# High Oxygen as an additional factor in Food Preservation

Promotor:
**Prof. Dr. ir. F.M. Rombouts**
Hoogleraar in de Levensmiddelenhygiëne en microbiologie, Wageningen Universiteit

Copromotors:
**Dr. L.G.M. Gorris**
SEAC, Unilever, Colworth House, Verenigd Koninkrijk
**Dr. E.J. Smid**
Groupleader Natural Ingredients, NIZO Food Research, Ede

Samenstelling promotiecommissie:

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| **Prof. Dr. J.T.M. Wouters** | (Wageningen Universiteit) |
| **Dr. J. Hugenholtz** | (NIZO Food Research, Ede) |

Athina Amanatidou

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**Proefschrift**

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*To my parents,*
*my brother and to Erik*

## Abstract

In this thesis, the efficacy of high oxygen as an additional hurdle for food preservation is studied. At high oxygen conditions and at low temperature, significant impairment of growth and viability of bacterial cells is found to occur as the result of free radical attack. The imposed oxidative stress leads to an increase of intracellularly generated reactive oxygen species (mainly $O_2^-$, $H_2O_2$ and HO·), which disturbs the cellular homeostasis due to catabolic imbalance and results in growth inhibition. The so-called "free radical burst" probably is responsible for the induction of a host defence mechanism against the destructive impact of high oxygen. Different *Lactobacillus sake* strains possess endogenous levels of antioxidative properties (metal chelating capacity, hydroxyl radical scavenging and reducing properties) which play an important role in protection against oxidative stress. Superoxide dismutase is demonstrated to be, among others, a main antioxidative enzyme in oxygen insensitive strains and mutants of *Lactobacillus sake*.

Although most aerobic organisms possess biochemical mechanisms that contribute to their resistance to oxygen stress conditions, high oxygen is an efficient preservation strategy if used in combination with other antimicrobial factors (hurdles). The impact of the combined application of high oxygen with refrigeration, carbon dioxide and/or high pressure on microbial stability and physicochemical properties of minimally processed fresh produce (carrots and salmon) is investigated.

# Contents

## Outline of this thesis

The topic of this thesis is the use of high concentrations of gaseous oxygen (high $O_2$) in the context of the food preservation strategy referred to as Hurdle Technology (Combination Preservation) in order to control growth of food associated spoilage and pathogenic micro-organisms. The biological effects of elevated $O_2$ concentrations are studied, with specific attention to the mechanisms underlying $O_2$ toxicity in the case of *Lactobacillus sake* strains. Combined application of high $O_2$ with other hurdles to extend the shelf life of two food products is evaluated.

The concept of hurdle technology, the phenomenon of oxidative stress and the potential of high $O_2$ to serve as an additional barrier to growth and survival of food-associated micro-organisms are discussed in **Chapter 1**.

In **Chapter 2** the impact of high $O_2$ (>40% $O_2$) on growth characteristics of selected pathogenic and spoilage micro-organisms able to grow at refrigeration temperature is studied in laboratory media.

In **Chapter 3,** the effect of high $O_2$ as an inimical factor in *L. sake* is investigated. It is shown that *L. sake* isolates derived from a variety of food sources differ in their ability to cope with oxidative stress due to differences in their antioxidative capabilities. An increase in the intracellular level of $H_2O_2$ is the direct result of the exposure to high $O_2$. The role of $H_2O_2$ in $O_2$ toxicity in *L. sake* is therefore investigated in detail.

In **Chapter 4** the importance of superoxide dismutase (SOD) as a primary defence mechanism against $O_2$ toxicity in *L. sake* is demonstrated. Using two strains of *L. sake* with different responses to high $O_2$, the role of SOD for survival under $O_2$ toxicity conditions is evaluated in cell-free extracts by correlating the level of intracellularly generated superoxide radicals with that of SOD.

In **Chapter 5** the effect of high $O_2$ in combination with high $CO_2$ modified atmospheres (MA) and refrigeration on growth of food spoilage micro-organisms and on physicochemical characteristics of carrot slices is assessed. The advantages and drawbacks of high $O_2$-MA versus the commonly used low $O_2$ -MA are discussed.

In **Chapter 6** it is shown that combinations of $O_2$ and $CO_2$ under pressure can be used to effectively reduce initial microbial contamination of heat-sensitive products such as salmon and microbiological spoilage during refrigerated storage.

In **Chapter 7** the main findings of this thesis are summarised. The significance of high $O_2$ as an additional hurdle for preservation of minimally processed products is discussed.

# Chapter 1
# Introduction

## 1.1 Background

In the past, prolonging shelf-life of foods was attained mainly by relatively harsh preservation technologies based on thermal treatment (e.g. sterilisation, pasteurisation, drying). Although these processes are relatively simple and inexpensive, their application for preservation of sensitive commodities, like minimally processed fruit, vegetables and fish, is limited due to adverse effects on nutritional, sensory and rheological properties. The appreciation of consumers for such products has declined as they expect foods to be natural and wholesome. Alternative preservation technologies (e.g. modified atmosphere packaging, high pressure treatment) have therefore received great attention as they may allow better to maintain or improve the sensory attributes and total quality of heat sensitive commodities. Additionally, the demand for convenient products that fit into the modern consumers' life-style and habits has made it evident that new preservation strategies and innovative preservation technologies need to be explored that assure food safety but still satisfy consumer expectations. A preservation strategy that potentially fulfils this demand is called *Hurdle Technology* (Leistner and Rödel, 1976). A significant amount of scientific literature has been published on hurdle technology in recent years, indicating the enormous popularity and potential of this concept that seeks to combine the benefits of traditional and more novel methods for food preservation. A method frequently used in hurdle technology strategies for the preservation of perishable commodities and heat-sensitive processed foods is Modified Atmosphere packaging. Since $O_2$ is known to exert significant inhibitory effect on biological systems, this thesis investigates the potential benefits of high $O_2$ concentrations in modified atmospheres following the hurdle technology concept. The studies presented were undertaken on the basis of the hypothesis that high $O_2$ modified atmospheres, through applying sufficient oxidative stress for control of undesirable micro-organisms, could be a promising preservative factor when combined with other appropriate hurdles.
The following paragraphs introduce the hurdle technology concept, discuss existing knowledge with respect to oxidative stress in biological systems and evaluate future prospects for the use of high $O_2$ for food preservation.

## 1.2 Hurdle technology for preservation of minimally processed foods

### Definition and history

Minimal processing (MP) methods include all those processes that minimise unadvantageous changes in food quality, freshness and/or appearance during shelf-life but still provide the food product with a sufficient length of shelf-life to be transported from the production site to the consumer (Ohlsson, 1994). MP foods can be found in all food categories (fruit, vegetables, fish, dairy and meat) and encompass a great variety of new, renewed and improved product formulations and processing designs. Table 1 lists some preservation methods used to produce MP foods, some of which have been proposed for immediate use, while others are identified for future application. The preservations techniques presented in Table 1 inhibit or inactivate microbial growth and delay deterioration reactions to different extends and their application should be carefully tailored to the characteristics of the food product concerned.
Hurdle technology, sometimes also referred to as combined processes, combination preservation, combined methods or barrier technology (Leistner, 2000), is a strategy that fits well with the production of MP foods. It advocates that optimal preservation can be achieved by applying intelligent combinations of different hurdles that are suitable for the product and production process considered. While a heat treatment like pasteurisation relies on the application of a single, lethal hurdle to

inactivate the nonsporeforming micro-organisms, according to the hurdle technology concept it should be possible to obtain an equal or better preservation effect by applying a suitable combination of hurdles that individually have a sublethal effect. The number, intensity and sequence of hurdles required for safety and stability of the food product depend on the initial population of the micro-organisms present and on the characteristics of the food product. For example, in a food that is rich in nutrients, growth of micro-organisms will be fostered and therefore the intensity of some or all of the hurdles applied must be sufficiently high, but the impact of the hurdles on product quality characteristis may limit the intensity of the hurdles.

***Table 1*** *Preservation factors applied or having potential for use in minimally processed food products (adapted from Alzamora et al., 2000)*

| Currently in Use | Having potential for immediate use | Having potential for future use |
|---|---|---|
| Superficial cleaning | Ozonation | Irradiation |
| Treatment in water chlorine | Slight $a_w$ depression (0.95-0.99) | Electric pulses |
| Aseptic packaging | pH controls (3.5-4.4) | Ultrasound |
| Modified atmosphere packaging | Traditional antimicrobials (e.g. sorbate) | Natural antimicrobials |
| Refrigeration | Other additives (e.g. ascorbic acid) | Light pulses |
| Vacuum cooking | High pressure | Magnetic fields |
| Edible film covering | Biocontrol with LAB | Microstructure control |
| | Active packaging | |

The hurdle concept was initially applied to the meat sector (Leistner and Rödel, 1976) and was later extended for the development of new bakery, dairy and fish products, cheese spreads and processed vegetables and fruits (Leistner, 1992, 1999; Leistner and Gorris, 1995; Gorris, 2000). Over the years, the insight into the hurdle effect has been broadened and the application of hurdle technology extended. Up to now, over 60 different hurdles have been identified in food preservation. Apart from the most important and commonly used hurdles such as temperature, pH, and water activity, there are many others of potential value. Hurdles can be based on physical (high temperature, high pressure, ultrasonication, food microstructure, etc), physicochemical (pH, redox potential, $a_w$, ozone, oxygen, carbon dioxide, spices) or other mechanisms (competitive flora, bacteriocins, chitosan). Every day new data are published on methods, which affect microbial growth alone or in combination with other preservation techniques. According to Alzamora et al., (1998) the hurdle technology concept can be applied in the design of preservation systems for minimally processed foods in three ways:

- As a backup measure in existing minimally processed products with short shelf-life to further diminish pathogenic hazards during storage, processing and or packaging (e.g. natural antimicrobials in combination with refrigeration),
- As a tool for improving quality of long shelf-life products without compromising their microbial stability (e.g. mild heat treatment in combination with $a_w$ lowering solutes)
- As a new preservation procedure deliberately designed to obtain novel food products (e.g. use of ultra high pressure treatment for the manufacture of MP foods with new product structures)

A better understanding of the impact and interaction of different preservation factors in foods is the basis for further improvements in food preservation systems.

## Basic aspects of hurdle technology

A key factor for successful application of the hurdle concept for control of microbial growth is the understanding of the physiological responses of micro-organisms upon exposure to certain hurdles that

allow them to survive and/or grow dispite the stress conditions applied. The physiological response mechanisms are collectively referred to as *homeostasis* mechanisms. Homeostasis is the constant tendency of micro-organisms to maintain a stable and balanced (uniform) internal environment (such as $pH_{in}$, proton motive force, ATP levels, water content, osmolarity). Food preservation is achieved by succesfully disturbing the homeostasis of micro-organisms (Gould, 1988). Preservative factors functioning as hurdles can disturb one or more of the mechanisms of homeostasis, thereby preventing micro-organisms from multiplying and causing them to remain in the lag phase or even die before their homeostasis is restored. Interference with homeostasis at a number of different sites, or in a co-operative manner, forms the rational for some of the observed *additive* or *synergistic* effects of combination preservation procedures. Two or more substances or preservation factors acting in concert may have a synergistic effect when their toxic potency is greater than the sum of each of the potencies when acting alone. For example, pH may be used as an individual factor to reduce microbial growth; introducing low water activity ($a_w$) as an additional hurdle may produce a synergistic effect; relatively small changes to both hurdles together may be as effective as large changes to either hurdle in isolation. Synergistic actions have been observed upon simultaneous application of, for instance, heat and hydrostatic pressure; low pH and salt; low $a_w$, pH and antimicrobial compounds; low $a_w$, pH and modified atmosphere packaging (Chirife, 1993; Tapia de Daza et al., 1996). Another phenomenon of practical importance in food preservation is the so-called *metabolic exhaustion*, which is relevant for micro-organisms that survive but not grow in a product (Leistner, 1995a). When micro-organisms are sublethally injured but still metabolically active they will activate every possible repair mechanism to restall homeostasis in an effort to overcome the hostile environment. By doing so, they may completely consume all their metabolic energy reserves, become metabolically exhausted and, ultimately, die (Leistner, 1995b). Applying conditions that favour metabolic activity (e.g. storing a food product at ambient temperature rather than keeping it refrigerated) will speed up metabolic exhaustion and may thus influence the preservation process. However, the use of the metabolic exhaustion phenomenon in preservation systems should be considered with due care. Certainly in the case of temperature, the importance of low temperature as an important factor to assure the safety and to enhance the stability of MP products should not be disregarded (Baross and Morita, 1978; Gorris and Peck, 1998).

### Risks related to hurdle technology and minimal processing

It is generally recognised that heat treatment is a very effective way to achieve sterilisation of food products. However, the concepts of hurdle technology and minimal processing aim at creating sufficient shelf-life or, in other words, conditions where the outgrowth of harmful, pathogenic micro-organisms possibly present is well controlled. In all cases, it should be considered that apparently small variations in the raw material or the preservation system might lead to reduced control over microbial growth. The availability of carbon and energy sources differ between seemingly similar products. In certain foods, compounds such as osmoprotectants and vitamins may enable or strongly stimulate growth of specific micro-organisms when present (*booster effect*). An example of this booster effect is the osmoprotectant effect of carnitine in meat which has been studied in *Listeria monocytogenes* (Beumer et al., 1994; Gorris and Peck, 1998). With the increasing popularity of MP fresh foods that are preserved only by relatively mild preservation techniques, new ecological routes for microbial growth may emerge. A multitude of stresses may give rise to survivors that cannot be recovered by conventional techniques. Bacteria can become resistant or even more virulent after exposure to mild stress due to the possession of specific anti-stress proteins and genes. The use of mild techniques may result in the development of cross protection to hurdles that would otherwise be lethal or sublethal. Some examples of cross-protection to heat, acid, osmotic or oxidative stress and starvation have recently been reported for micro-organisms such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium*, *Lactococcus lactis*, etc. growing in laboratory

medium or real food (Layer and Johnson, 1993; Blackburn and Davies, 1994; Lou and Yousef, 1996; O'Sullivan and Condon, 1997; Rowe and Kirk, 1999). The direct and indirect physiological responses of micro-organisms to stress may lower the success of a combination processing strategy when it is not carefully considered in the design of the preservation system. Considering the range of homeostasis mechanisms in micro-organisms, Leistner (2000) suggested that "multitarget preservation" would be the best way to achieve effective preservation and to avoid cross protection. Best results should be obtained when different stresses that target at different sites of the micro-organism and/or impact on different response mechanisms are applied at the same time. This "multitarget" approach is expected to spcecially enhance the synergistic effect of different hurdles and thus allow for optimal microbial stability.

## 1.3 Oxidative stress as an additional hurdle

### Oxidative stress and food preservation

When the level of Reactive Oxygen Species (ROS) generated in a living organism is higher than the ROS scavenging capacity of that organism, ROS may accumulate to levels that cells cannot cope with and a situation referred to as "oxidative stress" condition develops. The main areas of food preservation where the manipulation of ROS levels, whether deliberate or accidental, can have a practical relevance are the following:

- The use of ROS as preservatives or disinfectants; in this case, ROS are used to maximise oxidative stress with the intention of inactivating as many undesirable contaminating micro-organisms as possible.
- Accurate quantitative recovery of micro-organisms for evaluation of the efficacy of a preservation technique; an example is recovery of micro-organisms under anaerobic conditions and in the presence of catalase; in that case, ROS are minimised to avoid suboptimal recovery due to oxidative poisoning of the (usually already) stressed micro-organisms.
- The indirect use of oxidative stress to increase the effectiveness of traditional preservation techniques such as enhancement of "free radical burst" or maximisation of the suicide response (Dodd et al., 1997; Sigler et al., 1999; Aldsworth et al., 1999).

Free radicals in foods usually originate from interactions of food components with $O_2$ (Mohanan et al., 1997). UV light and other types of radiation are important causal agents for ROS, leading to the generation of oxidative stress. Chilling injury, freeze drying treatment and antibiotics are also believed to induce oxidative stress to some degree. Several researchers reported increased production of radicals during heating, ultra high pressure and manothermosonication treatments (George et al., 1998; Vercet et al., 1998; Robey et al., 1998). McMeekin et al., (2000) suggested that a new generation of mild food preservation techniques could be created, based on the observation that oxidative stress is developed at growth/no growth interfaces resulting in a cumulative damage and ultimate death of the microbial cells present. There are, at least, three ways to increase ROS levels and therefore increase oxidative stress in a food product:

1. By increasing the steady-state level of the radicals generated (e.g. directly by increasing the concentration of $O_2$ in the food or indirectly by increasing the environmental pressure which results in an increase of the $O_2$ concentration in the food),
2. By removing water from food products, or,
3. By adding radical generating agents (e.g. by adding chlorine).

Part of this thesis deals with the first of the three methods listed above, increasing ROS levels directly and/or indirectly by means of modified atmosphere packaging or high pressure. The following paragraphs will discuss the current understanding of $O_2$ as a factor that can generate or enhance oxidative stress and thus function as an antimicrobial hurdle in a food preservation system.

