V. 27.9327	Root MSE 8.5243	AIT Mean 30.517	

*, significantly different at $p < 0.05$.

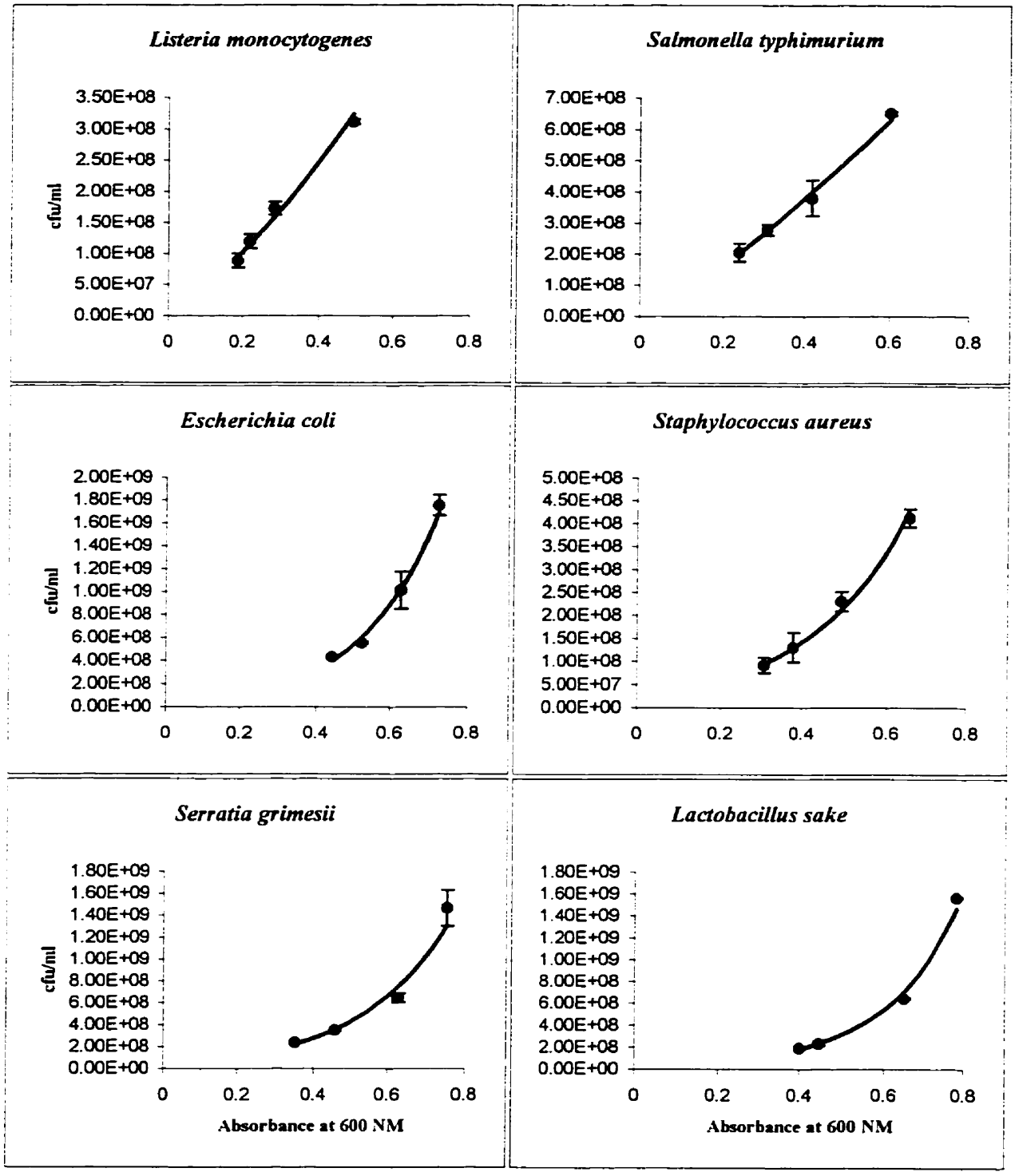
Appendix 11. Continued.

Day 7.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	857.3498	428.6749	9.71	0.0031*
Error	12	529.9561	44.1630		
Corrected Total	14	1387.3059			
	R-square 0.6180	C.V. 33.5249	Root MSE 6.6455		AIT Mean 19.8227

*, significantly different at $p < 0.05$.

Appendix 12. Standard curves relating bacterial numbers in phosphate buffer solution to absorbance measurements at 600 nm.



Appendix 13. Sample ballot used in the sensory analysis of pre-cooked, uncured roast beef stored under varying concentrations of horseradish distillate.

COOKED, UNCURED ROAST BEEF--NASAL EVALUATION

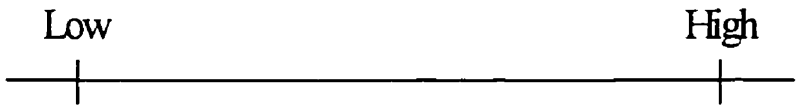
NAME_____ **DATE**_____**1997**

Session ___
Please evaluate the roast beef samples in the following order:

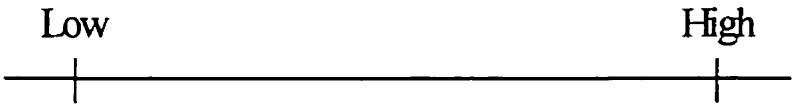
_____ _____ _____ _____ _____ _____

Please **SMELL** each of the samples. Remove the lid from the sample container and rate the intensity of the attributes listed below from low to high by placing a vertical mark on the scale. Please label each vertical line with the sample code number. **Replace the lid before moving on to the next sample.**

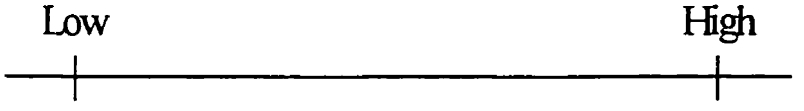
Please evaluate these roast beef samples for a **cooked, processed meat aroma.**



Please evaluate these roast beef samples for a **horseradish aroma** (watercress/celery).



Please evaluate these roast beef samples for **horseradish irritation.**



COMM **ENTS PLEASE:**

Appendix 13. Continued.