***Table 2**. Oxygen radicals of interest for food preservation*

| Free radical | | Mechanism of generation/sources in food | Physiological role |
|---|---|---|---|
| Oxides of nitrogen | $NO^{\cdot}$ | Reaction of L-arginine | Give rise to toxic $NO^{\cdot}_2$ upon reaction with $O_2$ |
| Thiyl radical | $RS^{\cdot}$ | Attack of proteins at low temperatures. Oxidation of R-SH in the presence of transition metals | Ascorbic acid oxidation |
| Peroxyl radical | $RO_2^{\cdot}$ | $O_2$ uptake by conjugated dienes | Propagates lipid peroxidation by abstracting $H^{\cdot}$ |
| Superoxide anion radical | $O_2^{-}$ | Spontaneous oxidation of ferredoxin, sulfite, thiols, flavoprotein and non-flavoprotein oxidases, oxidation of photo-reduced flavins | Reduction of ascorbic acid, catechols, thiols. Indirect involvement in damage by generation of other radicals |
| Ozone | $O_3$ | Photochemical reactions<br>No biological systems known | Reaction with polyunsaturated fatty acids, lipid oxidation, off-flavours of spray-dried milk, nitro compounds developments in dried protein foods, discoloration |
| Hydrogen peroxide | $H_2O_2$ | (Flavoprotein) oxidases, autoxidation of ascorbic acid (metal catalysed) | Low reactivity. Precursor for more ROS reactions with proteins |
| Singlet oxygen | $^1O_2$ | Photosensitized generation, Lactoperoxidase, $H_2O_2$, Cl-Chlorophyll, hematoporphyrins<br>Recombination of peroxyl radicals | Effect on natural pigments, killing of micro-organisms, cold pasteurization, autoxidation of vegetable oils, oxidation of histidine, tryptophan |
| Hydroxyl radical | $HO\cdot$ | Fenton reaction and Haber-Weiss reaction | Degradation of polysaccharides, DNA and other macromolecules |

## Oxidative stress and food quality

Oxidative changes in foods are important in terms of nutritional quality, palatability and possibly even toxicity. Free radicals occur in foods during processing and preservation as products of auto-oxidation of food components, due to transfer of electrons onto oxygen or through enzyme mediated synthesis. The interactions of ROS with biologically significant molecules are important for the chemical degradation of food (Thomas, 1995). Chilling injury is related to the increase in the free-radical content of stored fruits and vegetables. In Table 2, a list of oxidative processes related to food constituents is presented. Several physical and organoleptic changes in foods are the result of a pro-oxidative activity (activity of compounds that accelerate oxidation reaction) causing measurable changes in colour (e.g. Maillard reactions products), texture, taste and off-odours (Decker, 1998). Oxidation of fats results in the development of rancid off-flavours and affects the stability or nutritional value of many foods (Chan, 1987). Oxidative damages to lipids cause detectable food deterioration, whereas damages to proteins may not be organoleptically noticed. Lipid oxidation is a self-catalysed chemical reaction and the usual cause of deterioration of fat containing foods. When $O_2$ is present at a low concentration, the rate of oxidation is directly proportional to the concentrations and types of lipids that are oxidised.

Decker (1998) reviewed the strategies available to control free radicals generation and maximise oxidative stability of food products. Antioxidants are the means to ensure the quality of the food supply (Giese, 1996). Plant extracts such as garlic, ginger, green tea, thyme, oregano, clove, mango and onions possess antioxidant properties due to the presence of flavonoids and other polyphenols (Chang et al., 1977). Flavonoids occur naturally in several products (e.g. lard, butter, milk) and are components of many folk medicines (Aruoma, 1997). Types of antioxidants currently used are the so-called free radical terminators (i.e. butylated hydroxyanisole BHA, butylated hydroxytoluene BHT,

tocopherols, propyl gallate). Antioxidative enzymes (i.e. superoxide dismutase, catalase, oxidases, peroxidases) offer antioxidative protection to the biological systems in which they actively occur, but their effectiveness depends on a range of factors such as mechanism of action of the compound, target-biomolecules, distance of the antioxidant from the target, pH, temperature (Halliwell et al., 1995; Meyer and Isaksen, 1995).

## 1.4 High oxygen modified atmospheres and food preservation

### Modified atmosphere packaging

In modified atmosphere (MA) packaging, the proportions of the various gases or the pressure of the atmosphere inside the package has been changed as compared to that of ambient air. The proportion of gases inside a package can be changed passively, by using properly semi-permeable packaging materials, or actively, by using a specific gas mixture, together with semi-permeable or impermeable packaging material (Gorris and Peppelenbos, 1992; Church and Parson, 1994; Zagory, 1995). The main gases used in mixtures are $CO_2$, $N_2$ and $O_2$, but the potential of other gases (i.e. CO, Ar, $O_3$) has been extensively reviewed as well (Zagory and Kader, 1988; Kim et al., 1999). Food preservation systems using reduced pressure atmospheres (hypobaric storage or vacuum packaging) can be used in specific cases. The optimal gas atmosphere composition and storage conditions depend on the type of product and the desired effect, which is often a slowing down of the respiration rate to reduce produce metabolism and maturation (Kader et al., 1989; Phillips, 1996; Jay, 2000). For example, in minimally processed vegetables (either whole, segmented or cut), the respiration rate is reduced by employing a gas atmosphere with an $O_2$ level low enough to maintain basic produce metabolism and using storage of the packed product at as low as possible a refrigeration temperature (mostly between 2 and 8$^oC$) depending temperature sensitivity of the produce (Gorris and Taucher, 1999). Storage life at 8$^oC$ of low oxygen MA packaged minimally processed produce can be up to 5 to 7 days.

### Applications of high $O_2$ MA for food preservation

A high concentration (60 to 85%) of $O_2$ is recommended for poultry and fresh meat (Farber, 1991a; Church, 1993). The positive effects of high $O_2$ in muscle products is related to colour retention of red meat, minimised drip losses and inhibition of anaerobic micro-organisms. However, the shelf-life of meat products to a large extend depends on the presence of $CO_2$ (Nychas and Tassou, 1996; Jensen et al., 1998). $O_2$ concentrations above 30% are recommended for storage of white fish as it minimises the risk of growth of anaerobic pathogens (i.e. *Clostridium botulinum*) should they occur and to minimise drip losses *(*Lannelongue et al., 1982*)*. With fish, high $O_2$ is most often used in combination with $CO_2$. The recommended concentrations of $O_2$ and $CO_2$ depend on the type of fish and the storage conditions (Stammen et al., 1990). It has been suggested that $O_2$ should be excluded for fatty fish to minimise rancidity. Lipid peroxidation is generally increased in the presence of 80% $O_2$ + 20% $CO_2$ (Barnett et al., 1982). In Chapter 6 of this thesis it is demonstrated that lipid oxidation should not be attributed solely to the high $O_2$ concentration in these products. Brown et al., (1980) failed to demonstrate increased lipid peroxidation for high $O_2$ MA packed mullets and salmon in relation to control (vacuum packed) products.

The effect of elevated (>40%) levels of $O_2$ for packaging fresh fruits and vegetables has been studied with oranges, potato tubers, shredded lettuce and tomatoes, where it was found that $O_2$ levels affected respiration rate, colour, texture, and metabolism of these products. In addition, elevated $O_2$ was found to reduce mould infections and microbial spoilage (Aharoni and Houck, 1982; Heimdal et al., 1995, Chapter 5, this thesis). El-Goorani and Sommer (1981) showed that the response of bacteria and fungi occurring on vegetables and fruits to high $O_2$ tensions is variable. With the appropriate type of products, high $O_2$ (>40%) could be used in combinations with other preservation methods to inhibit spoilage and pathogenic micro-organisms.

## 1.5 Antimicrobial activity of high oxygen

Micro-organisms differ greatly in their sensitivity to $O_2$ present in ambient (20%) concentration. However, a common feature of many micro-organisms is the toxicity to excess $O_2$ which has been shown by Bert already in 1878. Studies of bacterial chemotaxis to $O_2$ (aerotaxis) show that several strains swim away from regions of high $O_2$ concentrations. Obligate anaerobes are injured even by 0.1 kPa $O_2$ concentrations. Inactivation of *Bacillus* and *Clostridium* spores by $O_2$ gas plasma has been demonstrated and the feasibility of applying this method for inactivation of micro-organisms that are resistant to other sterilising methods has been suggested. For instance, 80% $O_2$ at 5 atm can cause significant damage and growth inhibition of *E. coli* cells (Halliwell and Gutteridge, 1995). The purpose of the following paragraphs is to consider the mechanisms that enable some organisms to overcome exposure to $O_2$ concentrations that prove fatal to others.

### $O_2$ chemistry - Free radical generation and $O_2$ toxicity

A free radical is any atom or molecule that contains one unpaired electron occupying an outer orbital. The reactivity of a radical depends on the affinity of its unpaired electron to participate in covalent bonding. Radicals can initiate "chain reactions" which lead to consequent generation of new reactive species. The $O_2$ molecule as it occurs naturally, with two unpaired electrons with the same spin, qualifies as a radical. Due to the spin restriction, $O_2$ has low reactivity but it can be genuinly toxic to anaerobic microoorganisms (where the effect can range from bacteriostatic to bactericidal) and rather toxic for many other living organisms. The reason is that $O_2$ reacts exothermically with many substances after activation (Green and Hill, 1984). The one-electron reduction of $O_2$ ("univalent pathway") predominates and the complete reduction of oxygen involves the addition of four electrons and four protons to each $O_2$ molecule, resulting in the generation of ROS or free radicals: superoxide anion radical ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical (HO·). The series of these reactions is presented in Figure 1.

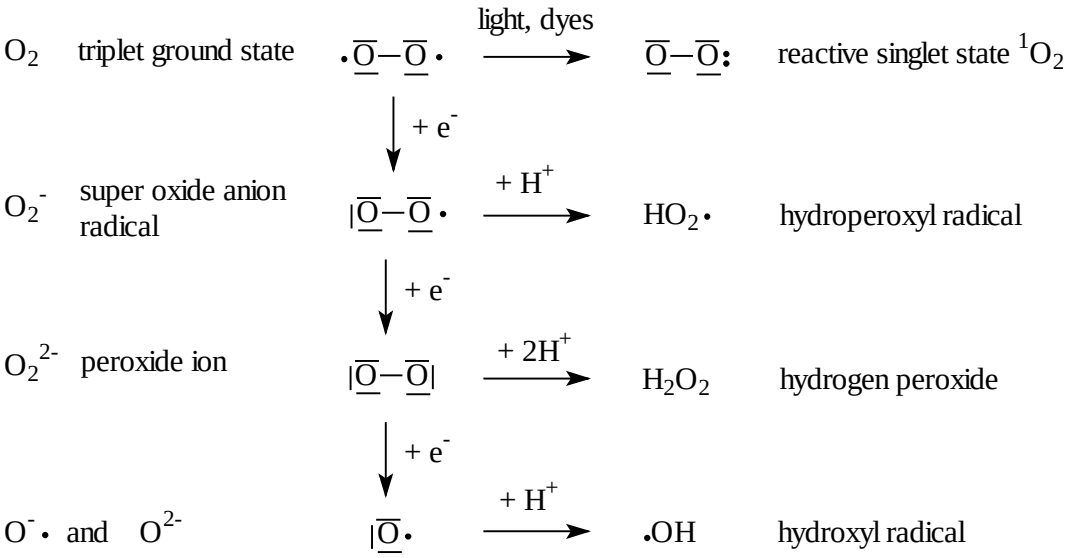


***Figure 1**. The products derived from the successive one-electron reduction of dioxygen (adapted from Bast* et al., *1991)*

Respiratory metabolism in living organisms requires biosynthetical degradation of ROS. Already in the 60's it was recognised that radicals are a product of normal cell metabolism (McCord and Fridovich, 1969). During $O_2$ metabolism, ROS accumulate in the intracellular space up to a steady state level. When the level of free radicals generated in a living organism exceeds the scavenging

capacity of the organism, free radicals may accumulate to levels that the cell cannot cope with and oxidative stress develops.

### Biochemical effects of high $O_2$ on micro-organisms

The concentration of ROS that causes oxidative stress in a population of bacteria is determined by factors such as growth phase (elevated stress resistance occurs in the stationary phase), population density, nutritional status and environmental conditions (Demple and Halbrook, 1983; Kolter et al., 1993). Heterotrophic micro-organisms can possess adaptive mechanisms that allow them to selectively adapt to enriched $O_2$ environments (Mikell et al., 1986). Hoffman et al., (1979) showed that inhibition of micro-organisms could be caused by the toxic $O_2$ by-products evolving endogenously or exogenously from interactions between $O_2$ and constituents of the medium. However, conflicting data about the significance of exogenous $O_2$ by-products on microbial growth inhibition and metabolism can be found in the literature (Mikell et al., 1983; Duwat et al., 1995b).
Although the importance of exogenous ROS on $O_2$ generated stress is under debate, the importance of intracellular generation of ROS is well recognised (Imlay and Fridovich, 1991; this thesis). All bacterial species with a functional respiratory chain produce intracellular harmful by-products such as ROS. Stortz and Imlay (1999) proposed a model describing intracellular oxidative damage in *E. coli* upon exposure to $O_2$ (Figure 2). The reactions of activated $O_2$ with organic substrates are complex even when studied *in vitro* with homogenous solutions. In biological systems there are even more complications and the nature of the oxidative injury that causes cell death is not always obvious. Lipid peroxidation is considered an indication of late oxidative injury in prokaryotes while it is less deleterious in procaryotes than in eukaryotes, due to the increased levels of monounsaturated and saturated fatty acids in the membranes of the former (Bradley and Minn, 1992). Activated forms of $O_2$ also degrade proteins and nucleic acids, reactions that can be very lethal. In the sections below some of the major reactions of activated $O_2$ with lipids, proteins and nucleic acids are reviewed.

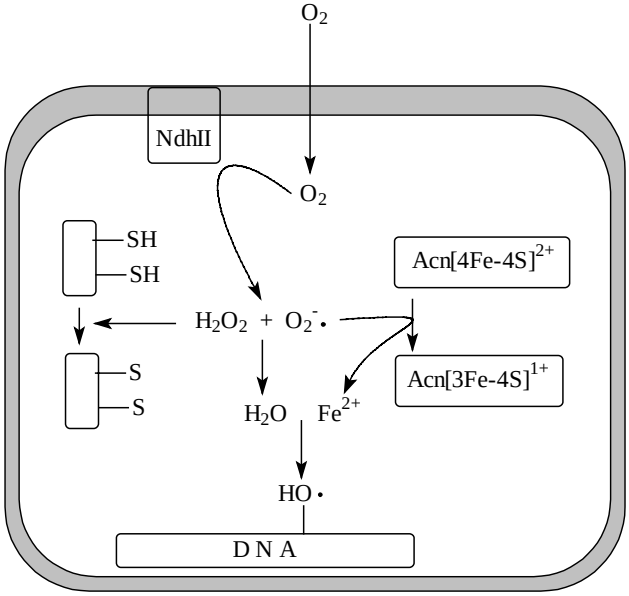


***Figure 2*** *A model for $O_2$ damage in* E. coli *(Storz and Imlay, 1999) Symbols: NdhII: NADH dehydrogenase II; $Acn[4Fe\text{-}4S]^{2+}$: aconitase; $Acn[3Fe\text{-}4S]^{1+}$: oxidised aconitase*

*Superoxide anion radical ($O_2^-$)*

The ability of $O_2^-$ to diffuse over relatively long distances combined with its ability to reduce transition metals strategically located on or near important macromolecules, such as DNA, may result in a selectivity of tissue macromolecular injury known as “site-specific injury” (Halliwell and Gutteridge, 1995). $O_2^-$ radical is lipophilic and moderately reactive at physiological conditions, but fast and

efficient elimination is of great importance for the cells because it is a potential source of more deleterious ROS. Due to the ability of $O_2^-$ to cross plasma membranes via anion gaps, extracellularly generated $O_2^-$ may penetrate cell membranes reaching intracellular targets.

The term "respiratory burst" or "oxidative burst" is used to describe the toxic phenomena that are induced in cells and tissues with the onset of phagocytosis. Activation of the membrane-bound NADPH enzyme complex of phagocytically active cells, which is accompanied by a rise in $O_2$ consumption, results in a dramatic increase of the level of $O_2^-$ radical which significantly effects physiological activities of eukaryotic cells (Cross and Jones, 1991; Mehdi, 1994). In analogy to the eukaryotic "respiratory burst", researchers have introduced the term "free radical burst" to explain the "suicide phenomenon" observed when rapidly growing and respiring bacterial cells are subjected to relatively mild oxidative stresses. These cells will suffer growth arrest but continue to metabolise. The uncoupling of biosynthetic and catabolic processes will ultimately result in activation of microbial death mechanisms (Dodd et al., 1997; Sigler et al., 1999; Aldsworth et al., 1999).