COOKED, UNCURED ROAST BEEF–ORAL EVALUATION

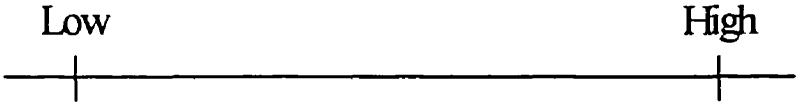
NAME_____ DATE_____1997

Session ___
Please evaluate the roast beef samples in the following order:

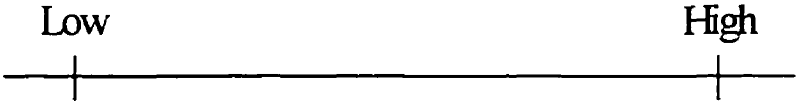
_____ _____ _____ _____ _____ _____

Please **TASTE** each of the samples. Remove the lid from the sample container and place the **entire sample** in your mouth (you may either swallow the sample or dispose of it in the provided waste container). Rate the intensity of the attributes listed below from low to high by placing a vertical mark on the scale. Please label each vertical line with the sample code number. **Replace the lid before moving on to the next sample.**

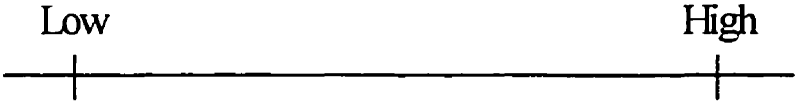
Please evaluate these roast beef samples for a **cooked, processed meat flavour.**



Please evaluate these roast beef samples for a **horseradish flavour** (watercress/celery).



Please evaluate these roast beef samples for **horseradish irritation**.



COMMENTS PLEASE:

Appendix 13. Continued.

COOKED, UNCURED ROAST BEEF–COLOUR EVALUATION

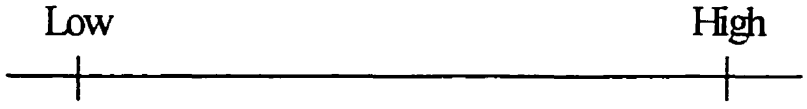
NAME_____ **DATE**_____**1997**

Session ___
Please evaluate the roast beef samples in the following order:

_____ _____ _____ _____ _____ _____

Please evaluate each sample for **COLOUR** only. Remove the lid from the sample container and rate the intensity of the attribute listed below from low to high by placing a vertical mark on the scale. Please label each vertical line with the sample code number. A low and high reference for each attribute is provided. **Replace the lid before moving on to the next sample.**

Please evaluate the intensity of **pink colour** for these roast beef samples.



COMMENTS PLEASE:

Thank You!!!

Appendix 14. Equations used in calculating moisture, ash, protein, fat, salt and phosphate contents of pre-cooked, uncured roast beef.

Determination of Moisture Content:

$$\% \ Moisture = \frac{A - B}{A} \ x \ 100$$

where: A = weight of wet sample plus dish (mg)
B = weight of vacuum dried sample plus dish (mg)

Determination of Ash Content:

$$\% \ Ash = \frac{A - B}{A} \ x \ 100$$

where: A = weight of wet sample plus dish (mg)
B = weight of dried sample (muffle furnace) plus dish (mg)

Determination of Protein Content:

$$\% \ Protein = \% \ nitrogen \ x \ 6.25$$

Determination of Fat Content:

$$\% \ Fat = 100 - (\% \ moisture + \% \ ash + \% \ protein)$$

Determination of Salt Content:

$$\% \ NaCl = \frac{[A - B] \ x \ 0.584}{weight \ of \ sample \ (mg)}$$

where: A = ml of 0.1N $AgNO_3$ added to sample
B = ml of 0.1N NH_4SCN titrated

Determination of Phosphate Content:

$$\% \ P_2O_5 = \frac{[A - B] \ x \ 0.309}{weight \ of \ sample \ (mg)}$$

where: A = ml of 1.0N NaOH titrated
B = (ml of 0.5N H_2SO_4 back titrated)/2

Appendix 15a. Analysis of variance of inhibition of *Listeria monocytogenes* on roast beef exposed to varying concentrations of vaporized horseradish distillate at 12°C.

Day 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	3	2.9408	0.9803	26.23	0.0001*
Error	10	0.3737	0.0374		
Corrected Total	13	3.3145			
	R-square 0.8873	C.V. 5.0881	Root MSE 0.1933	log cfu/cm² Mean 3.7993	

[1]treatment - 2 000 nL/L, 4 000 nL/L and 20 000 nL/L.
*, significantly different at $p < 0.05$.

Day 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	11.2606	3.7535	19.89	0.0002*
Error	10	1.8875	0.1887		
Corrected Total	13	13.1481			
	R-square 0.8564	C.V. 8.8432	Root MSE 0.4345	log cfu/cm² Mean 4.9129	

*, significantly different at $p < 0.05$.

Day 4.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	31.2438	10.4146	11.82	0.0013*
Error	10	8.8090	0.8809		
Corrected Total	13	40.0528			
	R-square 0.7801	C.V. 13.0719	Root MSE 0.9386	log cfu/cm² Mean 7.1800	

*, significantly different at $p < 0.05$.

Day 7.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	38.5870	12.8623	16.52	0.0003*
Error	10	7.7873	0.7787		
Corrected Total	13	46.3743			
	R-square 0.8321	C.V. 10.4645	Root MSE 0.8825	log cfu/cm² Mean 8.4329	

*, significantly different at $p < 0.05$.

Appendix 15b. Analysis of variance of inhibition of *Salmonella typhimuirum* on roast beef exposed to varying concentrations of vaporized horseradish distillate at 12°C.

Day 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	3	2.1435	0.7145	20.70	0.0001*
Error	12	0.4141	0.0345		
Corrected Total	15	2.5576			

R-square	C.V.	Root MSE	log cfu/cm² Mean
0.8381	5.6326	0.1858	3.2981

[1]treatment - 2 000 nL/L, 4 000 nL/L and 20 000 nL/L.
*, significantly different at $p < 0.05$.

Day 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	11.7275	3.9092	24.24	0.0001*
Error	12	1.9352	0.1613		
Corrected Total	15	13.6627			

R-square	C.V.	Root MSE	log cfu/cm² Mean
0.8584	10.6715	0.4016	3.7631

*, significantly different at $p < 0.05$.

Day 4.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	56.6615	18.8872	22.71	0.0001*
Error	12	9.9805	0.8317		
Corrected Total	15	66.6420			

R-square	C.V.	Root MSE	log cfu/cm² Mean
0.8502	16.7375	0.9120	5.4488

*, significantly different at $p < 0.05$.

Day 7.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	41.8310	13.9437	35.89	0.0001*
Error	12	4.6618	0.3885		
Corrected Total	15	46.4928			

R-square	C.V.	Root MSE	log cfu/cm² Mean
0.8997	8.7524	0.6233	7.1213

*, significantly different at $p < 0.05$.

Appendix 15c. Analysis of variance of inhibition of *Escherichia coli* on roast beef exposed to varying concentrations of vaporized horseradish distillate at 12°C.

Day 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	3	4.1012	1.3671	9.61	0.0027*
Error	10	1.4222	0.1422		
Corrected Total	13	5.5234			

R-square	C.V.	Root MSE	log cfu/cm² Mean
0.7425	10.9150	0.3771	3.4550

[1]treatment - 2 000 nL/L, 4 000 nL/L and 20 000 nL/L.
*, significantly different at $p < 0.05$.