The electron transport chains located in the plasma membrane of several aerobic bacteria, e.g. *E. coli* and *Paracoccus denitrificans*, have been shown to produce $O_2^-$, however, the contribution of these oxidation reactions to $O_2^-$ formation *in vivo* is difficult to assess (Figure 3). In extracts of *Enterococcus* spp. incubated with NADH, 17% of the $O_2$ consumed was used to form $O_2^-$, but it is unclear whether these findings apply to intact cells. 20% of the total $O_2$ consumed by cells at 8°C was responsible for the steady state $O_2^-$ in *Lactobacillus sake* upon exposure to 90% $O_2$ + 10% $N_2$ (Amanatidou et al., 2001). Several enzymes that reduce $O_2$ to $O_2^-$ have been discovered (Messner and Imlay, 1999). $O_2^-$ can be generated in the mitochondia (transfer of electrons from ubiquinone to the NADH dehydrogenase portion of the electron transport chain as well as malate and succinate and glutamate), or by the action of several intracellular enzymes (non specific peroxidases, cellobiose oxidase, xanthin oxidase, galactose oxidase, several dioxygenases, etc). Undoubtedly, these are the most important sources of $O_2^-$, for most aerobic cells. Auto-oxidation of biological molecules such as glyceraldehyde and thiol compounds and the reduced forms of biologically active flavoproteins has also been shown to release $O_2^-$ in the presence of $O_2$. *In vitro* oxidation of biologically active components such as iron-sulphur centres by $O_2^-$ is accelerated by the presence of metal ions (Gardner and Fridovich, 1991).

Enzymes directly inactivated by $O_2^-$ are the hydroxyacid dehydratase, 6-phosphogluconate dehydratase, fumarases A and B and aconitase (Flint et al., 1993a, b; Gardner et al., 1994, 1995).

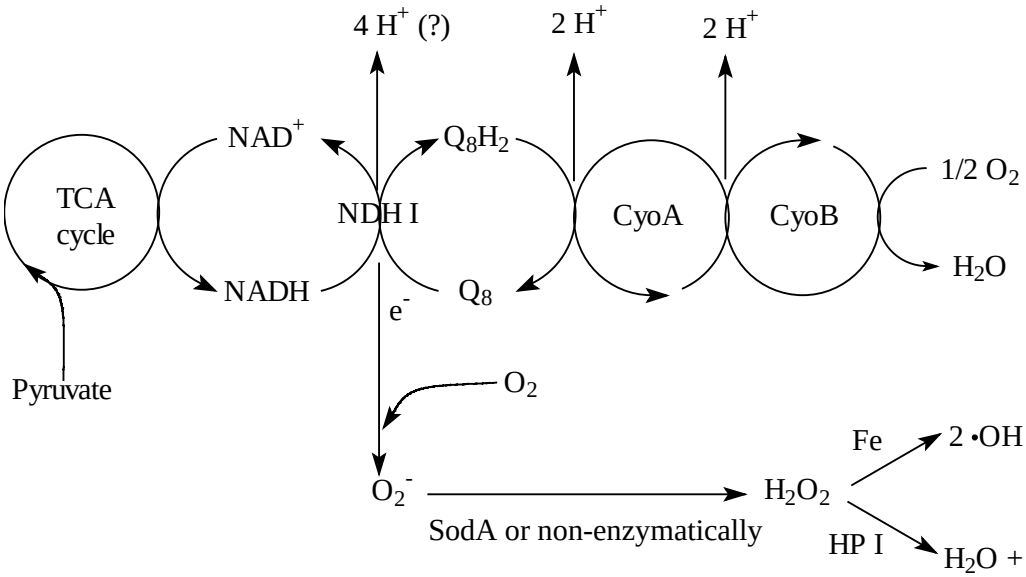


***Figure* 3**. *A simplified presentation of the electron transport chain and $O_2^-$ metabolism in* E. coli *(adapted from Iuchi and Weiner, 1996). TCA: Tricarboxylic acid cycle; NdhI: NADH dehydrogenase; Q8H2: reduced ubiquinone Q8: ubiquinone; CyoA, CyoB: aerobic terminal oxidases; HPI: hydroperoxidase*

*Hydrogen peroxide ($H_2O_2$)*

$H_2O_2$ arises from a variety of chemical and metabolic reactions. Intracellular sources of $H_2O_2$ include reactions catalysed by oxidases and dehydrogenases as well as the oxidation of thiols, flavins and ascorbate. Extracellular $H_2O_2$ can penetrate the cell membrane providing easy access to cellular components, it can inactivate enzymes and react with metal ions. $H_2O_2$ reactivity is limited in aqueous solutions. Scott et al., (1987) demonstrated that $H_2O_2$, as a product of SOD, can be at least as hazardous as the $O_2^-$ radical. The mechanism of $H_2O_2$ toxicity has been elucidated for *E. coli*. Cantoni et al., (1989) reported a dual mechanism of $H_2O_2$ toxicity in wild type *E. coli*: at lower concentrations (less than 5 mmol.$l^{-1}$) a "mode one" killing is attributed to an enzyme which generates $O_2^-$. At concentrations between 5 and 10 mmol.$l^{-1}$ this enzyme is inactivated by the $O_2^-$ radical, resulting in resistance at intermediate concentrations. "Mode two" killing could be related to the action of HO· radical in concert with $O_2^-$ (Cantoni et al., 1989). Indeed, the major reaction pathway is the formation of highly reactive HO· radicals through the Fenton or Haber-Weiss reaction (see equations 1 and 2 below).

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO\cdot + OH^- \quad \textbf{(1)}$$

$$O_2^- + H_2O_2 \xrightarrow{Fe} O_2 + HO\cdot + OH^- \quad \textbf{(2)}$$

Consequently, the major damage from $H_2O_2$ arises from these reactions as will be described in the following paragraph.

*Hydroxyl radical (HO·)*

According to the model of Stortz and Imlay (1999) presented in Figure 2, $O_2^-$ radicals play a role in $O_2$ toxicity in *E. coli* by elevating free iron pools available for catalysing the production of HO·. Hydroxyl radicals can react with virtually every molecule in a cell function as a "spark" that starts a fire (Halliwell and Gutteridge, 1992).

Oxidative attack on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis (Stadtman and Levine, 2000). In general, sulphur containing amino acids, specifically thiol groups, are very susceptible sites. Some amino acids (e.g. tryptophan) undergo specific irreversible modifications when a protein is oxidised (Davies and Lin, 1988). Histidine, lysine, proline, arginine, and serine form, among others, carbonyl groups upon exposure to high $O_2$ (Stadtman, 1986, 1990). The oxidative degradation of protein is enhanced in the presence of metal cofactors which bind to a divalent cation-binding site on the protein. The metal cofactors then react with $H_2O_2$ in a Fenton reaction to form a HO· radical that rapidly oxidises an amino acid residue at or near the cation binding site of the protein (Stadtman, 1986). This site-specific alteration of an amino acid usually inactivates the enzyme by destruction of the cation-binding site.

The main types of damage caused by HO· radicals on DNA are single strand breaks, and cross-linkings to protein (Oleinick et al., 1986). *In vitro*, neither $H_2O_2$ alone nor $O_2^-$ cause strand breaks under physiological conditions. Therefore, their toxicity *in vivo* is most likely the result of Fenton reactions with a metal cofactor which acts as a catalyst. Characterisation of this damage of DNA has indicated that both the sugar and the base moieties are susceptible to oxidation, causing base degradation by the HO· radical (Imlay and Linn, 1986). Degradation of the base moieties will produce numerous products, including 8-hydroxyguanine, hydroxymethyl urea, urea, thymine glycol, thymine and adenine open-ring and saturated products. At least in *E. coli*, Fenton reactions can be driven by NADH (Imlay and Linn, 1988). Generation of HO· radical can be avoided by preventing intracellular $O_2^-$ or $H_2O_2$ production (Duwat et al., 1995a).

## Cellular defences against damage in bacteria

In the case of facultative aerobes such as *E. coli* or *Salm.typhimurium*, positive aerotaxis ensures that the organism positions itself in an environment of optimal $O_2$ (Taylor, 1983). Most aerobes can tolerate mild oxidative stress because additional antioxidant defence mechanisms are activated upon exposure. The responses of *E. coli*, *Salm. typhimurium* and *Bacillus subtilis* to high $O_2$ or to oxidative stress generating agents (e.g. $H_2O_2$ or paraquat), UV radiation and oxidative burst of phagocytes are well studied. They also display enhanced resistance towards $H_2O_2$ induced oxidative stress (Imlay and Linn, 1988; Storz and Imlay, 1999). It is generally recognised that adaptive resistance to oxidative stress is different for $H_2O_2$ and $O_2^-$ (Foster and Spector, 1995). In the obligate anaerobe *Bacteroides fragilis* physiological responses to $H_2O_2$ toxicity and to $O_2$ toxicity are overlapping and complex. In *E. coli*, exposure to 60 $\mu mol.l^{-1}$ $H_2O_2$ induces production of 40 different proteins that aid survival at higher levels (15 $mmol.l^{-1}$) whereas exposure to $O_2^-$ generating reagents increases the level of about 30 proteins. Evidently, the stress responses entail large efforts to prevent or repair oxidative damage as much as possible (Demple, 1991; Stortz et al., 1990; Eisenstark et al., 1996). Extensive investigation has revealed the complex nature of the molecular processes involved in stress responses in *E. coli*, which center around the induction of regulons expressing both protective and repair enzymes. One such a regulon is the soxRS regulon, which directs the global response induced by $O_2^-$ (Nunoshiba, 1996; Iuchi and Weiner, 1996). The functions controlled by this system consist of a wide variety of enzymes such as manganese-containing SOD (Mn-SOD), glucose-6-phosphate dehydrogenase (G6PD), the DNA repair enzyme endonuclease IV, NADPH:ferredoxin oxidoreductase, fumarase C, and aconitase. The role and significance of some of these enzymes will be discussed in the following paragraphs.

*Superoxide dismutase*

The enzyme superoxide dismutase (SOD) helps organisms to survive in the presence of $O_2$. The discovery of SOD in aerobic cells led to the hypothesis that $O_2^-$ is a major factor in $O_2$ toxicity and that SOD is important in counteracting the impact of $O_2^-$ (McCord and Fridovich, 1969; Harth and Horwitz, 1999). Exposure of bacteria to elevated $O_2$ results in an increased formation of $O_2^-$ and, when the prevailing level of SOD is insufficient to cope with the $O_2^-$ generated, in synthesis of more SOD. This theory has been substantiated in studies with *E. coli* where it was shown that cells pre-grown under elevated $O_2$ levels contained increased SOD levels and were much more resistant to the toxic effects of high $O_2$ than cells grown under air (Touati et al., 1995).

A Mn-SOD has been isolated from *Lactococcus lactis* (Sanders et al., 1995) after exposure to aeration or other stresses. For induction of SOD in lactic acid bacteria (LAB), the presence of increased $Mn^{2+}$ in the growth medium is required (Gonzalez et al., 1989). An alternative for SOD in some LAB is a high internal level of $Mn^{2+}$ (Archibald and Fridovich, 1981a). *Listeria monocytogenes* exposed to high $O_2$ levels showed significantly higher concentrations of SOD as compared to catalase, indicating the importance of the former enzyme for the protection of this micro-organism (Fisher et al., 2000). In *Bacillus subtilis*, a micro-organisms that is equally sensitive to high $O_2$ whether pre-grown under air or 100% $O_2$, exposure to elevated $O_2$ increased catalase but not SOD activity (Halliwell and Gutteridge, 1989).

Although increased tolerance to oxidative stress is associated with induction of SOD, the relative importance of this enzyme in $O_2$ tolerance is not clear because conditions that promote the synthesis of SOD may affect other antioxidant enzymes and substances. SOD is also involved in the defence towards several stresses other than oxidative stress (Purdy et al., 1999).

*Catalases*

The $O_2$ response in different types of aerobic bacteria includes the generation of catalases and peroxidases, the main antioxidative enzymes upon exposure to high $O_2$ (equation 3). As with oxidative stress generated by for instance antimicrobial drugs or starvation, elevated $O_2$ levels

increase the catalase activity in *E. coli*. Exposure of anaerobically grown *E. coli* K12 cells to $O_2$ causes induction of catalase and peroxidase activities only at much higher $O_2$ concentrations than those necessary to increase synthesis of Mn-SOD.

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2 \quad \textbf{(3)}$$

Ma and Eaton (1992) suggested that bacterial catalase might rather be a colonial and therefore a multicellular defence system, because catalase in individual cells of *E. coli* failed to protect against exogenous $H_2O_2$. $O_2$-induced catalases have been identified in many bacteria (Loewen, 1997). Catalase is generally absent in LAB, but its presence was demonstrated in a few *Lactobacillus* spp. in the presence of hematine (Wolf et al., 1991; Knauf et al., 1992). Non-heme catalase in LAB is restricted to *Lactobacillus plantarum, Lactobacillus mali* and *Pediococcus pentosaceous* (Engesser and Hammes, 1994). Several reports supply evidence of the operation of a general, well-orchestrated defence system against oxidative stress at least in some types of bacteria. Both catalase and SOD are components of this system (Iuchi and Weiner, 1996). For example, *Pseudomonas aeruginosa* in biofilms showed higher levels of survival upon exposure to $H_2O_2$ when Mn- and Fe-SOD as well as catalase were present (Hassett et al., 1999).

*NADH oxidase and peroxidase*

As compared to current insight into the responses of *E. coli* to $O_2$ and oxidative stress, only few studies give insight to the responses of LAB to oxidative stress (Lin and Yen, 1999; Chapter 3). Significant differences have been found in the degree of $O_2$ tolerance among different LAB species but also between subpopulations of the same LAB species (Condon, 1987; this thesis). A unique property of LAB is that they contain flavoproteins involved in oxidative metabolism that are very different from the respiratory redox enzymes of cytochrome containing bacteria like *E. coli*. Among the flavoproteins in LAB, NADH oxidase and NADH peroxidase (Table 3) form the first line of defence to oxidative stress, as they result in a steady-state production of radicals. The system of NAD(P)H oxidase/peroxidase is responsible for the removal of toxic $H_2O_2$ in LAB. In *Enterococcus faecalis*, NADH peroxidase uses $H_2O_2$ as an electron acceptor providing enzymatic defence against peroxide stress. Sakamoto et al., (1998) have performed a broad screening in LAB with respect to enzyme induction during aeration and they concluded that NADH peroxidase plays a role in detoxification of $H_2O_2$ as does catalase. Several other bacteria such as *Lactobacillus casei* or *Streptococcus faecalis* contain a peroxidase which uses $H_2O_2$ to oxidise NADH into NAD+ (Table 3). This enzyme should not be confused with the NADH oxidase enzyme found in some bacteria in which NADH is oxidised to NAD+ while $O_2$ is simultaneously reduced to water. NADH oxidase catalyses the direct four electron reduction of $O_2$ to $H_2O$ (Ross & Claiborne, 1992) and it is always induced when $O_2$ is used as an electron acceptor (Warriner and Morris, 1995). Several aerotolerant anaerobic bacteria synthesise NADH oxidase on exposure to $O_2$ (Matsumoto et al., 1996). Aeration does not change the homolactic character of lactose fermentation by *Lactobacillus delbrueckii* subsp. *bulgaricus* and most of the NADH was re-oxidised by lactate dehydrogenase with pyruvate in the presence of NADH oxidase. This indicates that in these bacteria, NADH oxidase had no (or a very small) role in energy metabolism and its action is restricted to the protection of cells against $O_2$ stress (Teysset et al., 2000). Hence, the level of NADH oxidase is not regulated directly by the level of $O_2$. In *Lact. lactis*, the importance of the NADH oxidase was demonstrated under constant supply of 90% $O_2$ to sterile milk after the action of the lactoperoxidase system. Exposure to this system reduced the biomass of a *Lact. lactis* sensitive mutant which had a lower ratio of NADH oxidase/NADH peroxidase as compared to the wild type strain (Roginski et al., 1991). Gibson et al., (2000) demonstrated that in *Streptococcus pneumoniae*, NADH oxidase acts as an $O_2$ sensor and, although the metabolism in these bacteria is strictly fermentative, global cellular response and virulence are affected by $O_2$ levels. The activities of NADH oxidase in extracts from aerobically grown *Streptococcus* spp. showed a positive correlation with the growth rate under aerobic conditions (Schmidt et al., 1986). Several anaerobic bacteria have

gained a NADH oxidase which is useful because it allows the cells to survive exposure to limited amounts of $O_2$ but the NADH it requires must be provided by metabolic pathways such as glycolysis. If this enzyme is exposed to high concentrations of $O_2$, irreversible damage may occur (Halliwell and Gutteridge, 1989).