Day 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	14.5392	4.8464	14.33	0.0006*
Error	10	3.3811	0.3381		
Corrected Total	13	17.9203			

R-square	C.V.	Root MSE	log cfu/cm² Mean
0.8113	14.2493	0.5815	4.0807

*, significantly different at $p < 0.05$.

Day 4.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	62.3311	20.7770	63.82	0.0001*
Error	10	3.2557	0.3256		
Corrected Total	13	65.5868			

R-square	C.V.	Root MSE	log cfu/cm² Mean
0.9504	10.6155	0.5706	5.3750

*, significantly different at $p < 0.05$.

Day 7.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	101.8806	33.9602	255.63	0.0001*
Error	10	1.3285	0.1329		
Corrected Total	13	103.2091			

R-square	C.V.	Root MSE	log cfu/cm² Mean
0.9871	5.5484	0.3645	6.5693

*, significantly different at $p < 0.05$.

Appendix 15d. Analysis of variance of inhibition of *Staphylococcus aureus* on roast beef exposed to varying concentrations of vaporized horseradish distillate at 12°C.

Day 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	3	1.6038	0.5346	3.08	0.0906
Error	8	0.3903	0.1738		
Corrected Total	11	2.9941			

R-square	C.V.	Root MSE	log cfu/cm² Mean
0.5356	13.1717	0.4169	3.1650

[1]treatment - 2 000 nL/L, 4 000 nL/L and 20 000 nL/L.

Day 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	9.1720	3.0573	11.47	0.0029*
Error	8	2.1324	0.2666		
Corrected Total	11	11.3044			

R-square	C.V.	Root MSE	log cfu/cm² Mean
0.8114	13.5153	0.5163	3.8200

*, significantly different at $p < 0.05$.

Day 4.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	33.7202	11.2401	21.32	0.0004*
Error	8	4.2179	0.5272		
Corrected Total	11	37.9381			

R-square	C.V.	Root MSE	log cfu/cm² Mean
0.8888	15.5652	0.7261	4.6650

*, significantly different at $p < 0.05$.

Day 7.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	81.0503	27.0168	49.49	0.0001*
Error	8	4.3676	0.5459		
Corrected Total	11	85.4179			

R-square	C.V.	Root MSE	log cfu/cm² Mean
0.9488	12.3576	0.7389	5.9792

*, significantly different at $p < 0.05$.

Appendix 15e. Analysis of variance of inhibition of *Serratia grimesii* on roast beef exposed to varying concentrations of vaporized horseradish distillate at 12°C.

Day 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	3	4.8092	1.6031	3.56	0.0672
Error	8	3.6055	0.4507		
Corrected Total	11	8.4148			
	R-square 0.5715	C.V. 16.7346	Root MSE 0.6713	log cfu/cm^2 Mean 4.1007	

[1]treatment - 2 000 nL/L, 4 000 nL/L and 20 000 nL/L.

Day 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	24.4100	8.1367	6.97	0.0127*
Error	8	9.3378	1.1672		
Corrected Total	11	33.7479			
	R-square 0.7233	C.V. 18.8988	Root MSE 1.0804	log cfu/cm^2 Mean 5.7167	

*, significantly different at $p < 0.05$.

Day 4.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	65.4709	21.8236	17.01	0.0008*
Error	8	10.2659	1.2832		
Corrected Total	11	75.7368			
	R-square 0.8645	C.V. 14.6878	Root MSE 1.1328	log cfu/cm^2 Mean 7.7125	

*, significantly different at $p < 0.05$.

Day 7.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	84.7856	28.2619	106.02	0.0001*
Error	8	2.1326	0.2666		
Corrected Total	11	86.9183			
	R-square 0.9755	C.V. 6.0790	Root MSE 0.5163	log cfu/cm^2 Mean 8.4933	

*, significantly different at $p < 0.05$.

Appendix 15f. Analysis of variance of inhibition of *Lactobacillus sake* on roast beef exposed to varying concentrations of vaporized horseradish distillate at 12°C.

Day 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	0.2251	0.0750	0.38	0.7679
Error	8	1.5656	0.1957		
Corrected Total	11	1.7907			

R-square	C.V.	Root MSE	log cfu/cm^2 Mean
0.1257	14.3124	0.4424	3.0908

Day 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	1..9831	0.6610	3.55	0.0672
Error	8	1.4876	0.1859		
Corrected Total	11	3.4707			

R-square	C.V.	Root MSE	log cfu/cm^2 Mean
0.5714	10.9562	0.4312	3.9358

Day 4.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	4.0744	1.3581	2.11	0.1778
Error	8	5.1595	0.6449		
Corrected Total	11	9.2338			

R-square	C.V.	Root MSE	log cfu/cm^2 Mean
0.4412	11.9107	0.8031	6.7425

Day 7.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	3	0.5794	0.1931	4.09	0.0494*
Error	8	0.3779	0.0472		
Corrected Total	11	0.9573			

R-square	C.V.	Root MSE	log cfu/cm^2 Mean
0.6053	2.6181	0.2173	8.3008

*, significantly different at $p < 0.05$.

Appendix 16. Effect of horseradish distillate concentration and storage time on bacterial growth at the surface of roast beef slices stored at 12°C[1, 2].

Vapour Concentration	Log colony forming units / cm²							
	Control	2 000 nL/L	4 000 nL/L	20 000 nL/L	Control	2 000 nL/L	4 000 nL/L	20 000 nL/L
	1 day storage				2 days storage			
L. monocytogenes	4.22 ± 0.19[a]	3.73 ± 0.15[b]	3.37 ± 0.18[bc]	3.04 ± 0.28[c]	5.63 ± 0.43[a]	5.34 ± 0.47[a]	3.98 ± 0.24[b]	3.38 ± 0.65[b]
S. aureus	3.50 ± 0.55[a]	2.99 ± 0.00[ab]	3.11 ± 0.27[ab]	2.47 ± 0.32[b]	4.61 ± 0.69[a]	3.25 ± 0.21[a]	3.02 ± 0.27[a]	2.62 ± 0.07[b]
E. coli	3.95 ± 0.42[a]	3.39 ± 0.51[ab]	2.94 ± 0.15[b]	2.56 ± 0.23[b]	5.04 ± 0.71[a]	3.85 ± 0.50[b]	3.03 ± 0.15[bc]	2.54 ± 0.26[c]
S. typhimurium	3.64 ± 0.22[a]	3.11 ± 0.17[b]	3.03 ± 0.09[b]	2.66 ± 0.08[c]	4.60 ± 0.48[a]	3.30 ± 0.50[b]	2.93 ± 0.19[b]	2.55 ± 0.01[b]
S. grimesii	4.31 ± 0.68[a]	4.15 ± 0.55[ab]	3.94 ± 0.95[ab]	2.66 ± 0.06[b]	6.40 ± 1.33[a]	5.69 ± 0.15[a]	5.97 ± 0.43[a]	2.65 ± 0.10[b]
L. sake	3.00 ± 0.41[a]	3.09 ± 0.50[a]	3.16 ± 0.59[a]	2.81 ± 0.06[a]	4.16 ± 0.52[a]	3.77 ± 0.36[ab]	4.03 ± 0.56[ab]	3.11 ± 0.16[b]
	4 days storage				7 days storage			
L. monocytogenes	8.02 ± 1.12[a]	8.53 ± 0.04[a]	6.48 ± 0.52[b]	3.93 ± 0.83[c]	9.07 ± 1.12[a]	9.66 ± 0.42[a]	8.80 ± 0.11[a]	4.42 ± 0.16[b]
S. aureus	6.25 ± 0.79[a]	3.93 ± 1.27[b]	3.23 ± 0.19[bc]	2.07 ± 0.28[c]	8.25 ± 0.65[a]	5.57 ± 1.54[b]	3.59 ± 0.27[c]	1.72 ± 0.21[d]
E. coli	7.30 ± 0.72[a]	5.28 ± 0.28[b]	3.10 ± 0.12[c]	2.15 ± 0.21[c]	8.88 ± 0.16[a]	7.15 ± 0.98[b]	3.62 ± 0.19[c]	2.26 ± 0.36[d]
S. typhimurium	7.26 ± 1.17[a]	4.56 ± 0.31[b]	3.68 ± 0.26[b]	2.60 ± 0.28[b]	8.39 ± 0.69[a]	7.54 ± 0.87[a]	6.11 ± 0.27[b]	3.62 ± 0.61[c]
S. grimesii	8.63 ± 1.49[a]	8.77 ± 0.28[a]	8.53 ± 0.29[a]	2.49 ± 0.13[b]	9.74 ± 0.69[a]	9.79 ± 0.15[a]	9.14 ± 0.05[a]	2.57 ± 0.02[b]
L. sake	7.24 ± 1.00[a]	6.61 ± 0.43[a]	6.35 ● 0.27[a]	5.71 ± 0.85[b]	8.45 ± 0.29[a]	8.20 ± 0.03[ab]	8.37 ± 0.16[ab]	7.85 ± 0.12[b]

[1] each value represents the logarithm of mean cell density/cm² ± standard deviation (two replicates; exception - three replicates with *E.coli* and *L. monocytogenes* @ 4 000 nL/L and four replicates with *S. typhimurium* @ 4 000 nL/L).

[2] log cfu/cm² at day 0: *L. monocytogenes* - 3.29 ± 0.07; *S. typhimurium* - 3.02 ± 0.14; *E. coli* - 3.15 ± 0.15; *S. aureus* - 2.77 ± 0.20; *S. grimesii* - 3.17 ± 0.08; *L. sake* - 3.02 ± 0.15.

[a-d] means within each row and each time period with different superscripts are significantly different ($P<0.05$).

Appendix 17. Analysis of variance of AIT from horseradish distillate in a roast beef model system at 12°C.

Day 1

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	2	8678.4429	4339.2214	50.65	0.0001*
Error	17	1456.4278	85.6722		
Corrected Total	19	10134.8707			

R-square	C.V.	Root MSE	AIT Mean
0.8563	32.0679	9.2559	28.8635

[1] treatment - 2 000 nL/L, 4 000 nL/L and 20 000 nL/L.
*, significantly different at $p < 0.05$.

Day 2

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	5091.1199	2545.5599	91.86	0.0001*
Error	16	443.3991	27.7124		
Corrected Total	18	5534.5190			

R-square	C.V.	Root MSE	AIT Mean
0.9199	27.5601	5.2643	19.1010

*, significantly different at $p < 0.05$.

Day 4

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	4406.9089	2203.4544	70.14	0.0001*
Error	17	534.0426	31.4143		
Corrected Total	19	4940.9515			

R-square	C.V.	Root MSE	AIT Mean
0.8919	40.4507	5.6048	13.8560

*, significantly different at $p < 0.05$.

Day 7

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	2	1554.3528	777.1764	99.85	0.0001*
Error	16	124.5388	7.7837		
Corrected Total	18	1678.8916			

R-square	C.V.	Root MSE	AIT Mean
0.9258	35.2427	2.7899	7.9163

*, significantly different at $p < 0.05$.

Appendix 18a. Analysis of variance of log cfu/cm^2 on PCA for pre-cooked, uncured roast beef pieces stored under 0 or 20 000 nL horseradish distillate / L air or nitrogen gas.

Replicate 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Block[1]	1	0.1186	0.1186	0.74	0.4096
Treatment[2]	1	48.6941	48.6941	304.11	0.0001*
Time[3]	5	59.9280	11.9856	74.85	0.0001*
Treat*Time	4	37.7713	9.4428	58.97	0.0001*
Error	10	1.6012	0.1601		
Corrected Total	21	176.2052			

R-square	C.V.	Root MSE	Log cfu/cm^2 Mean
0.9909	8.6330	0.4001	4.6351

[1] Block -initial atmosphere (nitrogen flushed and air).
[2] Treatment - concentration of horseradish distillate vapours (0 and 20 000 nL/L).
[3] Time - days 1, 3, 7, 14, 21 and 28.
*, significant at $p < 0.05$.

Replicate 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Block	1	0.0043	0.0043	0.01	0.9047
Treatment	1	6.9408	6.9408	24.13	0.0005*
Time	5	151.5961	30.3192	105.39	0.0001*
Treat*Time	5	1.9360	0.3872	1.35	0.3156*
Error	11	3.1646	0.2877		
Corrected Total	23	163.6419			

R-square	C.V.	Root MSE	Log cfu/cm^2 Mean
0.9807	8.5767	0.5364	6.2538

*, significant at $p < 0.05$.

Replicate 3.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Block	1	0.5885	0.5885	0.46	0.5125
Treatment	1	68.0851	68.0851	53.01	0.0001*
Time	5	135.9515	27.1903	21.17	0.0001*
Treat*Time	5	35.9442	7.1888	5.60	0.0083*
Error	11	14.1295	1.2845		
Corrected Total	23	254.6987			

R-square	C.V.	Root MSE	Log cfu/cm^2 Mean
0.9445	28.1778	1.1334	4.0022

*, significant at $p < 0.05$.

Appendix 18b. Analysis of variance of log cfu/cm^2 on VRBG for pre-cooked, uncured roast beef pieces stored under 0 or 20 000 nL horseradish distillate / L air or nitrogen gas.

Replicate 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Block[1]	1	0.0079	0.0079	0.05	0.8273
Treatment[2]	1	87.7583	87.7583	559.47	0.0001*
Time[3]	5	67.4984	13.4997	86.06	0.0001*
Treat*Time	4	63.4151	15.8538	101.07	0.0001*
Error	10	1.5686	0.1569		
Corrected Total	21	261.2695			

R-square	C.V.	Root MSE	Log cfu/cm^2 Mean
0.9940	12.1802	0.3961	3.2516

[1] Block -initial atmosphere (nitrogen flushed and air).
[2] Treatment - concentration of horseradish distillate vapours (0 and 20 000 nL/L).
[3] Time - days 1, 3, 7, 14, 21 and 28.
*, significant at $p < 0.05$.