***Table 3*** *Enzymes of LAB involved in oxygen metabolism (adapted from Condon, 1987)*

| Reactants | (Co)Enzyme/catalyst | Products |
|---|---|---|
| $NADH + H^+ + O_2$ | $NADH:H_2O_2$ oxidase | $NAD^+ + H_2O_2$ |
| $2NADH + 2H^+ + O_2$ | $NADH:H_2O$ oxidase | $2NAD^+ + 2H_2O$ |
| $NADH + H^+ + H_2O$ | NADH peroxidase | $2NAD^+ + H_2O$ |
| $2O_2^- + 2H^+$ | Superoxide dismutase | $H_2O_2 + O_2$ |
| $2H_2O_2$ | $Mn^{2+}$ pseudocatalase | $2H_2O_2 + O_2$ |
| Pyruvate + phosphate + $O_2$ | Pyruvate oxidase, TPP, FAD | Acetyl-phosphate $H_2O_2 + CO_2$ |
| α-glycerophosphate + $O_2$ | α-glycerophosphate oxidase | dihydroxyacetone-phosphate + $H_2O_2$ |
| Lactate + $O_2$ | Lactate oxidase | pyruvate + $H_2O_2$ |
| Lactate + $O_2$ | D-lactate dihydrogenase | $H_2O_2 + O_2$ |

*Other defence mechanisms*

Except for the antioxidative enzymes mentioned in the previous paragraphs, several other compounds produced by bacteria that remain present intracellularly or are subsequently excreted are involved in the detoxification of ROS. Although no enzymes with HO· scavenging capacity are known, several compounds (e.g. mannitol, formate, carotenoids, vitamins A, C and E) can protect against damage caused by HO·. These compounds may be present in the cytosol or the membrane and protect the cells either by scavenging HO· radicals, by preventing their formation or by repairing damage. Enzymes such as glutathione reductase have been purified from Gram(+) and Gram(-) micro-organisms upon exposure to elevated $O_2$ as part of the SOS response or an SOS-like response (Pebay et al., 1995; Patel et al., 1998). SOS response and DNA repair system have been verified in several bacterial species (Huang et al., 1995; Hartke et al., 1995). The repair system of membranes has only been studied in *E. coli* for which researchers have shown that the response pathway is under control of the $H_2O_2$ stress response. Lipid peroxidation of membranes can induce DNA damage and may affect cell-envelope integrity (Marnett et al., 1985).

## 1.6 Combination of high oxygen MA with other hurdles

There are several reports suggesting that exposure to increased $O_2$ levels enhances the sensitivity of food related micro-organisms to other antimicrobial factors. Recovery of *E. coli* O157:H7, *Salmonella enteritidis* and *List. monocytogenes* in an $O_2$ enriched environment after a mild heat treatment was poor, especially for micro-organisms that were isolated from products that were packed under vacuum or low $O_2$ atmospheres (George et al., 1998). Cells of *Campylobacter* spp. have been found to be more sensitive to freeze-thaw in the presence than in the absence of $O_2$, indicating that radicals contribute to cell death in the latter case (Stead and Park, 2000). Irradiation of food in an atmosphere containing high $O_2$ generally produces a greater variety and greater amount of radiolytic products than there would be produced in a low $O_2$ atmosphere. Irradiation initiates certain oxidation reactions, some of which are also produced for instance whith heating under aerobic condition (Food and Drug Administration, 1997), and these occur in greater frequency in foods with high fat content. The synergistic effect of light and molecular $O_2$ has been previously demonstrated (Kerr et al., 1995). The

mechanism of the photodynamic inactivation of micro-organisms is based on the interaction of an excited sensitizer with the substrate which results in reaction with $O_2$. Energy transfer to $O_2$ is generating the powerful singlet molecular oxygen $^1O_2$ that can cause significant cellular damage to bacteria. Photodynamic inactivation can be used for disinfection of surfaces, materials or interfaces between solid-liquids or gas liquids systems. Although some powerful photosensitisers (e.g. chlorophyll) are food grade, there are regulatory issues to be resolved before commercial application would be feasible.
In the following paragraphs the impact of the application of elevated levels of $O_2$, in combination with $CO_2$ or high pressure processing on food related micro-organisms are discussed. Advantages and disadvantages of the combined applications of elevated $O_2$ with the two preservation factors are studied in this thesis using two model food products (Chapters 5 and 6).

### Combined applications of $O_2$ MA with $CO_2$

Combined application of $CO_2$ with $O_2$ is intended to serve different purposes, for instance inhibition of microbial growth and retention of a desirable colour. A synergistic effect between these gases in relation to microbial inactivation has been reported (Ogihara et al., 1993; this thesis). $CO_2$ may have bacteriostatic or bactericidal properties, but its effect is complex and still poorly understood. King and Nagel (1967) suggest that $CO_2$ affects several metabolic processes through enzymatic decarboxylation, alteration of membrane functionality, inhibition of respiration and decrease of the intracellular pH. Wolfe (1980) proposed that the synergistic effect of $CO_2$ with low temperature is probably due to increased solubility of $CO_2$ in water at these temperatures, which allows penetration of more bicarbonate into microbial cells. The bicarbonate ion is a dissociation product of $CO_2$ in water, which changes cell permeability and metabolic processes. However, it is very difficult to determine whether the antimicrobial effect arising from an atmosphere enriched in $CO_2$ is either due to $CO_2$, bicarbonate or a combination of both. Jones and Greenfield (1982) and more recently also Devlieghere (2000) found $CO_2$ and not bicarbonate to be the major inhibitor of microbial growth. However, it should not be assumed that this is always the case. Gram(-) bacteria are more sensitive to $CO_2$ than Gram(+) bacteria, which may be explained by the capacity of $CO_2$ to inactivate amino acid binding proteins within the periplasm (Phillips, 1996). $CO_2$ reduces the growth rate of many bacteria. An important aspect with respect to the $CO_2$ sensitivity of micro-organisms is the lack of adaptation to enriched $CO_2$ atmospheres. Pseudomonads seem to be the most $CO_2$ sensitive micro-organisms and clostridia the most $CO_2$ resistant (Molin, 1983). The effect of elevated levels of $CO_2$ on growth and toxin formation of *Clostridium perfringens* and pathogens such as *Listeria monocytogenes, Yersinia enterocolitica* and *Aeromonas hydrophila* depends on factors such as storage temperature, pH, type of tissue (Berrang et al., 1989; Bennik et al., 1998; Barakat and Harris, 1999). $CO_2$ applied under pressure is considerably more antimicrobial than $CO_2$ applied under ambient pressure (Doyle, 1983). At ambient pressure, excessive $CO_2$ delays the production of toxins by *Clostridium botulinum* as compared to an atmosphere consisting of 100% $N_2$. $CO_2$ in a supercritical state can destroy bacteria and fungi under different conditions (Wei et al., 1991; Lin et al., 1992a).

### Combined applications of $O_2$ MA with high pressure

The patterns of inactivation by high pressure (HP) processing observed with different micro-organisms are variable but a two-phase inactivation phenomenon has been proposed (Cheftel, 1995). Pressure level, temperature, and composition of the medium all influence the inactivation kinetics. The possibility of cell recovery exists and in many cases pressure treated micro-organisms may not be detected with plate count methods because they fail to start to grow when they are plated immediately after treatment. With respect to the inactivation of micro-organisms by HP processing, the membrane is most probable the key site of disruption as cell death is often attributed to permeabilization of the cell membrane. A decrease in pH due to enhancement of ionic dissociation during high pressure treatment has been reported as well (Palou et al., 1999). Protein denaturation and volume changes

during decompression affect many different physiological functions and could, in addition, contribute to microbial inactivation. Inactivation of key enzymes, including those involved in DNA replication and transcription, has been observed by Hoover et al., (1989). Robey et al., (1998) investigated the potential use of HP processing for food pasteurisation. They concluded that microbial inactivation by HP processing may be mediated by the production of ROS, because antioxidative enzymes such as SOD and catalase seemed to have a protecting effect on pressure treated cells of *E. coli*. The antimicrobial effects of $O_2$ and $CO_2$ under pressure and the mechanisms possibly underlying these effects have been reported (ZoBell and Hittle, 1967; Wei et al., 1991; this thesis). The impact of treatment with several other types of compressed gases ($N_2O$, $O_2$, Kr and Xe at 1.7 MPa) has been studied for *E. coli*, *Saccharomyces cerevisiae* and *Tetrahymena thermophila* (Thom and Marquis, 1984).

# Chapter 2
## Effect of elevated oxygen and carbon dioxide on the surface growth of vegetable-associated micro-organisms

A. Amanatidou, E.J. Smid & L.G.M. Gorris
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## Abstract

The impact of a novel type of Modified Atmosphere (MA), referred to as high $O_2$-MA, on micro-organisms associated with the spoilage of minimally-processed vegetables was studied. Pure cultures of *Pseudomonas fluorescens, Enterobacter agglomerans, Aureobacterium* strain 27, *Candida guilliermondii, C. sake, Salmonella typhimurium, Salm. enteritidis, Escherichia coli, Listeria monocytogenes, Leuconostoc mesenteroides* var. *mesenteroides, Lactobacillus plantarum* and *Lactococcus lactis* were cultured on an agar-surface model system and incubated at 8°C under an atmosphere composed of $O_2$ (80 or 90%, balanced with $N_2$), $CO_2$ (10 or 20%, balanced with $N_2$), or a combination of both gases. In general, exposure to high $O_2$ alone did not inhibit microbial growth strongly, while $CO_2$ alone reduced growth to some extent in most cases. Consistently strong inhibition was observed only when the two gases were used in combination. With minimally-processed vegetables, where $CO_2$ levels of around 20% or above cannot be used because of physiological damage to the produce, the combined treatment of high $O_2$ and 10-20% $CO_2$ may provide adequate suppression of microbial growth, allowing a safe, prolonged shelf-life.

## 2.1 Introduction

Modified atmosphere packaging (MAP) is used to extend shelf-life and maintain high quality of minimally-processed fruits and vegetables. The rapid increase in the market share of MAP of "ready-to-eat vegetables" reflects the trends of today's consumers for fresh, additive-free foods.

Respiring products, like raw or processed vegetables and ready-made salads, generate equilibrium gas conditions inside the package that are typically very low in $O_2$ (2-3%) and moderately high in $CO_2$ (5-20%). These conditions reduce deterioration by limiting product respiration and maturation (Kader, 1986; Day, 1992; Gorris and Peppelenbos, 1992) as well as by slowing down the proliferation of aerobic spoilage micro-organisms (Hotchkiss, 1988; Kader et al., 1989; Moleyar and Narasimham, 1994). The antimicrobial effect of $CO_2$ on micro-organisms has been intensively documented (Molin, 1983; Eklund, 1984; Daniels et al., 1985; Dixon and Kell, 1989).

Although MA packaging of respiring produce has come to substantial use in practice, some potential problems with regard to product quality and safety remain to be solved. The $O_2$ and $CO_2$ levels in a MA package are achieved mostly by the active respiration of the produce and are often difficult to predict and control (Ahvenainen, 1996). All too frequently, $O_2$ is completely depleted, resulting in the production of off-odours and rapid deterioration of the product (Zagory and Kader, 1988). In addition, excessive levels of $CO_2$ (generally over 20%) cause specific disorders such as the development of brown stains (Lougheed, 1987; Kader et al., 1989). Bennik et al., (1995) showed that $CO_2$ levels from 20 to 50%, in combination with low $O_2$ (1.5%), affect the growth rate of spoilage bacteria relevant to minimally processed vegetables but have no effect on the maximum population densities or the lag phase duration.

Concerning product safety, psychrotrophic pathogens such as *Listeria monocytogenes* are not suppressed under MA conditions that are optimal for respiring produce (Berrang et al., 1989; Brackett, 1994; Bennik et al., 1995; Carlin et al., 1996; Francis and O'Beirne, 1997). On the contrary, growth

may be enhanced in certain because of suppression of the natural flora (Hotchkiss, 1988; Bennik et al., 1996). Evidently, alternatives to the current, low oxygen, MA packaging need to be investigated to better assure the safety of MA-packaged respiring produce.
Recent experimental trials on fresh commodities have indicated that high $O_2$ (70-90%) may be advantageous for product quality (Day, 1996a,b). The use of high $O_2$-MAP packaging for respiring produce in practice is still in its infancy and needs to be supported by research. At present, little literature is available documenting a possible inhibitory effect of high $O_2$ on surface growth of micro-organisms (Ogihara et al., 1993). In the present study, therefore, the effect of high $O_2$ alone, and in combination with moderately high $CO_2$ (10-20%), on the growth of pure cultures of a number of yeasts and bacteria relevant to MA-packaged vegetables was investigated. A surface model system was used in which micro-organisms are exposed to controlled gas conditions at 8$^o$C, a refrigeration temperature generally used for retail storage.

## 2.2 Material and methods

### Micro-organisms

*Salmonella typhimurium* DSM 4780, *Salm. enteritidis* DSM 13076, non-pathogenic *Escherichia coli* DSM 11755, *Leuconostoc mesenteroides* subsp. *mesenteroides* DSM 20343, *Lactobacillus plantarum* ATCC 8014 and *Lactococcus lactis* subsp. *lactis* NCDO 495 are type strains obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSM, Braunsweig, Germany). *Listeria monocytogenes* Scott A was from the culture collection of the Department of Food Science, University of Wageningen (The Netherlands). Strain 27 was isolated from ready-made salads incubated under 90% $O_2$ : 10% $N_2$ at 8$^o$C and identified by DSM as *Aureobacterium* spp. Two yeast strains also isolated from ready-made salads stored under modified atmospheres (10% $O_2$ : 5% $CO_2$) were identified by the Centraal Bureau voor Schimmelcultures (CBS, Baarn, The Netherlands) as *Candida sake* (Saito & Ota) van Uden & Burkley and *Candida guilliermondii* (Castelalani) Langeron & Guerra, respectively. *Pseudomonas fluorescens* and *Enterobacter agglomerans* were isolated from fresh produce and identified with the Biolog MicroPlate 2N system (Biolog®, Hayward, USA) in combination with classical identification methods.

### Growth conditions

*Listeria monocytogenes* was grown on Palcam Listeria Selective Medium (PLSM, Oxoid, Hampshire, UK) and *Leuc. mesenteroides, L.. plantarum* and *Lact. lactis* on de Man Rogosa Sharp agar (MRS agar, Oxoid, Hampshire, UK). All other strains were cultivated on Nutrient Agar (NA, Oxoid, Hampshire, UK). All media were prepared according to the manufacturers' instructions.

### Storage and preparation

*List. monocytogenes* was routinely stored at -80$^o$C in Brain Heart Infusion broth (BHI, Oxoid, Hampshire, UK), lactic acid bacteria in MRS broth, yeasts in Yeast Nitrogen Base (YNB, Difco Kansas City, MO, USA), and all other strains in Nutrient broth (NB, Oxoid, Hampshire, UK) supplemented with 20% glycerol. A 0.1% inoculum (v/v) of a 5 ml full-grown culture was sub-cultured in MRS broth (lactic acid bacteria) or NB (all other strains) for 24 hours at 25$^o$C, and subsequently transferred to agar plates of the same medium for further incubation. *List. monocytogenes* was transferred on PLSM. Stationary phase cells were harvested from plates, washed twice in 0.1 mol $l^{-1}$ sodium phosphate buffer (pH 7.0) and then resuspended in the same buffer to an optical density of 0.5 at 660 nm. From this suspension, serial dilutions were made in saline and 50 µl samples of the diluted cultures were spread on 60 mm petri dishes containing 9 ml medium, which gave an initial population on the agar surface of $10^3$-$10^4$ cfu.cm$^{-2}$.

### Experimental set-up

The surface model system employed here was previously described by Bennik et al., (1995). In short, agar plates were prepared as described above and incubated in a series of 1 litre jars. The jars were placed in a temperature-controlled room at 8°C and connected in sequence to a gas flow-through system. The following mixtures of $O_2 + N_2 + CO_2$ (% v/v) were used: a) 90 + 10 + 0, b) 90 + 0 + 10, c) 80 + 20 + 0, d) 80 + 0 + 20, e) 0 + 90 + 10 and f) 0 + 80 + 20. A mixture of 20% $O_2$ and 80% $N_2$ was used as the control. The compositions were prepared from pure gases using mass-flow controllers (5850 TR series Brooks Instruments B.V., Veenendaal, The Netherlands), humidified to a level close to saturation by passage through a water bottle, and finally introduced into the jars at a flow-rate of 200 $ml.min^{-1}$. The composition was checked daily with a gas analyser (Servomex Series 1400, ADC 7000, ThIS Analytical B.V., Breda, The Netherlands).

### Enumeration of viable counts

Plate samples were taken in duplicate at each sampling time by removing the plates from two jars that were last in their sequence. The agar from one plate was removed aseptically and homogenised for 1 min in a stomacher bag containing 50 ml saline. The drop count method was used to estimate the viable counts in $cfu.ml^{-1}$, which were converted to $cfu.cm^{-2}$ assuming that all counts originated from micro-organisms growing on the surface of the agar.

The pH of uninoculated medium was measured on each day of sampling in order to detect acidification of the medium. In none of the incubations was the pH lower than 6.6 at the end of the incubation time. The pH of uninoculated MRS was 5.9 at the end of the incubation time.

### Quantification of microbial growth parameters

Viable count data were fitted to the mathematical model described by Baranyi et al., (1993) and reparameterized with the DMFit program (1996, IFR, Reading Laboratory, U.K.) kindly provided by Dr. J. Baranyi. The model was used to estimate the lag phase (h, hours), the growth rate ($h^{-1}$) and the maximum population density $y_{max}$ (log $cfu.cm^{-2}$) as well as the associated standard error (S.E.) and correlation coefficient $r^2$.