Replicate 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Block	1	8.8660	8.8660	2.67	0.1308
Treatment	1	65.1447	65.1447	19.58	0.0010*
Time	5	142.6442	28.5288	8.58	0.0016*
Treat*Time	5	39.3766	7.8753	2.37	0.1085*
Error	11	36.5923	3.3266		
Corrected Total	23	292.6239			

R-square	C.V.	Root MSE	Log cfu/cm^2 Mean
0.8750	50.9127	1.8239	3.5824

*, significant at $p < 0.05$.

Replicate 3.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Block	1	2.3437	2.3437	1.30	0.2781
Treatment	1	81.2304	81.2304	45.13	0.0001*
Time	5	47.6392	9.5278	5.29	0.0101*
Treat*Time	5	47.6392	9.5278	5.29	0.0101*
Error	11	19.7981	1.7998		
Corrected Total	23	198.6506			

R-square	C.V.	Root MSE	Log cfu/cm^2 Mean
0.9003	72.9225	1.3416	1.8397

*, significant at $p < 0.05$.

Appendix 18c. Analysis of variance of log cfu/cm^2 on PCFC for pre-cooked, uncured roast beef pieces stored under 0 or 20 000 nL horseradish distillate / L air or nitrogen gas.

Replicate 1

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Block[1]	1	0.1061	0.1061	0.53	0.4851
Treatment[2]	1	94.1869	94.1869	466.50	0.0001*
Time[3]	5	69.8710	13.9742	69.21	0.0001*
Treat*Time	4	59.1287	14.7822	73.22	0.0001*
Error	10	2.0190	0.2019		
Corrected Total	21	271.8889			

R-square	C.V.	Root MSE	Log cfu/cm^2 Mean
0.9926	13.5086	0.4493	3.3263

[1] Block -initial atmosphere (nitrogen flushed and air).
[2] Treatment - concentration of horseradish distillate vapours (0 and 20 000 nL/L).
[3] Time - days 1, 3, 7, 14, 21 and 28.
*, significant at $p < 0.05$.

Replicate 2

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Block	1	0.0001	0.0001	0.00	0.9891
Treatment	1	150.4434	150.4434	397.99	0.0001*
Time	5	45.8222	9.1644	24.24	0.0001*
Treat*Time	5	74.4213	14.8843	39.38	0.0001*
Error	11	4.1581	0.3780		
Corrected Total	23	274.8451			

R-square	C.V.	Root MSE	Log cfu/cm^2 Mean
0.9849	18.1070	0.6148	3.3955

*, significant at $p < 0.05$.

Replicate 3

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Block	1	0.1052	0.1052	0.62	0.4463
Treatment	1	188.3630	188.3630	1116.98	0.0001*
Time	5	52.3597	10.4719	62.10	0.0001*
Treat*Time	5	93.6856	18.7371	111.11	0.0001*
Error	11	1.8550	0.1686		
Corrected Total	23	336.3685			

R-square	C.V.	Root MSE	Log cfu/cm^2 Mean
0.9945	12.4898	0.4107	3.2879

*, significant at $p < 0.05$.

Appendix 18d. Analysis of variance of log cfu/cm^2 on MRSA for pre-cooked, uncured roast beef pieces stored under 0 or 20 000 nL horseradish distillate / L air or nitrogen gas.

Replicate 1.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Block[1]	1	0.4739	0.4739	1.06	0.3276
Treatment[2]	1	15.1629	15.1629	33.90	0.0002*
Time[3]	5	124.8553	24.9711	55.82	0.0001*
Treat*Time	4	9.4172	2.3543	5.26	0.0152*
Error	10	4.4733	0.4473		
Corrected Total	21	168.9747			

R-square	C.V.	Root MSE	Log cfu/cm^2 Mean
0.9735	16.9875	0.6688	3.9371

[1] Block -initial atmosphere (nitrogen flushed and air).
[2] Treatment - concentration of horseradish distillate vapours (0 and 20 000 nL/L).
[3] Time - days 1, 3, 7, 14, 21 and 28.
*, significant at $p < 0.05$.

Replicate 2.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Block	1	0.0297	0.0297	0.09	0.7654
Treatment	1	0.7605	0.7605	2.40	0.1500
Time	5	218.4009	43.6802	137.58	0.0001*
Treat*Time	5	1.5510	0.3102	0.98	0.4731
Error	11	3.4924	0.3175		
Corrected Total	23	224.2345			

R-square	C.V.	Root MSE	Log cfu/cm^2 Mean
0.9844	10.0584	0.5635	5.6019

*, significant at $p < 0.05$.

Replicate 3.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Block	1	0.0535	0.0535	0.20	0.6671
Treatment	1	21.0576	21.0576	76.83	0.0001*
Time	5	215.2423	43.0485	157.07	0.0001*
Treat*Time	5	16.2857	3.2571	11.88	0.0004*
Error	11	3.0149	0.2741		
Corrected Total	23	255.6539			

R-square	C.V.	Root MSE	Log cfu/cm^2 Mean
0.9882	13.1146	0.5235	3.9919

*, significant at $p < 0.05$.

Appendix 19a. Analysis of variance of nitrogen present in the head space of a roast beef model system at 4°C.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	1	22.8085	22.8085	7.41	0.0131*
Time[2]	5	18.5108	3.7021	1.20	0.3434
Treat*Time	5	42.1269	8.4254	2.74	0.0484*
Replicate	2	139.5105	69.7553	22.67	0.0001*
Error	20	61.5392	3.0770		
Corrected Total	33	297.5688			

R-square	C.V.	Root MSE	Nitrogen Mean
0.7932	1.8851	1.7541	93.05

[1] Treatment -concentration of horseradish distillate vapours (0 and 20 000 nL/L).
[2] Time - days 1, 3, 7, 14, 21 and 28.
*, significant at $p < 0.05$.

Appendix 19b. Analysis of variance of oxygen present in the head space of a roast beef model system at 4°C.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	1	81.8815	81.8815	24.71	0.0001*
Time	5	5.3486	1.0697	0.32	0.8933
Treat*Time	5	90.2579	18.0516	5.45	0.0025*
Replicate	2	105.9556	52.9778	15.99	0.0001*
Error	20	66.2788	3.3139		
Corrected Total	33	329.7788			

R-square	C.V.	Root MSE	Oxygen Mean
0.7990	29.6287	1.8204	6.1441

*, significant at $p < 0.05$.