## 2.3 Results

### Effect of high $O_2$ and $CO_2$ on the growth characteristics of selected micro-organisms

This study evaluated the potential of high $O_2$-MAP to reduce the growth of a number of spoilage micro-organisms and pathogenic bacteria typically associated with minimally-processed vegetables as compared with storage under an ambient atmosphere. The possible antimicrobial effect of traditional low $O_2$-MA packaging was assessed as well. Pure cultures of selected isolates were subjected to different controlled atmosphere conditions in an agar-surface model system incubated at 8°C. The impact of gas atmosphere compositions on microbial growth was evaluated quantitatively on the basis of general growth characteristics and on three specific growth parameters (lag phase duration, growth rate and maximum yield) with the D-Model (Baranyi et al., 1993).

### *Listeria monocytogenes, Salmonella enteritidis, Salmonella typhimurium* and *Escherichia coli*

Growth of the pathogens was monitored in the presence of 90% $O_2$, or 10% $CO_2$, or the combination of the two gases (90% $O_2$ plus 10% $CO_2$), and compared with growth under normal air (Table 1). All pathogens could grow at 8°C under air but growth of *Salm. typhimurium* was extremely slow. Growth rates of *E. coli* and *Salm. enteritidis* were found to be inhibited in the presence of 90% $O_2$ by 22 % and 44%, respectively. On the other hand, growth rates of *List. monocytogenes* and *Salm. typhimurium* were hardly affected under these conditions. All four pathogens showed a lag phase in the presence of 90% oxygen which was longer than that under normal air. High oxygen alone reduced the final yield of *Salmonella* and slightly reduced the final yield of *E. coli.*

In the presence of 10% $CO_2$, growth of *E. coli* was hardly affected (Figure 1A). Growth rate of *Salm. enteritidis* was inhibited by approximately 91%, while growth of *Salm. typhimurium* and *List. monocytogenes* was stimulated under these conditions (Table 1). An increase in the lag phase in the presence of 10% $CO_2$ was observed only for *List. monocytogenes* and *Salm. typhimurium.* On the other hand, no lag phase was observed for *E. coli* and *Salm. enteritidis*. For the latter strain, the effect on the final yield (4.5) was significant.

The combination of 90% $O_2$ and 10% $CO_2$ significantly reduced growth rates of *E. coli* and *Salm. enteritidis* (Table 1). Slight increases in the growth rate of the other two pathogens *(List. monocytogenes* and *Salm. typhimurium*) were observed. The lag phase of *E. coli* and *Salm. typhimurium,* increased moderately to strongly, respectively, compared with the control. As all four pathogens grew at relatively slow rates at 8°C, unequivocal conclusions about the effects of the gas conditions on the final yield cannot be drawn from the data presented.

***Table 1.*** *Estimated parameters of lag time (λ), specific growth rate (μ) and maximum population densities ($y_{max}$), for the growth of* Listeria monocytogenes, Salmonella enteritidis, Salmonella typhimurium *and* Escherichia coli *derived from fitting data to the D-model. The standard errors (S.E.) of lag are also presented*

| Micro-organism | $O_2$ (%) | $CO_2$ (%) | Lag λ (h) | (S.E.) (lag) | Specific growth rate μ ($h^{-1}$) | $Y_{max}$ (log cfu.$cm^{-2}$) |
|---|---|---|---|---|---|---|
| *Listeria monocytogenes* | 20 | 0 | 62 | 20 | 0.031 | (7.2)* |
| | 90 | 0 | 82 | 21 | 0.039 | (7.8) |
| | 90 | 10 | 75 | 9 | 0.041 | (8.1) |
| | 0 | 10 | 123 | 16 | 0.052 | (8.1) |
| *Salmonella typhimurium* | 20 | 0 | 101 | 0 | 0.011 | (6.5) |
| | 90 | 0 | 353 | 32 | 0.015 | (5.3) |
| | 90 | 10 | 371 | 64 | 0.023 | (5.6) |
| | 0 | 10 | 123 | 17 | 0.022 | (7.8) |
| *Salmonella enteritidis* | 20 | 0 | 0 | 0 | 0.091 | (7.8) |
| | 90 | 0 | 91 | 4 | 0.051 | 6.1 |
| | 90 | 10 | 0 | 0 | 0.021 | 4.5 |
| | 0 | 10 | 0 | 0 | 0.008 | 4.1 |
| *Escherichia coli* | 20 | 0 | 0 | 0 | 0.027 | 9.3 |
| | 90 | 0 | 31 | 18 | 0.021 | 8.9 |
| | 90 | 10 | 65 | 24 | 0.009 | (7.2) |
| | 0 | 10 | 0 | 0 | 0.024 | 9.1 |

** Numbers in brackets indicate population densities at the end of the sampling period for the cases in which $y_{max}$ was not detected due to slow growth*

### *Lactobacillus plantarum, Leuconostoc mesenteroides* and *Lactococcus lactis*

All three lactic acid bacteria tested grew very well under ambient $O_2$ conditions at 8°C. The growth rate of *Lact. lactis* and *Leuc. mesenteroides* was stimulated by the presence of 90% $O_2$ alone, while that of *L. plantarum* was significantly (63%) reduced (Table 2). *Lact. lactis* (Figure 1B) showed a prominent lag phase (71 h) and a significant reduction in the final yield (6.6) with high $O_2$ alone

(Table 2). A reduction in the final yield was also observed with *L. plantarum* but not with *Leuc. mesenteroides.*

In the presence of 10% $CO_2$, all three strains grew very well. No effect on growth rate was observed with *Lact. lactis* and *Leuc. mesenteroides* compared with the control. With *L. plantarum,* a 20% increase in growth rate was found (Table 2). A lag phase (17h) was only apparent with *L. plantarum.* Final maximum population densities were not affected in any case. All growth characteristics of the three strains were affected by the combination of 90% $O_2$ and 10% $CO_2$. Moderate reduction of growth rates was observed for *Leuc. mesenteroides* and *L. plantarum* but not for *Lact. lactis*. A lag phase of ± 30 h was observed with all three strains. Maximum population densities were reduced for all three strains.

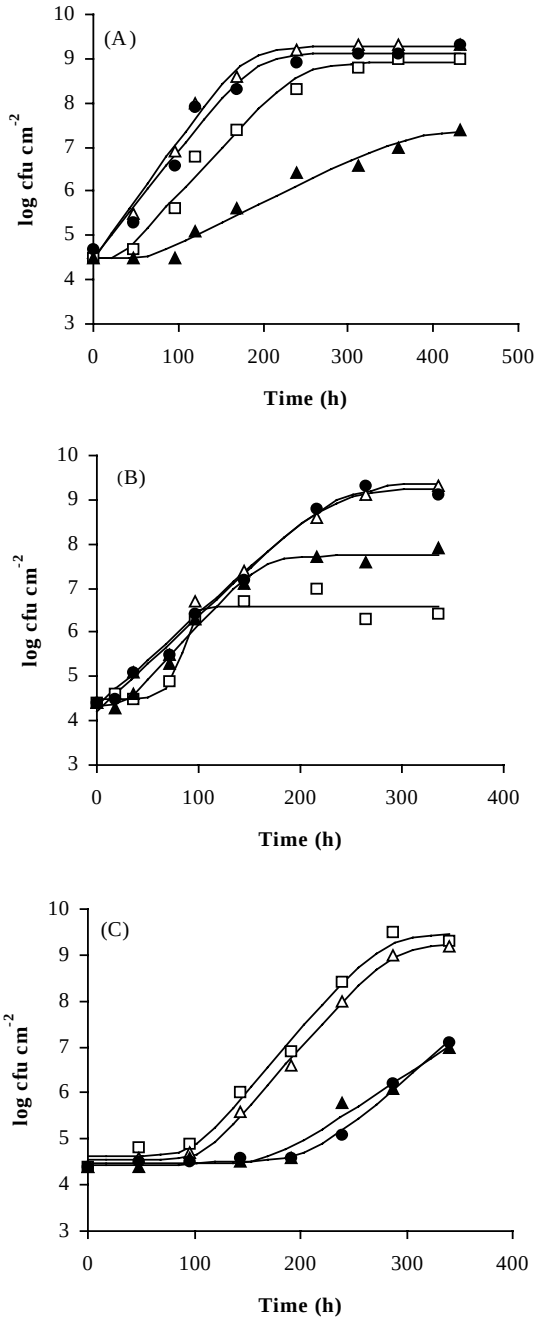


***Figure 1***. *Growth of* Escherichia coli *(A),* Lactococcus lactis *subsp* lactis *(B) and strain 27 (C) on the surface of Nutrient Agar or MRS broth under the following combinations of gases: (Δ) 20% $O_2$ : 80% $N_2$ , (□) 90% $O_2$ +10% $N_2$, (●) 10% $CO_2$ + 90% $N_2$, (▲) 90% $O_2$ + 10% $CO_2$. Data are fitted with the D-Model*

***Table 2.*** *Estimated parameters of lag time (λ), specific growth rate (μ) and maximum population densities ($y_{max}$), for the growth of* Lactococcus lactis, Leuconostoc mesenteroides *var* mesenteroides *and* Lactobacillus plantarum *derived from fitting data to the D-model. The standard errors (S.E.) of lag are also presented*

| Micro-organism | $O_2$ (%) | $CO_2$ (%) | Lag λ (h) | (S.E.) (lag) | Specific growth rate, μ ($h^{-1}$) | $Y_{max}$ (log cfu.$cm^{-2}$) |
|---|---|---|---|---|---|---|
| *Lactococcus lactis* | 20 | 0 | 0 | 0 | 0.021 | 9.2 |
| | 90 | 0 | 71 | 15 | 0.057 | 6.6 |
| | 90 | 10 | 27 | 8 | 0.025 | 7.7 |
| | 0 | 10 | 0 | 0 | 0.022 | 9.3 |
| *Leuconostoc mesenteroides* | 20 | 0 | 0 | 0 | 0.036 | 9.2 |
| | 90 | 0 | 10 | 5 | 0.044 | 9.2 |
| | 90 | 10 | 30 | 13 | 0.023 | 7.5 |
| | 0 | 10 | 0 | 0 | 0.038 | 9.2 |
| *Lactobacillus plantarum* | 20 | 0 | 0 | 0 | 0.047 | 9.3 |
| | 90 | 0 | 0 | 0 | 0.030 | 7.4 |
| | 90 | 10 | 30 | 6 | 0.032 | (8.1)* |
| | 0 | 10 | 17 | 6 | 0.056 | 9.5 |

- *As in table 1*

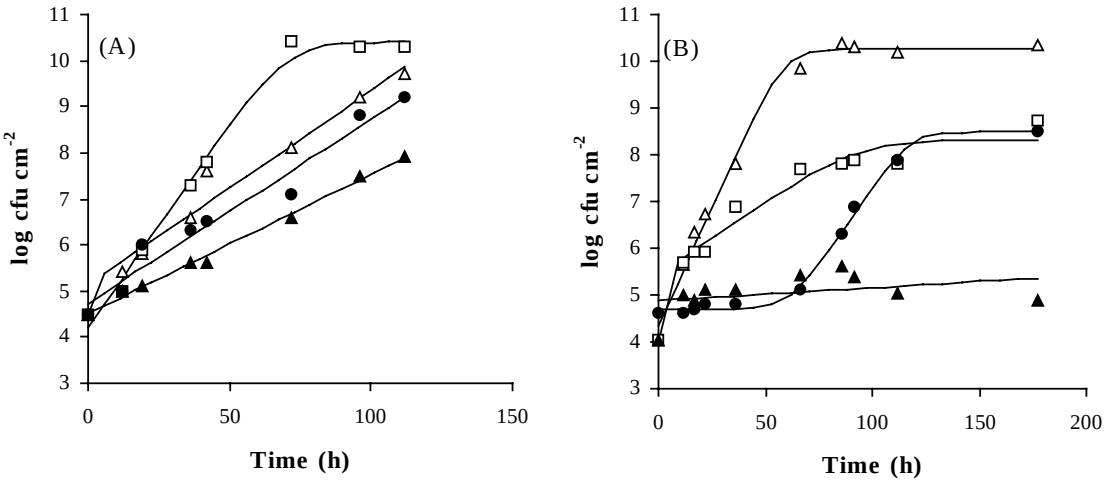


***Figure 2****. Growth of* Pseudomonas fluorescens *(A) and* Candida guilliermondii *(B) on the surface of Nutrient Agar under the following combinations of gases: (Δ) 20% $O_2$ + 80% $N_2$ , (□) 80% $O_2$ + 20% $N_2$, (●) 20% $CO_2$ + 80% $N_2$, (▲) 80% $O_2$ + 20% $CO_2$. Data are fitted with the D-Model*

### *Pseudomonas fluorescens, Enterobacter agglomerans* and *Aureobacterium* strain 27

Growth of the three strains of spoilage bacteria tested was rapid under ambient conditions at 8°C; 80% $O_2$ alone increased the growth rates of *Ps. fluorescens* (Figure 2A) and *Ent. agglomerans* by 200% and 185%, respectively. Final yields were also increased. With strain 27, incubation under 90% $O_2$ resulted in a slight extension of the lag phase, while there was no effect on either growth rate or final yield (Figure 1C, Table 3).

Under 20% $CO_2$, growth rate and lag phase of *Ent. agglomerans* were not affected (Table 3). An increase in the lag phase (26 h) was only observed with *Ps. fluorescens*. With strain 27, 10% $CO_2$ resulted in a reduction in the growth rate (28%) and a significant increase in the lag phase (211h compared to 97h at ambient conditions). Carbon dioxide alone did not affect apparent final yields with *Ps. fluorescens* and *Ent. agglomerans*, while strain 27 did not reach stationary phase within the incubation period.

The combination of the gases significantly affected growth rates of all three strains; 80% $O_2$ and 20% $CO_2$ together caused a reduction in the growth rates of *Ps. fluorescens* and *Ent. agglomerans* (Table 3). The combination of 90% $O_2$ and 10% $CO_2$ almost halved the growth rate of strain 27. An increase in lag phase duration was apparent for all three strains, most prominently with strain 27 (170 h).

***Table 3.*** *Estimated parameters of lag time ($\lambda$), specific growth rate ($\mu$) and maximum population densities ($y_{max}$), for the growth of* Aerobacterium *strain 27,* Pseudomonas fluorescens, *and* Enterobacter agglomerans *derived from fitting data to the D-model. The standard errors (S.E.) of lag are also presented*

| Micro-organism | $O_2$ (%) | $CO_2$ (%) | Lag $\lambda$ (h) | (S.E.) (lag) | Specific growth rate $\mu$ ($h^{-1}$) | $Y_{max}$ (log cfu.$cm^{-2}$) |
|---|---|---|---|---|---|---|
| *Aureobacterium* strain 27 | 20 | 0 | 97 | 12 | 0.061 | 9.5 |
| | 90 | 0 | 106 | 16 | 0.059 | 9.3 |
| | 90 | 10 | 170 | 18 | 0.035 | (7.4)* |
| | 0 | 10 | 211 | 6 | 0.048 | (7.3) |
| *Pseudomonas fluorescens* | 20 | 0 | 16 | 3 | 0.097 | 9.9 |
| | 80 | 0 | 24 | 5 | 0.200 | 10.4 |
| | 80 | 20 | 37 | 10 | 0.069 | (7.9) |
| | 0 | 20 | 26 | 9 | 0.092 | (9.6) |
| *Enterobacter agglomerans* | 20 | 0 | 0 | 0 | 0.141 | 9.9 |
| | 80 | 0 | 17 | 11 | 0.261 | 10.4 |
| | 80 | 20 | 5 | 3 | 0.110 | 9.3 |
| | 0 | 20 | 6 | 1 | 0.130 | 9.6 |

**As in table 1*

### *Candida sake* and *Candida guilliermondii.*

High $O_2$ (80%) considerably stimulated the growth rate of both *C. guilliermondii* (Figure 2B) and *C. sake*. High oxygen alone did not affect the lag phase of the two strains compared with the ambient control condition (Table 4). With *C. guilliermondii*, the growth yield under ambient $O_2$ was higher than under 80% $O_2$. The increase in viable counts of *C. sake* did not reach a maximum value under the test conditions.

With *C. guilliermondii* under 20% $CO_2$, a slightly reduced growth rate and a strongly increased lag phase were found. Also with *C. sake*, a decreased growth rate was evident.
The combined application of 80% $O_2$ and 20% $CO_2$ almost completely blocked growth of *C. guilliermondii* (Table 4). An apparent maximum yield was not reached due to the virtual absence of growth. For the same reason, a lag phase could not be deduced. Similar growth characteristics were observed with *C. sake*, although to a lesser extend.

***Table 4.*** *Estimated parameters of lag time (λ), specific growth rate (μ) and maximum population densities ($y_{max}$), for the growth of* Candida sake, *and* Candida guilliermondii *derived from fitting data to the D-model. The standard errors (S.E.) of lag are also presented*

| Micro-organism | $O_2$ (%) | $CO_2$ (%) | Lag λ (h) | (S.E.) (lag) | Specific growth rate μ ($h^{-1}$) | $Y_{max}$ (log cfu.$cm^{-2}$) |
|---|---|---|---|---|---|---|
| *Candida guilliermondii* | 20 | 0 | 0 | 0 | 0.100 | 10.3 |
| | 80 | 0 | 0 | 0 | 0.190 | 8.3 |
| | 80 | 20 | - | - | 0.002 | 4.9 |
| | 0 | 20 | 61 | 12 | 0.070 | 8.4 |
| *Candida sake* | 20 | 0 | 10 | 5 | 0.029 | 8.8 |
| | 80 | 0 | 10 | 9 | 0.040 | (9.3)* |
| | 80 | 20 | 31 | 10 | 0.010 | (7.4) |
| | 0 | 20 | - | - | 0.018 | (7.3) |

**As in table 1*

## 2.4 Discussion

The micro-organisms tested here have been reported in the literature as possible contaminants of minimally-processed vegetables (Lund, 1992; Marchetti et al., 1992; Nguyen-The and Carlin, 1994; Fain, 1996). Under ambient $O_2$ conditions (control conditions), full growth in the model system was detected between 120 and 500 h at 8°C in most cases. Growth of *List. monocytogenes* and *Salm. typhimurium* was rather slow. On the bases of the current evaluation, the following general observations can be made regarding the effects of high $O_2$ (80 or 90%) or $CO_2$ (20 or 10%), applied alone (balanced to 100% with $N_2$) and in combination:

(i) 80 or 90% $O_2$ alone did not inhibit growth of most test micro-organisms, but caused a significant reduction in the growth rate and/or maximum yield of *Salm. typhimurium*, *Salm. enteritidis* and *C. guilliermondii*. Growth of all other strains tested here was stimulated;

(ii) 10 or 20% $CO_2$ alone was found to reduce growth rates of *Ps. fluorescens*, *C. guilliermondii* and *Salm. enteritidis* significantly. A prolonged lag phase was noticed with strain 27 and *C. guilliermondii*. Growth rates of the lactic acid bacteria, *Salm. typhimurium*, *List. monocytogenes* and the non-pathogenic *E. coli* were either not affected or stimulated;

(iii) The combined application of high $O_2$ and $CO_2$ had an inhibitory effect on the growth rate of all micro-organisms, except for two of the strains that grew only poorly in the experimental set-up, *i.e. List. monocytogenes* and *Salm. typhimurium*. In most cases, a notable prolongation of the lag phase and a reduction in the final population density was observed. The most prominent effect of the combined application of high $O_2$ and $CO_2$ was found with the yeast strains that were almost completely inhibited in their growth.

Although only a limited number of prokaryotic and eukaryotic micro-organisms was evaluated in this study, it may be concluded that exposure of micro-organisms to high $O_2$ alone has an inhibitory effect on growth in a few cases only, and may occasionally have a stimulatory effect. It would be expected that the high $O_2$ levels applied would lead to intracellular generation of reactive oxygen species (ROS, $O_2^-$, $H_2O_2$, HO·) that would affect vital cell components and reduce cell viability (Halliwell and Gutteridge, 1984; Fridovich, 1986). Evidently, micro-organisms have developed strategies such as induction of $O_2$-decomposing enzymes (catalase, peroxidase, superoxide dismutase) or radical scavengers (e.g. glutathione) in order to avoid lethal damage by oxygen. Studies on the effects of oxygen stress on *Salm. typhimurium, E. coli* and *Lact. lactis* have identified the presence of inducible multi-gene systems which destroy ROS. Other proteins such as the SOS system serve to repair oxidative damage at 30-37°C (Demple and Halbrook, 1983; Farr and Kogoma, 1991; Sanders, 1997). However, to our knowledge, no information is available in the literature about the responses of pure cultures of micro-organisms to oxidative stress at lower temperatures. The ability of *Salm. typhimurium* to respond to oxidative stress is reduced as metabolic processes are slowed down at 8°C. This observation is, to a lesser degree, valid for *Salm. enteritidis* that can adapt easily at low temperature. Considering the sensitivity of the salmonellas to high oxygen, as found in the present study, incubation temperature will play a role in the ability of a micro-organisms to counteract oxidative stress.

Lactic acid bacteria can tolerate oxygen, or even use oxygen, in the presence of a carbon source by a closely coupled NADH oxidase/peroxidase system and, in some cases, superoxide dismutase (Archibald and Fridovich, 1981b; Condon, 1987; Piard and Desmazeaud, 1991). *L. plantarum* ATCC 8014 growing in flasks with APT broth could tolerate oxygen but its growth rate was reduced when incubation was under a stream of 100% $O_2$ (Gregory and Fridovich, 1974). Pure oxygen reportedly had a positive effect on growth and biomass production of *Leuc. mesenteroides* in batch cultures (Plihon et al., 1995). Similar results were obtained for *Ps. fluorescens* (Onken, 1990).

Our results concerning the effect of high oxygen on yeasts are in agreement with previous reports. Whereas an "excess oxygen state" (60% or higher) has been found to initiate high cell yields and low levels of fermentation products of *Saccharomyces cerevisiae*, the cellular response of eukaryotic micro-organisms to oxidative stress remains to be resolved (Brown and Johnson, 1971; Moradas-Ferreira et al., 1996; Pinheiro et al., 1997).

High levels of $CO_2$ have frequently been reported to reduce microbial growth significantly, although results were quite variable when comparing different spoilage and pathogenic bacteria (Dixon and Kell, 1989; Eyles et al., 1993; Ogihara et al., 1993). Extension of the lag phase and reduction of the growth rate is often considered to be a prominent effect of $CO_2$ (Farber, 1991a). Bennik et al., (1995) have shown that this effect is consistent only at very high $CO_2$ concentrations but $CO_2$-enriched modified atmospheres are not a reliable way to control the fate of *List. monocytogenes* in vegetable products (Carlin et al., 1996). *List. monocytogenes* growing on fresh produce was not inhibited by $CO_2$ up to 70% (Kallander et al., 1991). Several studies showed that *Pseudomonas* spp. expressed a rather high sensitivity towards $CO_2$ (also at 20%), although total inhibition may not be achieved if $O_2$ is reduced to 0.2-0.6% but not fully depleted (Gill and Tan, 1979; Enfors and Molin, 1980; Molin, 1983). *Enterobacteriaceae* were reported to be unaffected by 20% $CO_2$ at chill temperatures (Gill and Tan, 1980; Bennik et al., 1998). In general, growth of *Enterobacteriaceae*, including *Salmonella* spp., is only retarded at very high levels of $CO_2$ but the inhibitory effect is temperature dependent (Sawaya et al., 1995; Woolfe et al., 1995). Most reports for growth of *Salmonella* at elevated $CO_2$ levels concern highly proteinaceous foods. Prolongation of the lag phase by $CO_2$ of *Salm. typhimurium* at low temperature (10°C) has been reported by Silliker and Wolfe (1980). The high sensitivity of *Salm. enteritidis* to only 10% $CO_2$ is probably a strain-specific phenomenon. Lactic acid bacteria were generally favoured by elevated $CO_2$ or reduced $O_2$ concentrations (Roth and Clark, 1975), while the effect on yeasts is variable (Jones and Greenfield, 1982; Eklund and Jarmund, 1983; Ison and Gutteridge, 1987; Barriga et al., 1991).

In this study, growth characteristics of all pathogenic micro-organismswere significantly reduced at combinations of high $O_2$ and $CO_2$ compared with air. In the case of *Listeria*, the growth rate was stimulated compared with air but was retarded compared with 10% $CO_2$. Ogihara et al., (1993) have found that pure cultures of facultative anaerobic bacteria such as *Enterobacter* spp., *List. monocytogenes* and *Salm. typhimurium* are not completely inhibited by high $O_2$ or $CO_2$ or a mixture of both, although the growth rate was reduced in proportion to the increase in the ratio of $CO_2$ in the gas mixtures.

From the results of the current study, as well as from the available literature, it is concluded that when high $O_2$ and $CO_2$ are applied alone, the inhibitory effect on microbial growth is highly variable. Stronger and much more consistent inhibition of microbial growth can be obtained when the two gases are used in combination. This result is very relevant because it enables a further improvement of the low-$O_2$ MA packaging systems currently used for minimally-processed vegetables; $CO_2$ levels in the combined treatment need not be higher than 20% to give consistent suppression of microbial growth, although this finding remains to be validated in product studies.

**Acknowledgements**

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# Chapter 3
## Antioxidative properties of *Lactobacillus sake* upon exposure to elevated levels of oxygen

A. Amanatidou, E.J. Smid, M.H.J. Bennik & L.G.M. Gorris
(FEMS Microbiology Letters, *in press*)

## Abstract

The ability of bacteria to overcome oxidative stress is related to the levels and types of antioxidative mechanisms which they possess. In this study, the antioxidative properties in *Lactobacillus sake* strains from different food origins were determined at low temperature (8$^{o}$C) and upon exposure to oxygen levels between 20 and 90% $O_2$. The *L. sake* strains tested grew well at 8$^{o}$C and in the presence of 20% $O_2$, however, most of the strains could not grow at $O_2$ levels as high as 50 and/or 90%. Cell-free extracts of all strains possessed certain levels of hydroxyl radical scavenging, metal chelating and reducing capacities essential for growth of cells at ambient $O_2$. At elevated $O_2$ conditions, high $H_2O_2$ splitting capacity and low specific rate of $H_2O_2$ production were demonstrated in the $O_2$ insensitive strain *L. sake* NCFB 2813. Although $H_2O_2$ was generated in the $O_2$-sensitive *L. sake* DSM 6333, at levels which were not directly toxic to the cells (<0.2 mmol.l$^{-1}$), it is concluded that its removal is essential for cell protection at elevated $O_2$ conditions.

## 3.1 Introduction

It is well known that the $O_2$ molecule has low reactivity and its toxicity stems mostly from its excited state (singlet oxygen) or its semi-reduced radical forms which can cause deleterious or lethal oxidative damage to the cells (Gille and Sigler, 1995). The four electron reduction of $O_2$ to $H_2O$ gives rise successively to the formation of reactive oxygen species (ROS), i.e. superoxide radical anion ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical (HO·). Several models have been proposed to explain the mechanism of $O_2$ sensitivity in micro-organisms. $O_2^-$ and $H_2O_2$ are moderately reactive in aqueous solutions, but they both contribute to the formation of the highly reactive oxidant HO· via the Haber-Weiss or Fenton reactions (Imlay and Linn, 1986; Kehrer, 2000). In bacteria, generation of the HO· radical is facilitated by the presence of $O_2^-$, $H_2O_2$ and free iron (Britigan et al., 1996; McCormick et al., 1998). Many components like hemoproteins, lipids and DNA are targets for HO· radicals**,** which are formed by a site-specific Fenton-driven mechanism.

Many micro-organisms possess enzymatic and non-enzymatic antioxidative mechanisms and minimise generation of reactive oxygen species (ROS) to levels that are not harmful to the cells. These antioxidative mechanisms of bacteria are well studied in *Escherichia coli* and *Salmonella typhimurium* (Farr and Kogoma, 1991).

Lactic acid bacteria lack many of the components of the respiratory chain which facilitate the utilisation of $O_2$ as a terminal electron acceptor (Morishita and Yajima, 1995). However, many lactic acid bacteria synthesise the NADH oxidase/NADH peroxidase system which balances the NAD+/NADH ratio, catalyses the reduction of $O_2$ to $H_2O_2$ and decomposes $H_2O_2$ to $H_2O$ for the purpose of protection (Gotz et al., 1980; Condon, 1987; Lopez de Fellipe et al., 1997; Yi et al., 1998). The effect of elevated $O_2$ (>40%) on growth and metabolism of lactic acid bacteria is much less studied (Condon 1987, Warriner and Morris, 1995; Lin and Yen, 1999). Duwat et al., (1995a) and Sanders et al., (1999) suggested that oxygen-induced toxicity in *Lactococcus lactis* upon aeration occurs due to the formation of ROS.

Elevated $O_2$ can be used in combination with other preservation methods to inhibit growth of micro-organisms and increase shelf-life of food products (Amanatidou et al., 2000a,b). The objective of this

study was to investigate the responses of *Lactobacillus sake* strains from different origins to elevated $O_2$ concentrations in more detail. The focus was on the importance of the antioxidative capability of different strains for their protection dupon exposure to elevated $O_2$. *L. sake* is a fermentative, Gram(+) micro-organism and does not require strict anaerobic conditions for growth. *L. sake* strains are involved in numerous food fermentation processes as starters or as protective cultures, but are also associated with spoilage of, for instance, meat. Because of the economical importance for preservation, but also because the effect of elevated $O_2$ is enhanced at low temperature (Amanatidou et al., 1999), all experiments presented here, were performed at 8°C.

## 3.2 Material and methods

### Reagents

Horseradish peroxidase was obtained from Boehringer GmbH (Mannheim, Germany). Hydrogen peroxide was obtained from Merck GmbH (Darmstadt, Germany). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

### Strains and growth conditions

All the strains used in this study were original food isolates obtained from international culture collections. *Lactobacillus sake* NCFB 2814, *Lactobacillus sake* NCFB 2813 and *Lactobacillus sake* NCFB 2812 originate from meat; *Lactobacillus sake* NCFB 20498 originates from fermented vegetables; *Lactobacillus sake* DSM 6333 is a starter culture; *Lactobacillus sake* IFO 3541, *Lactobacillus sake* IFO 18450 and *Lactobacillus sake* DSM 15521 originate from vegetable products.
Cultures were routinely grown overnight in 100 ml MRS broth (Oxoid, Hampshire, UK) up to $OD_{660}$ ± 0.6. Aliquots of the cultures were washed twice in 0.1 M potassium phosphate buffer (KPi, pH 6.4) and transferred to modified MRS medium, in which magnesium sulphate, manganese sulphate and sodium acetate were not added in the formula. 200 µl aliquots were incubated in 16 wells of a 96-well microtiter plate. Microtiter plates were covered with a lid and wrapped with parafilm to allow gas exchange while avoiding evaporation. Plates were incubated at 8°C in a controlled atmosphere environment which was continuously flushed to maintain obtain anaerobic (10% $CO_2$ + 90% $N_2$), aerobic (20% $O_2$ + 80% $N_2$) or high oxygen (either 50% $O_2$ + 50% $N_2$ or 90% $O_2$ + 10% $N_2$) conditions. Each gas composition was prepared by mixing pure gases ($O_2$, $CO_2$, $N_2$ ) using mass-flow controllers (5850 TR series Brooks Instruments B.V., Veenendaal, The Netherlands), humidified to a level close to saturation by passage through a water bottle, and finally introduced into the chambers at a flow rate of 200 $ml.min^{-1}$. Growth under the different gas conditions was assessed by determining the mean of the optical densities in 12 wells. An initial inoculum level of $10^2$ to $10^3$ $cfu.ml^{-1}$ was used. For physiological studies, cells were inoculated in flasks containing 100 ml of MRS pre-conditioned for 48h with the desired gas conditions. Lactobacilli were enumerated by plating on MRS agar after incubation at 25°C for 3 days. The absorbance values were fitted to the mathematical model described by Baranyi et al., (1993) and reparameterised with the DMFit program (1996, IFR, Reading, UK), kindly provided by Dr J. Baranyi, to estimate the specific growth rate µ ($h^{-1}$) of each culture under the different gas conditions.

### Preparation of cell-free extracts

Cells were cultured up to exponential phase ($10^7$ $cfu.ml^{-1}$) under aerobic conditions (20% $O_2$ + 80% $N_2$) at 8°C. Subsequently, cells were harvested and centrifuged at 5500 x *g* for 10 min at 4°C. Immediately after removal, chloramphenicol was added to all samples at a final concentration of 170 µg $ml^{-1}$, in order to inhibit further enzyme activity while preparing cell-free extracts. Cells were concentrated up to about $OD_{660}$ = 1 (equivalent to $\pm 10^{10}$ $cfu.ml^{-1}$), washed twice and resuspended in lysis buffer (potassium phosphate $K_2HPO_4/KH_2PO_4$ 30 $mmol.l^{-1}$ and sucrose 0.4 M, pH 7.4). Mutanolysin (25 $U.ml^{-1}$) and lysozyme (500 $\mu g.ml^{-1}$) were added. Cells were incubated at 37°C for 45

min, washed again, resuspended in KPi and disrupted with a sonifier cell disrupter (Brandson Sonic Power Co., Plainview, NY, USA) in an ice bath, for three cycles of 0.5 min, with 1 min intervals. Cell debris was removed by centrifugation at 40000 x *g* for 20 min at 4°C.

### Determination of anti-oxidative properties of cells and cell-free extracts

Unless stated otherwise, all determinations of the anti-oxidative properties of whole cells or cell-free extracts were performed using cultures in exponential growth phase under 20% $O_2$ + 80% $N_2$ at 8°C and concentrated to about $OD_{660}$ = 1.