Appendix 19c. Analysis of variance of carbon dioxide present in the head space of a roast beef model system at 4°C.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	1	22.6001	22.6001	25.09	0.0041*
Time	2	7.6296	3.8148	4.24	0.0839
Treat*Time	2	4.8746	2.4373	2.71	0.1598
Replicate	2	5.2042	2.6021	2.89	0.1466
Error	5	4.5029	0.9006		
Corrected Total	12	50.8326			

R-square	C.V.	Root MSE	Carbon Dioxide Mean
0.9114	50.5197	0.9490	1.8785

*, significant at $p < 0.05$.

Appendix 19d. Analysis of variance of head space gases present in a roast beef model system exposed to 0 nL horseradish distillate / L nitrogen at 4°C.

1. Nitrogen

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Time	5	29.3417	5.8683	1.79	0.2118*
Replicate	2	77.9946	38.9973	11.87	0.0030*
Error	9	29.5663	3.2851		
Corrected Total	16	134.8033			

R-square	C.V.	Root MSE	Nitrogen Mean
0.7807	1.9327	1.8125	93.7794

*, significant at $p < 0.05$.

2. Oxygen

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Model	7	111.0588	15.8655	6.26	0.0070*
Error	9	22.8268	2.5363		
Corrected Total	16	133.8856			

R-square	C.V.	Root MSE	Oxygen Mean
0.8295	33.4576	1.5926	4.76

Source	DF	Type III SS	Mean Square	F value	Pr > F
Time	5	63.6705	12.7341	5.02	0.0179*
Replicate	2	63.8929	31.9464	12.60	0.0025*

*, significant at $p < 0.05$.

3. Carbon Dioxide

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Time	2	20.1623	10.0811	7.50	0.0680
Replicate	2	5.4021	2.7010	2.01	0.2793
Error	3	4.0299	1.3433		
Corrected Total	7	34.0477			

R-square	C.V.	Root MSE	Carbon Dioxide Mean
0.8816	41.8602	1.1590	2.7688

Appendix 19e. Analysis of variance of head space gases present in a roast beef model system exposed to 20 000 nL horseradish distillate / L nitrogen at 4°C.

1. Nitrogen

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Time	5	31.5338	6.3068	4.10	0.0321*
Replicate	2	79.6615	39.8308	25.93	0.0002*
Error	9	13.8274	1.5364		
Corrected Total	16	144.6761			

R-square	C.V.	Root MSE	Nitrogen Mean
0.9044	1.3426	1.2395	92.3206

*, significant at $p < 0.05$.

2. Oxygen

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Time	5	28.0499	5.6100	3.65	0.0166*
Replicate	2	71.6766	35.8383	23.31	0.0341*
Error	9	13.8381	1.5376		
Corrected Total	16	130.7566			

R-square	C.V.	Root MSE	Oxygen Mean
0.8942	16.4712	1.2400	7.5283

*, significant at $p < 0.05$.

3. Carbon Dioxide

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Model	3	0.02936	0.0979	19.97	0.1627
Error	1	0.0049	0.0049		
Corrected Total	4	0.2985			

R-square	C.V.	Root MSE	Carbon Dioxide Mean
0.9836	15.4185	0.0700	0.4540

Source	DF	Type III SS	Mean Square	F value	Pr > F
Time	2	0.0152	0.0076	1.55	0.4935
Replicate	1	0.2704	0.2704	55.18	0.0852

Appendix 20. Analysis of variance of L*, a* and b* value for roast beef pieces exposed to varying atmospheres of nitrogen and vaporized horseradish distillate at 4°C.

1. L* value.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Atmosphere[1]	1	11.5560	11.5560	4.25	0.0465*
Treatment[2]	1	3.0388	3.0388	1.12	0.2975
Time[3]	5	8.3567	1.6713	0.61	0.6893
Atm.*Trt	1	3.0251	3.0251	1.11	0.2956
Trt*Time	4	2.5641	0.6410	0.24	0.9163
Atm.*Time	5	6.6916	1.3383	0.49	0.7799
Atm.*Trt*Time	3	4.8021	1.6007	0.59	0.6264
Error	36	97.8828	2.7190		
Corrected Total	56	145.4931			

R-square	C.V.	Root MSE	L* Mean
0.3272	3.3499	1.6489	49.2226

[1] Atmosphere - air and nitrogen flushed.
[2] Treatment - 0 nL/L or 20 000 nL/L.
[3] Time - 0, 3, 7, 14, 21, 28 days.
*, significantly different at $p < 0.05$.

2. a* value.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Atmosphere	1	0.0237	0.0237	0.19	0.6699
Treatment	1	31.9372	31.9372	250.57	0.0001*
Time	5	19.8666	3.9733	31.17	0.0001*
Atm.*Trt	1	0.0120	0.0120	0.09	0.7604
Trt*Time	4	11.3628	2.8407	22.29	0.0001*
Atm.*Time	5	0.1104	0.0221	0.17	0.9709
Atm.*Trt*Time	3	0.1766	0.0589	0.46	0.7107
Error	36	4.5884	0.1275		
Corrected Total	56	80.2850			

R-square	C.V.	Root MSE	a* Mean
0.9428	3.7396	0.3570	9.5468

*, significantly different at $p < 0.05$.

Appendix 20. Continued.

3. b* value.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Atmosphere	1	0.2385	0.2385	0.46	0.5039
Treatment	1	0.4102	0.4102	0.78	0.3818
Time	5	5.8109	1.1622	2.22	0.0734
Atm.*Trt	1	0.4921	0.4921	0.94	0.3387
Trt*Time	4	3.5250	0.8812	1.68	0.1751
Atm.*Time	5	3.1558	0.6312	1.21	0.3260
Atm.*Trt*Time	3	0.3501	0.1167	0.22	0.8798
Error	36	18.8382	0.5233		
Corrected Total	56	35.3058			

R-square	C.V.	Root MSE	b* Mean
0.4664	9.6251	0.7234	7.5156

Appendix 21a. Analysis of variance of aroma attributes for pre-cooked, uncured roast beef exposed to varying levels of vaporized horseradish distillate at 4°C.

Beef aroma

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	5	606.9126	121.3825	31.18	0.0001*
Time[2]	1	0.0012	0.0012	0.00	0.9858
Treatment*Time	5	19.2464	3.8493	0.99	0.4250
Error	271	1055.0649	3.8932		
Corrected Total	282	1681.6544			

R-Square	C.V.	Root MSE	Beef aroma Mean
0.3726	38.6326	1.9731	5.1074

[1] treatment - 0 nL/L-3 days; 0 nL/L-15 days; 4 000 nL/L-3 days; 4 000 nL/L-15 days; 20 000 nL L-3 days; 20 000 nL L-15 days.
[2] time - am/pm evaluations.
*, significantly different at $p < 0.05$.

Horseradish aroma

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	5	1172.5306	234.5061	77.78	0.0001*
Time	1	0.1467	0.1467	0.05	0.8256
Treatment*Time	5	3.1839	0.6368	0.21	0.9576
Error	276	832.1788	3.0151		
Corrected Total	287	2008.0400			

R-Square	C.V.	Root MSE	Horseradish aroma Mean
0.5856	48.9179	1.7364	3.5497

*, significantly different at $p < 0.05$.