*Hydroxyl radical scavenging ability*

Hydroxyl radicals were generated by UV treatment of 1 ml of de-ionised water (265 nm, 30 min, UV radiation flux 0.75 $mW.cm^{-2}$). The hydroxyl radical scavenging capacity of 50 µl of cell-free extract was determined using the method of Gutteridge (1987). Uric acid was used as a standard.

*$Fe^{2+}$ chelating capacity*

0.5 ml of cell-free extract was mixed with 0.1 ml of ascorbate (1% w/v), 0.1 ml $FeSO_4$ (0.4% w/v) and 1 ml NaOH (0.2 M). Proteins in the cell-free extracts were precipitated by adding 10% trichloroacetic acid (TCA, 10% w/v) whereupon the mixture was incubated in a water bath at 37°C for 20 min. The supernatant was obtained by centrifugation at 3000 x *g* for 10 min at 4°C. $Fe^{2+}$ chelating capacity in the deproteinized supernatant was measured spectrophotometrically by determining the increase in absorption at 510 nm after 10 min of reaction time with 0.5 ml of 0.1% *o*-phenanthroline (Yamauchi et al., 1984).

*Reducing activity*

A reaction mixture was prepared by mixing 0.5 ml of a cell-free extract with 0.5 ml of potassium ferricyanide (1% w/v) and 0.5 ml KPi buffer (0.02 M, pH 6.4), according to Wolfe (1962). The reaction mixture was incubated at 50°C for 20 min and rapidly cooled down to 20°C. 0.5 ml of TCA (10% w/v) was added in the reaction mixture. A deproteinized supernatant was obtained by centrifugation at 3000 x *g* for 5 min at 4°C. Aliquots of the supernatant (0.1 and 0.2 ml) were mixed with 0.2 ml of $FeCl_3$ (0.1% w/v). Reducing activity of cell-free extracts was expressed as equivalent amount of µM cysteine, which demonstrated the same absorbance at 700 nm.

### Extracellular $H_2O_2$ toxicity

Whole cells from cultures which had grown to exponential phase were washed; 1 ml of culture was inoculated in a petri dish into which 15 ml MRS agar was added with the pour-plate technique. A filter disk was immersed in a solution of $H_2O_2$ (1.5 $mmol.l^{-1}$) and subsequently placed in the centre of the gelated MRS agar. The petri dish was incubated under aerobic conditions at 8°C. The zone of growth inhibition was measured after 10 days of storage. Three independent experiments were performed with each strain to determine the toxicity of $H_2O_2$.

### Extracellular generation of $H_2O_2$

Extracellular generation of $H_2O_2$ was measured for cultures of *L. sake* NCFB 2813 and *L.* s*ake* DSM 6333 growing at 8°C under 10% $CO_2$ + 90% $N_2$ , 20% $O_2$ + 80% $N_2$ or 90% $O_2$ + 10% $N_2$. At given time intervals, 1 ml aliquots were taken from the cultures and centrifugated (1300 x *g*, 10 min, 4°C); 0.5 ml aliquots of supernatant were then mixed with 0.5 ml 4-aminoantipyrine (6 $mmol.l^{-1}$, pH 7) and 0.5 ml 3,5 dichloro-2-hydroxybenzene sulfonate (21 $mmol.l^{-1}$, pH 7.0); 50 mU horseradish peroxidase was added to catalyse the reaction. The absorbance decrease was assayed spectro-photometrically at 546 nm. The $H_2O_2$ content was determined by comparing the absorbance of the sample with a standard curve. The $H_2O_2$ generated was expressed either as $mmol.l^{-1}$ of $H_2O_2$ in the extracellular growth medium or as nmol $H_2O_2$ $mg^{-1}$ bacterial protein.

### Intracellular generation of $H_2O_2$

The specific intracellular $H_2O_2$ generation rate was determined for *L. sake* NCFB 2814, *L. sake* NCFB 2813, *L. sake* NCFB 20498, *L. sake* DSM 6333 and *L. sake* IFO 3541. Cells grown up to exponential phase under 10% $CO_2$ + 90% $N_2$, 20% $O_2$ + 80% $N_2$ or 90% $O_2$ + 10% $N_2$ were concentrated to $10^{10}$ cfu.ml$^{-1}$, washed twice in fresh PBS and resuspended in PBS previously equilibrated for 48h at 8$^o$C with the same gas mixture. At regular time intervals, 0.5 ml samples were taken for measurement of the $H_2O_2$ generation rate using the method described above. After about 15 min of incubation a plateau level was reached. The intracellular production rate of $H_2O_2$ was calculated on the assumption that $H_2O_2$ can freely move across the cytoplasmic membrane until an equilibrium is reached between the extracellular and intracellular concentration. The specific rate of $H_2O_2$ generation was expressed as pmol $H_2O_2$ s$^{-1}$ .mg$^{-1}$ bacterial protein.

### Effect of extracellular $H_2O_2$ on *L. sake* NCFB 2813 and *L. sake* DSM 6333

Cultures of *L. sake* NCFB 2813 and *L. sake* DSM 6333, grown in MRS under 90% $O_2$ + 10% $N_2$ at 8$^o$C, were washed in phosphate buffered saline (PBS, 0.05 M, pH 6.4) whereupon aliquots of the culture were transferred into fresh PBS containing 0.2, 0.4 or 0.6 mmol.l$^{-1}$ $H_2O_2$; prior to inoculation, the fresh PBS was equilibrated for 48h using the same gas mixture. At regular time intervals during 30 min incubation, 1 ml aliquots were taken from the cultures and viable counts determined to assess the effect of $H_2O_2$ on viability. The effect of $H_2O_2$ on growth during 10 d at 8$^o$C in the absence of $O_2$ was determined in fresh MRS medium, equilibrated for 48h at 10% $CO_2$ + 90% $N_2$. $H_2O_2$ was added to the MRS medium prior to inoculation with a culture aliquot to an initial inoculum level of $10^2$ to $10^3$ cfu.ml$^{-1}$; cultures were pre-grown under anaerobic conditions.

### Other methods

Protein concentration was measured using bovine serum albumin as a standard according to Lowry et al., (1959).

## 3.3 Results

### Growth of *L. sake* strains under different gas conditions

Eight different strains of *L. sake* were grown at low temperature under four different gas atmospheres to evaluated their ability to respond to oxidative stress induced by incubation under elevated $O_2$ conditions. The maximum specific growth rates ($\mu_{max}$) recorded under anaerobic, ambient and high $O_2$ conditions are summarised in Table 1. All strains could grow at 8$^o$C under anaerobic conditions (10% $CO_2$ + 90% $N_2$); the highest growth rate was observed with *L. sake* NCFB 20498, whereas the lowest growth rates were recorded with *L. sake* IFO 3541, IFO 18450 and DSM 15521. The latter three strains also displayed the lowest growth rates under ambient $O_2$ conditions (20% $O_2$ + 80% $N_2$); the growth rates of *L. sake* NCFB 2814, NCFB 20498, DSM 6333 and DSM 15521 were lower under ambient compared with under anaerobic conditions, while growth of *L. sake* NCFB 2813 and IFO 18450 was about equal under both conditions; growth of *L. sake* NCFB 2812 and IFO 3541 under aerobic conditions was slightly faster than under anaerobic condition. The various strains tested displayed different levels of sensitivity to elevated $O_2$ (present in the gas atmosphere either at 50% or at 90%); for *L. sake* NCFB 2812, NCFB 20498 and DSM 6333, growth rates under 90% $O_2$ + 10% $N_2$ were significantly lower compared with growth under 50% $O_2$ + 50% $N_2$; only strain *L. sake* NCFB 2813 grew consistently well under both high $O_2$ conditions tested.

***Table 1*** *Specific growth rates of different* L. sake *strains grown in MRS broth in microplates at 8°C under different gas conditions. Specific growth rates were calculated with the DMFit program from absorbance data assessed at* $OD_{660}$

| Strain | Specific growth rate μ ($h^{-1}$) | | | |
|---|---|---|---|---|
| | 10%$CO_2$+90% $N_2$ | 20%$O_2$+80% $N_2$ | 50%$O_2$+50% $N_2$ | 90%$O_2$+10% $N_2$ |
| *L. sake* NCFB 2814 | 0.171 ± 0.002 | 0.133 ± 0.009 | 0.064 ± 0.012 | 0.107 ± 0.014 |
| *L. sake* NCFB 2813 | 0.124 ± 0.030 | 0.119 ± 0.020 | 0.110 ± 0 | 0.117 ± 0.011 |
| *L. sake* NCFB 2812 | 0.088 ± 0.001 | 0.116 ± 0.021 | 0.066 ± 0 | 0.010 ± 0.002 |
| *L. sake* NCFB 20498 | 0.308 ± 0.040 | 0.246 ± 0.032 | 0.066 ± 0.009 | 0.017 ± 0.002 |
| *L. sake* DSM 6333 | 0.168 ± 0.022 | 0.124 ± 0.015 | 0.128 ± 0.027 | 0.032 ± 0.006 |
| *L. sake* IFO 3541 | 0.054 ± 0.004 | 0.078 ± 0.013 | 0.031 ± 0.002 | 0.016 ± 0.002 |
| *L. sake* IFO 18450 | 0.046 ± 0.010 | 0.050 ± 0.009 | 0.027 ± 0.002 | 0.010 ± 0.002 |
| *L. sake* DSM 15521 | 0.024 ± 0.003 | 0.009 ± 0.002 | 0.008 ± 0.002 | 0.001 ± 0.001 |

## Anti-oxidative properties of cells and cell-free extracts

*Hydroxyl radical scavenging activities in cell-free extracts of* L. sake *strains*

The HO· radical scavenging activities assessed in the various *L. sake* strains grown under ambient $O_2$ conditions are presented in Table 2. The lowest HO· scavenging capacity was found in cell- free extract of *L. sake* NCFB 2814 (0.7 ± 0.6 mmol.$l^{-1}$ equivalent uric acid) and the highest in cell-free extract of *L. sake* DSM 15521 (6.4 ± 0.5 mmol.$l^{-1}$ equivalent uric acid). The HO· radical scavenging capacities of cells grown under anaerobic or high oxygen conditions were similar to those under ambient $O_2$ conditions, indicating that this property is not induced by the environmental gas conditions (data not shown).

***Table 2*** *Antioxidative properties of cell-free extracts or whole cells derived from cultures of different* L. sake *strains grown to exponential phase under 20% $O_2$ + 80% $N_2$. The results presented are means of two independent measurements with two repetitions each*

| Strain | Scavenging of HO· (mmol.$l^{-1}$ equiv. Uric acid) | Chelating ability ($Fe^{2+}$ ppm) | Reducing activity (μmol.$l^{-1}$ cystein equiv.) | Extracellular $H_2O_2$ toxicity (inhibition zone mm) |
|---|---|---|---|---|
| *L. sake* NCFB 2814 | 0.7 ± 0.6 | 7.2 ± 0.6 | 59.8 ± 3.6 | 32 ± 5 |
| *L. sake* NCFB 2813 | 1.7 ± 0.4 | 17.5 ± 1.3 | 32.7 ± 2.2 | 11 ± 4 |
| *L. sake* NCFB 2812 | 1.3 ± 0.2 | 6.8 ± 0.5 | 56.6 ± 4.3 | 74 ± 9 |
| *L. sake* NCFB 20498 | 1.6 ± 0.4 | 5.7 ± 1.0 | 29.4 ± 2.8 | 56 ± 4 |
| *L. sake* DSM 6333 | 2.1 ± 0.9 | 11.8 ± 2.3 | 19.5 ± 3.1 | 81 ± 3 |
| *L. sake* IFO 3541 | 4.9 ± 0.5 | NM* | 77.9 ± 5.2 | 24 ± 6 |
| *L. sake* IFO 18450 | 1.7 ± 0.3 | 4.9 ± 1.5 | 2.7 ± 1.4 | NG* |
| *L. sake* DSM 15521 | 6.4 ± 0.5 | 5.6 ± 1.7 | 5.8 ± 2.6 | NG |

**NM: not measured; NG: no growth*

*$Fe^{2+}$ chelating capacities in cell-free extract of* L. sake *strains*

High $Fe^{2+}$ chelating capacities were found in cell-free extract of *L. sake* NCFB 2813 and DSM 6333, namely 17.5 ± 1.3 and 11.8 ± 2.3 ppm, respectively (Table 2). The capacities of the other *L. sake* strains tested were about 2 to 3 times lower.

*Reducing activities in cell-free extract of* L. sake *strains*

Relatively high values for the reducing activity were observed for 5 out of the 8 strains tested (Table 2). The reducing activity was highest in cell-free extract of *L. sake* IFO 3541 (77.9 ± 5.2 µM equivalent cystein) and lowest for *L. sake* IFO 18450 and DSM 15521 (2.7 ± 1.4 and 5.8 ± 2.6 µM equivalent cystein, respectively).

### Toxicity of extracellular $H_2O_2$ towards growing cells of *L. sake* strains

As presented in Table 2, the largest inhibition zones were observed on pour-plates inoculated with *L. sake* DSM 6333 (81 ± 3 mm) and *L. sake* NCFB 2812 (74 ± 9 mm), strains that are relatively $O_2$-sensitive; the smallest inhibition zone was observed for *L. sake* NCFB 2813 (11 ± 4 mm), a strain which is rather $O_2$-insensitive; the inhibition zones observed for *L. sake* NCFB 2814 (32 ± 5 mm) and *L. sake* IFO 20498 (56 ± 4 mm) were of intermediate size diameters. No growth was observed for the remainder two strains tested.

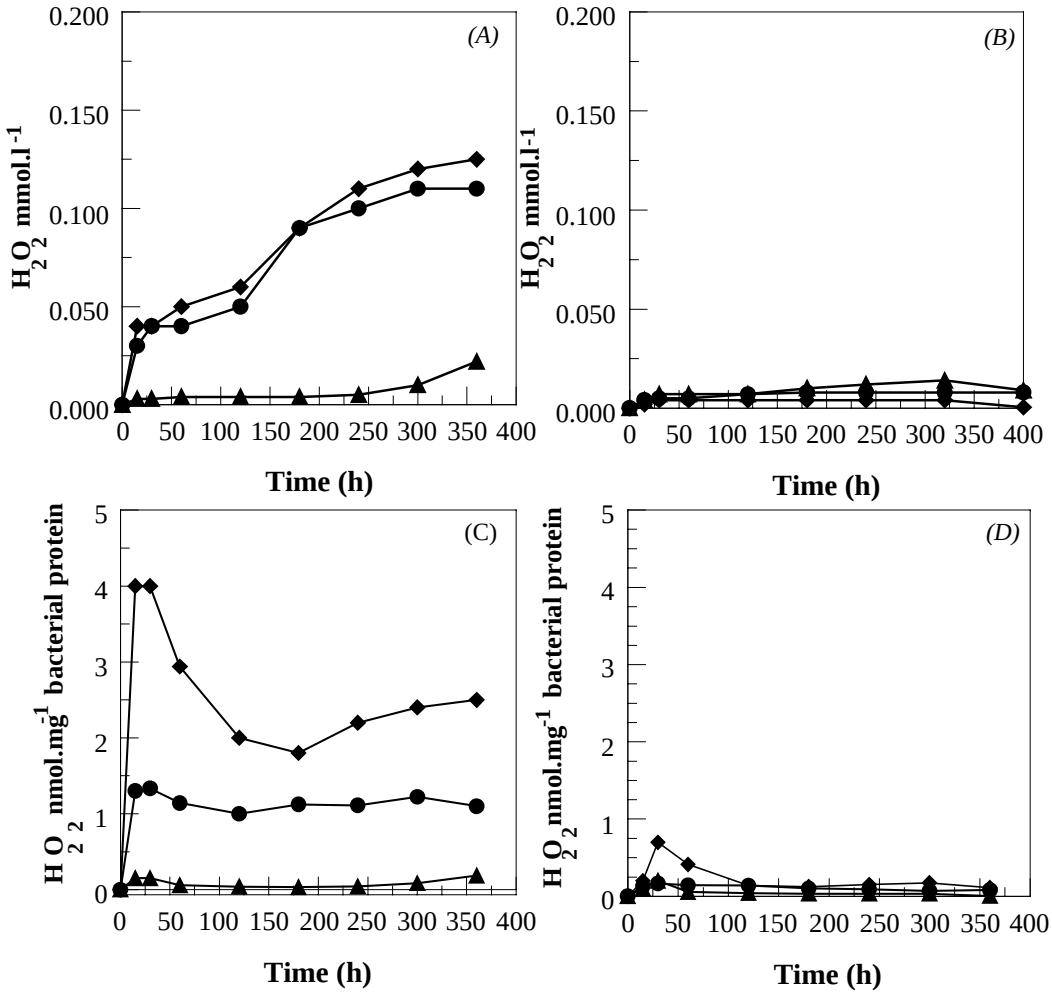


***Figure 1*** *$H_2O_2$ generation in* L. sake *DSM 6333 (A, C) and* L. sake *NCFB 2813 (B, D) in MRS broth at 8°C during exposure to 90% $O_2$ + 10% $N_2$ (♦), 20% $O_2$ + 90% $N_2$ (●) or 10% $CO_2$ + 90% $N_2$ (▲). Panel A and B depict the $H_2O_2$ pools measured in cell-free supernatanst. Panels C and D show the specific rate of $H_2O_2$ generation per mg of protein. Each measurement is the mean of two independent experiments*

### Determination of extracellular $H_2O_2$ generation by selected *L. sake* strains

Extracellular generation of $H_2O_2$ was assessed for two strains that displayed either $O_2$-sensitivity or $O_2$-insensitivity most distinctly and consistently, namely *L. sake* DSM 6333 ($O_2$-sensitive) and *L. sake* NCFB 2813 ($O_2$-insensitive). Results are shown in Figure 1. In the absence of $O_2$, accumulation of $H_2O_2$ was very low in both strains (panel A and B). After about 250 h incubation, $H_2O_2$ pools in the *L. sake* DSM 6333 culture reached 0.120 mmol.$l^{-1}$ both under ambient and high $O_2$ condition. The specific $H_2O_2$ generation values (panel C and D) show a low activity under all conditions for *L. sake* NCFB 2813 and under anaerobic condition for *L. sake* DSM 6333; for the latter strain, the value under high $O_2$ condition was two to four times higher than that under aimbient $O_2$ condition throughout the course of incubation.