Horseradish irritation aroma

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	5	764.3808	152.8762	63.07	0.0001*
Time	1	0.1058	0.1058	0.04	0.8346
Treatment*Time	5	1.2657	0.2531	0.10	0.9912
Error	275	666.5495	2.4238		
Corrected Total	286	1432.3291			

R-Square	C.V.	Root MSE	Horseradish irritation aroma Mean
0.5346	58.8695	1.5569	2.6446

*, significantly different at $p < 0.05$.

Appendix 21b. Analysis of variance of flavour attributes for pre-cooked, uncured roast beef exposed to varying concentrations of vaporized horseradish distillate at 4°C.

Beef flavour

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	4	585.5798	146.3950	57.66	0.0001*
Time[2]	1	1.1129	1.1129	0.44	0.5087
Treatment*Time	4	21.3810	5.3452	2.11	0.0183*
Error	213	540.8206	2.5391		
Corrected Total	222	1164.6326			

R-Square	C.V.	Root MSE	Beef flavour Mean
0.5356	31.5828	1.5934	5.0453

[1] treatment - 0 nL/L-3 days; 0 nL/L-15 days; 4 000 nL/L-3 days; 4 000 nL/L-15 days; 20 000 nL/L-3 days; 20 000 nL/L-15 days.
[2] time - am/pm evaluations.
*, significantly different at $p < 0.05$.

Horseradish flavour

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	4	1000.0339	250.0085	60.40	0.0001*
Time	1	0.2189	0.2189	0.05	0.8183
Treatment*Time	4	32.2630	8.0657	1.95	0.1036
Error	214	885.8298	4.1394		
Corrected Total	223	1945.6196			

R-Square	C.V.	Root MSE	Horseradish flavour Mean
0.5447	47.8868	2.0345	4.2487

*, significantly different at $p < 0.05$.

Horseradish irritation flavour

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	4	1210.0623	302.5156	104.34	0.0001*
Time	1	0.3751	0.3751	0.13	0.7194
Treatment*Time	4	7.1392	1.7848	0.62	0.6519
Error	214	620.4753	2.8994		
Corrected Total	223	1858.6886			

R-Square	C.V.	Root MSE	Horseradish irritation flavour Mean
0.6662	47.2054	1.7028	3.6071

*, significantly different at $p < 0.05$.

Appendix 21c. Analysis of variance of colour attribute for pre-cooked, uncured roast beef exposed to varying concentrations of vaporized horseradish distillate at 4°C.

Pink colour

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	5	836.7249	167.3450	81.97	0.0001*
Time[2]	1	6.9378	6.9378	3.40	0.0663
Treatment*Time	5	9.0149	1.8030	0.88	0.4928
Error	276	563.4746	2.0416		
Corrected Total	287	1416.1522			

R-Square	C.V.	Root MSE	Pink colour Mean
0.6021	30.3269	1.4288	4.7115

[1] treatment - 0 nL/L-3 days; 0 nL/L-15 days; 4 000 nL/L-3 days; 4 000 nL/L-15 days; 20 000 nL/L-3 days; 20 000 nL/L-15 days.
[2] time - am/pm evaluations.
*, significantly different at $p < 0.05$.

Appendix 22. Analysis of variance of L*, a* and b* value for roast beef pieces measured immediately after storage at 4°C.

1. L* value.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	5	3.3684	0.6737	0.53	0.7467
Time[2]	1	0.6240	0.6240	0.49	0.5136
Replicate	1	0.4187	0.4187	0.33	0.5898
Trt*Time	5	5.8232	1.1646	0.92	0.5346
Trt*Rep	5	3.1558	0.6312	0.50	0.7678
Time*Rep	1	2.3250	2.3250	1.84	0.2330
Error	5	6.3185	1.2637		
Corrected Total	23	22.0338			

R-square	C.V.	Root MSE	L* Mean
0.7132	2.2475	1.1241	50.01625

[1]treatment - 0 nL/L-3 days; 0 nL/L-15 days; 4 000 nL/L-3 days; 4 000 nL/L-15 days; 20 000 nL/L-3 days; 20 000 nL/L-15 days.
[2]time - am/pm.

2. a* value.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	5	19.1380	3.8276	28.06	0.0011*
Time	1	0.3626	0.3626	2.66	0.1639
Rep	1	0.4401	0.4401	3.23	0.1324
Trt*Time	5	0.4006	0.0801	0.59	0.7132
Trt*Rep	5	0.2490	0.0498	0.37	0.8535
Time*Rep	1	0.7315	0.7315	5.36	0.0684
Error	5	0.6819	0.1364		
Corrected Total	23	22.0038			

R-square	C.V.	Root MSE	a* Mean
0.9690	4.0253	0.3693	9.1746

Appendix 22. Continued.

3. b* value.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	5	1.3293	0.2659	2.11	0.2154
Time	1	0.04420	0.0442	0.35	0.5791
Rep	1	0.3480	0.3480	2.77	0.1571
Trt*Time	5	1.0772	0.2154	1.71	0.2846
Trt*Rep	5	0.6195	0.1239	0.99	0.5064
Time*Rep	1	0.8400	0.8400	6.68	0.0492*
Error	5	0.6289	0.1258		
Corrected Total	23	4.8870			

R-square	C.V.	Root MSE	b* Mean
0.8713	4.6493	0.3546	7.6279

Appendix 23. Analysis of variance of L*, a* and b* value for roast beef pieces measured 1.75 hours after storage at 4°C.

1. L* value.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment[1]	5	5.1186	1.0237	0.84	0.5736
Time[2]	1	0.7038	0.7038	0.58	0.4816
Replicate	1	1.1051	1.1051	0.91	0.3848
Trt*Time	5	8.6355	1.7271	1.42	0.3558
Trt*Rep	5	1.6587	0.3317	0.27	0.9102
Time*Rep	1	2.0945	2.0945	1.72	0.2469
Error	5	6.0952	1.2190		
Corrected Total	23	25.411			

R-square	C.V.	Root MSE	L* Mean
0.7601	2.2087	1.1041	49.9896

[1]treatment - 0 nL/L-3 days; 0 nL/L-15 days; 4 000 nL/L-3 days; 4 000 nL/L-15 days; 20 000 nL/L-3 days; 20 000 nL/L-15 days.
[2]time - am/pm.

2. a* value.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	5	17.2299	3.4460	38.09	0.0006*
Time	1	0.5046	0.5046	5.58	0.0646
Replicate	1	0.0122	0.0122	0.13	0.7290
Trt*Time	5	0.8602	0.1720	1.90	0.2488
Trt*Rep	5	0.3302	0.0660	0.73	0.6309
Time*Rep	1	0.2091	0.2091	2.31	0.1889
Error	5	0.4523	0.0905		
Corrected Total	23	19.5983			

R-square	C.V.	Root MSE	a* Mean
0.9769	3.5237	0.3008	8.5358

*, significantly different at $p < 0.05$.

Appendix 23. Continued.

3. b* value.

Source	DF	Sum of Squares	Mean Square	F value	Pr > F
Treatment	5	1.5863	0.3173	3.37	0.1044
Time	1	0.0067	0.0067	0.07	0.8009
Replicate	1	0.2817	0.2817	2.99	0.1444
Trt*Time	5	0.8336	0.1667	1.77	0.2732
Trt*Rep	5	0.4622	0.0924	0.98	0.5081
Time*Rep	1	0.4760	0.4760	5.05	0.0745
Error	5	0.4711	0.0942		
Corrected Total	23	4.1176			
	R-square 0.8856	C.V. 4.134	Root MSE 0.3070	b* Mean 7.4258	

Appendix 24. Composition of media used in the antimicrobial evaluation of horseradish distillate.

Pseudomonas Agar Base (Oxoid)

Component	Amount (g/L)
gelatin peptone	16.0
casein hydrolysate	10.0
K_2SO_4	10.0
$MgCl_2$	1.4
agar	11.0

Pseudomonas CFC Supplement (Oxoid)

Component	Amount (mg/vial)
cetrimide	5.0
fucidin	5.0

Trypticase Soy Broth (BBL)

Component	Amount (g/L)
pancreatic digest of casein	17.0
papaic digest of soybean meal	3.0
sodium chloride	5.0
dipotassium phosphate	2.5
dextrose	2.5

Plate Count Agar (Difco)

Component	Amount (g/L)
Bacto tryptone	5.0
yeast extract	2.5
dextrose	1.0
agar	15.0

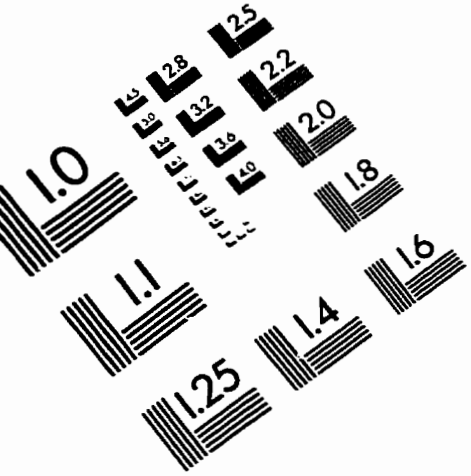
Violet Red Bile Glucose Agar (Oxoid)

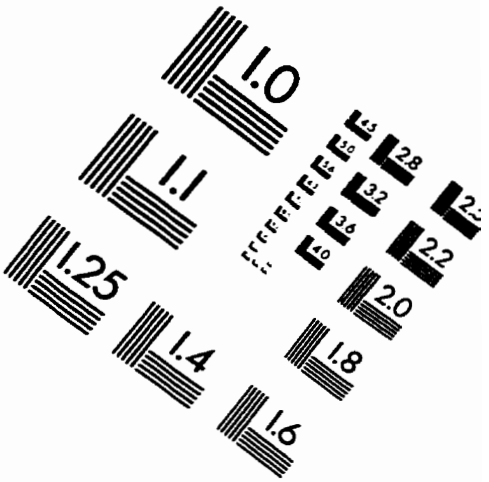
Component	Amount (g/L)
yeast extract	3.0
peptone	7.0
sodium chloride	5.0
bile salts No. 3	1.5
glucose	10.0
neutral red	0.03
crystal violet	0.002
agar	12.0

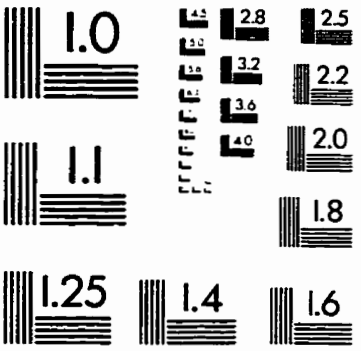
MRS Broth (Oxoid)

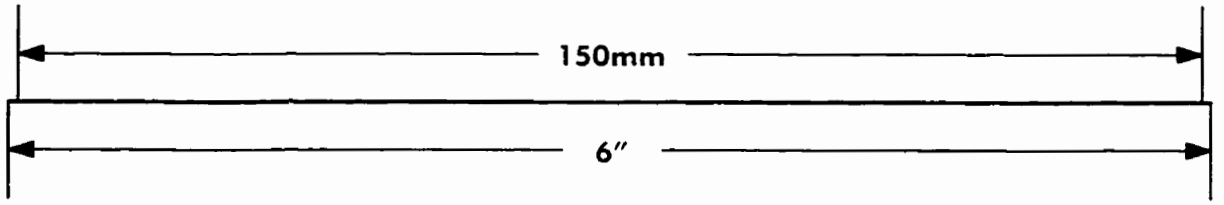
Component	Amount (g/L)
peptone	10.0
'Lab-Lemco' powder	8.0
yeast extract	4.0
glucose	20.0
'Tween' 80	1 ml
di-potassium hydrogen phosphate	2.0
sodium acetate $3H_2O$	5.0
tri-ammonium citrate	2.0
magnesium sulphate $7H_2O$	0.2
manganese sulphate $4H_2O$	0.05

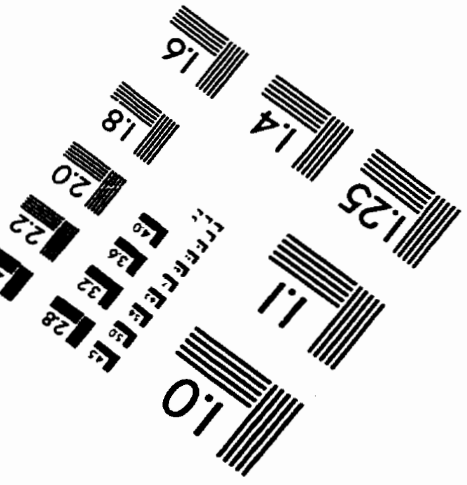
IMAGE EVALUATION TEST TARGET (QA-3)

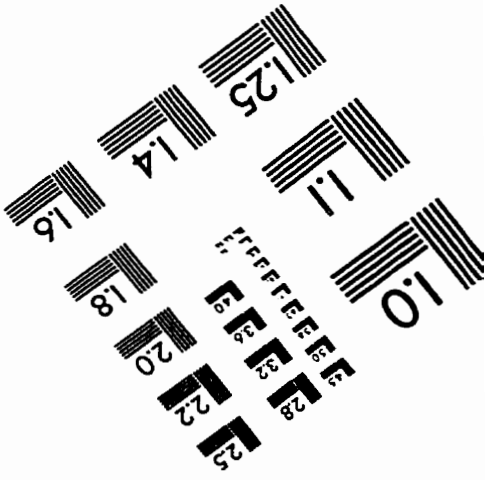












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