### Specific rates of intracellular $H_2O_2$ generation by selected strains of *L. sake*

Significant differences were observed in the levels of the specific intracellular $H_2O_2$ generation rate under different gas conditions of the four strains tested (Table 3). Under anaerobic conditions (10% $CO_2$ + 90% $N_2$ ), the rate of $H_2O_2$ accumulation was only measured in *L. sake* IFO 3541, for which it was found to be very low (0.07 ± 0.15 pmol.$s^{-1}$.$mg^{-1}$ bact protein). Under ambient $O_2$ conditions, the highest rate was found in *L. sake* DSM 6333 (1.9 ± 0.3 pmol.$s^{-1}$.$mg^{-1}$ bact protein) and the lowest in *L. sake* NCFB 2813 (0.41 ± 0.15 pmol.$s^{-1}$.$mg^{-1}$ bact protein). Also under high $O_2$ condition, the rate was highest in *L. sake* DSM 6333 (3.4 ± 0.2 pmol.$s^{-1}$.$mg^{-1}$ bact protein) and lowest in strains *L. sake* NCFB 2813 (0.77 ± 0.1 pmol.$s^{-1}$.$mg^{-1}$ bact protein).

***Table 3*** *Specific rates of intracellular $H_2O_2$ generation in cultures of selected strains of* L. sake *grown in MRS at 8°C under different gas conditions*

| Strain | $H_2O_2$ specific production rate (pmol.$s^{-1}$.$mg^{-1}$ bacterial protein) | | |
|---|---|---|---|
| | 10% $CO_2$ + 90% $N_2$ | 20% $O_2$ + 80% $N_2$ | 90% $O_2$ + 10% $N_2$ |
| *L. sake* NCFB 2814 | NM* | 1.24 ± 0.15 | 1.92 ± 0.1 |
| *L. sake* NCFB 2813 | NM | 0.41 ± 0.15 | 0.77 ± 0.1 |
| *L. sake* DSM 6333 | NM | 1.9 ± 0.3 | 3.4 ± 0.2 |
| *L. sake* IFO 3541 | 0.07 ± 0.15 | 1.09 ± 0.06 | 1.11 ± 0.32 |

**not measured*

### Effect of extracellular $H_2O_2$ on viability and growth of selected *L. sake* strains

Cells of *L. sake* DSM 6333 and NCFB 2813 were exposed different levels of $H_2O_2$ (0, 0.2, 0.4 and 0.6 mmol.$l^{-1}$) under high $O_2$ (90% $O_2$ + 10% $N_2$) or anaerobic (10% $CO_2$ + 90% $N_2$ ) conditions for 30 min at 8°C. During short-term incubation under high $O_2$, the number of surviving, viable cells of *L. sake* DSM 6333 declined significantly towards the end of the incubation period in the presence of 0.4 and 0.6 mmol.$l^{-1}$ $H_2O_2$, while cell numbers were hardly affected in the presence of 0 and 0.2 mmol.$l^{-1}$ $H_2O_2$ (Figure 2). During prolonged growth under anaerobic conditions, the presence of 0.2 mmol.$l^{-1}$ $H_2O_2$ resulted in a significantly lower growth rate of *L. sake* DSM 6333 compared with the control (no $H_2O_2$ added), while in the presence of 0.4 and 0.6 mmol.$l^{-1}$ $H_2O_2$ a gradual decline in the number of viable cells was observed from the start of the incubation (Figure 3). Viability and growth of *L. sake* NCFB 2813 were not affected by any concentration of $H_2O_2$, including 0.6 mmol.$l^{-1}$ (data not shown).

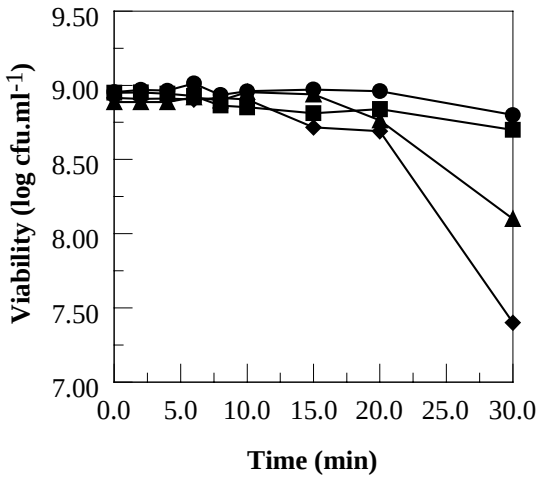


***Figure 2*** *Influence of $H_2O_2$ on viability of strain* L. sake *DSM 6333 at 8°C during 30 min under 90% $O_2$ + 10% $N_2$ in PBS supplemented with different concentrations of $H_2O_2$. Symbols ●: 0 mmol.l$^{-1}$ $H_2O_2$, ■: 0.2 mmol.l$^{-1}$ $H_2O_2$, ▲: 0.4 mmol.l$^{-1}$ $H_2O_2$, ♦:0.6 mmol.l$^{-1}$ $H_2O_2$*

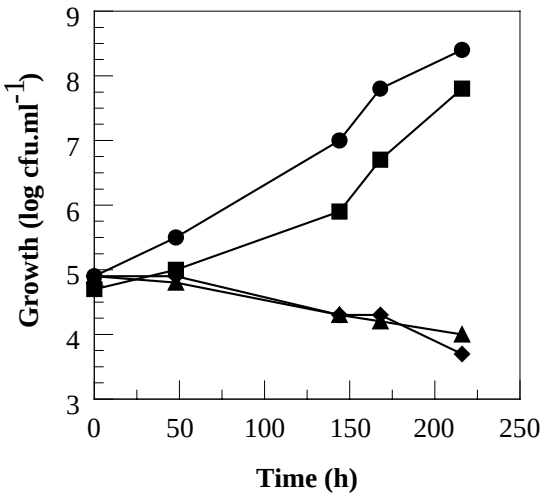


***Figure 3*** *Growth of strain* L. sake *DSM 6333 during incubation under 10% $CO_2$ + 90% $N_2$ in MRS broth supplemented with the different concentrations of $H_2O_2$. Symbols are as in Figure 2*

## 3.4 Discussion

The aim of this study was to investigate the effect of elevated oxygen on a range of *L. sake* strains originating from different food products. Five of the eight *L. sake* strains tested in this study could grow well at low temperatures under both anaerobic or ambient oxygen conditions, as indicated by the ralatively high specific growth rates observed. Most strains tested showed some degree of sensitivity to elevated (50% or 90%) $O_2$, with the exception of *L sake* NCFB 2813. The various strains were found to differ in their specific antioxidative properties, assessed on the basis of metal chelating capacity, hydroxyl radical scavenging ability and reducing activity. Only in a few cases these antioxidative properties were found to be clearly correlated with oxygen sensitivity or tolerance during growth under high $O_2$ conditions.

Antioxidative properties are important to overcome oxidative stress conditions that may develop during exposure to elevated oxygen levels; antioxidative compounds can inactivate HO· radicals or its precursors (i.e. $O_2^-$, $H_2O_2$). Bacteria have many non-enzymatic strategies to counteract the propagation of the very reactive HO· radical (Halliwell et al., 1995). The extent of HO· radical scavengering activity seems to be an indigenous property of each strain as it is apparently not regulated by changes in environmental conditions or the level of the imposed oxidative stress; in the present study, the levels of hydroxyl radical scavengers were similar whether cells of *L. sake* were exposed to anaerobic or high $O_2$ conditions. Although all *L. sake* strains tested had significant levels of HO· radical scavenger activity, this antioxidatie property alone does not explain the differences in oxygen sensitivity observed between the various strains.

Prevention of HO· radical generated toxicity can be through the reducing of the level of available ferrous ions, since iron is participating in the HO· radical generating Fenton and Haber-Weiss reactions. Release of Fe (II) from enzymes containing iron-sulphur clusters via oxidation reactions catalyses the formation of ROS and stimulates lipid peroxidation in biological systems (Halliwell and Gutteridge, 1990; Liochev and Fridovich, 1994). Iron chelators can protect oxygen-sensitive enzymes such as CuZnSOD against the action of $H_2O_2$. A high $Fe^{2+}$ chelating activity was found in the $O_2$-insensitive *L. sake* NCFB 2813 and this activity may contribute to the ability of this strain to withstand high levels of $O_2$. However, a relatively high iron chelating activity was found as well in the quite $O_2$-

sensitive strain *L. sake* DSM 6333. This may indicate that, whereas a high $Fe^{2+}$ chelating activity probably contributes to the ability of *L. sake* to overcome oxidative stress, its contribution to the overall antioxidative properties of *L. sake* NCFB 2813 is sufficient to allow this specific strain to grow well when exposed to high $O_2$ levels, while the contribution in the case of *L. sake* DSM 6333 may not be yielding sufficiently high antioxidative protection.

With respect to the reducing activity of the *L. sake*, which all strains possessed to different extends, again a direct correlation with protection towards reactive radicals generated under oxidative stress conditions was not apparent. Reducing activity is non-specific, and is the sum of all enzymatic (catalase, NADH oxidase, NADH peroxidase) and/or non-enzymatic compounds ($Mn^{2+}$, ascorbate, tocopherols, glutathione) capable to reduce oxygen radical or iron, rendering these compounds unavailable for oxidative reactions (Archibald and Fridovich, 1982; Gille and Sigler, 1995; Warriner and Morris, 1995).

The high specific rate of $H_2O_2$ generation found in the $O_2$-sensitive strains *L. sake* DSM 6333 and *L. sake* NCFB 2814 compared with the very low specific rate observed in the $O_2$-insensitive *L. sake* NCFB 2813 indicates that $H_2O_2$ contributes significantly to oxidative stress *in vivo*. $H_2O_2$ is a weak oxidant but it is highly diffusive and has a long lifetime. Due to these two basic characteristics $H_2O_2$ is an important factor in oxidative damage, either directly or as HO· precursor (Imlay et al., 1988). The intracellular concentration of $H_2O_2$ is determined by its rate of diffusion out of the cell, the rate of its destruction rate by NADH oxidases and peroxidases and its reaction with Fe(II) or other metals to produce HO·. Regulation of the intracellular level of $H_2O_2$ in *L. sake* is more critical under high $O_2$ conditions as compared to ambient $O_2$ conditions. Effective regulation to a low intracellular level may minimizs the impact of endogenous oxidative stress, as was previously shown for *E. coli* (Gonzalez-Flecha and Demple, 1997).

A correlation indeed exists between the ability of *L. sake* strains to grow in the presence of $O_2$ and the capacity of strains to minimise $H_2O_2$ toxicity. This was shown for instance by the size of the growth inhibiton zones around a filter-disk (loaded with 1.5 $mmol.l^{-1}$ $H_2O_2$) placed on pour-plates; the smaller the diameter of the inhibition zone, the higher the ability to inactivate $H_2O_2$. In this experiment, the smallest inhinition zone was found for the $O_2$-insensitive *L. sake* NCFB 2813, while inhibition zones were 2 to 8 times larger for the other, more $O_2$-sensitive strains.

*L. sake* NCFB 2813 also showed much higher $H_2O_2$ scavenging properties compared with the $O_2$-sensitive strain *L. sake* DSM 6333, as is evident from the finding that former strain is able to maintain very low intracellular levels of $H_2O_2$ and is much less sensitive to extracellular $H_2O_2$ than the latter strain. The 4-fold increase in the extracellular level of $H_2O_2$ observed in growing cultures of *L. sake* DSM 6333 within the first 24h of incubation under 90% $O_2$ compared to 20% $O_2$ can be attributed to the lower ability to effectively control the level of $H_2O_2$; this may be due to disorders of the overall metabolic status of the cells, upon the $O_2$ shock (Collins and Aramaki, 1980; Koshland, 1981). Many proteins are known to be sensitive to physiological levels of $H_2O_2$ as low as 100 nM.

Elimination and growth of *L. sake* NCFB 2813 under anaerobic conditions in the presence of levels of extracellular $H_2O_2$ as high as 0.6 $mmol.l^{-1}$, a level at which *L. sake* DSM 6333 cannot grow, is probably related to the presence of a cytoplasmic NADH:$H_2O$ oxidase. The presence of this enzyme in the former strain has been demonstrated (Amanatidou et al., 2001).

Although $H_2O_2$ accumulates in $O_2$-sensitive *Lactobacillus* spp. propagated in MRS at low temperatures (Villegas and Gilliland 1998*;* Jeroni and Brashears, 2000), it is unlikely that $H_2O_2$ toxicity as observed in the $O_2$-sensitive strain *L. sake* DSM 6333 was caused solely by the intracellular formation of $H_2O_2$ up to about 0.120 $mmol.l^{-1}$. At least under anaerobic conditions, the presence of 0.2 $mmol.l^{-1}$ $H_2O_2$ did not have a strong effect on growth of *L. sake* DSM 6333, although $H_2O_2$ at 0.4 and 0.6 $mmol.l^{-1}$ caused a gradual decline in viable cell number. In *E. coli* a “mode one killing” has been observed at concentrations of $H_2O_2$ between 1 and 3 $mmol.l^{-1}$ occurring via a Fenton type DNA damage (Imlay and Linn, 1986).

In conclusion, *L. sake* strains possess antioxidative abilities to maintain reactive oxygen species at levels that are not very toxic for aerobically growing cells. Under elevated oxygen conditions, $H_2O_2$ is apparently generated at sublethal levels even in a $O_2$-sensitive strain such as *L. sake* DSM 6333; the $O_2$-insensitive strain *L. sake* NCFB 2813, however, is much better capable of reducing the intracellular $H_2O_2$ concentration to a negligible level than *L. sake* DSM 6333, which results in superior tolerance to high $O_2$ conditions and the ability of *L. sake* NCFB 2813 to survive and grow well under these conditions.

The findings in this study have raised a number of issues that remain to be resolved through future research. One such issue is the molecular basis of activation and regulation of antioxidant properties in *L. sake* as compared to related LAB or other bacteria. Another issue is the role of $O_2^-$ in oxidative stress; it would be relevant to explore whether $O_2^-$ can cause damage to cells *in vivo* directly, thus without involvement of $H_2O_2$. In *E. coli*, levels of only $10^{-10}$ M $O_2^-$ can impose significant oxidative stress (Imlay and Fridovich, 1991). To obtain more insight into the mechanisms underlying toxicity of elevated $O_2$ in *L. sake*, it would be useful to determine the levels of $O_2^-$ that indirectly or directly cause damage to macromolecules and enzymes in different strains.

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# Chapter 4

## Superoxide dismutase plays an important role in the survival of *Lactobacillus sake* upon exposure to elevated oxygen

A. Amanatidou, M.H.J. Bennik, L.G.M. Gorris & E.J. Smid
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## Abstract

In this study, the responses of two *Lactobacillus sake* strains to elevated oxygen concentrations at 8°C were investigated. *L. sake* DSM 6333 (*L. sake*$^{sens}$), unlike *L. sake* NCFB 2813 (*L. sake*$^{ins}$), showed a low growth rate in the presence of 90% $O_2$ and a rapid loss in viability shortly after entry into stationary phase. The steady-state cytosolic superoxide radical ($O_2^-$) concentration in *L. sake*$^{sens}$ was 0.134 μM and in the oxygen-insensitive mutant LSUV4 it was 0.013 μM. The nine- to ten-fold decrease in the rate of $O_2^-$ elimination in *L. sake*$^{sens}$ indicates the significance of the $O_2^-$-scavenging system in protecting against elevated $O_2$. The superoxide dismutase (SOD) activity was 10- to 20-fold higher in *L. sake*$^{ins}$ than in *L. sake*$^{sens}$, depending on the growth phase. An oxygen-insensitive mutant of *L. sake*$^{sens}$, designated as strain LSUV4, had a ten-fold higher SOD activity than the wild-type strain, which likely restored its oxygen tolerance. Damage to proteins in *L. sake*$^{sens}$ was evidenced by the increased protein carbonyl content and reduced activities of the [Fe-S]-cluster-containing enzymes fumarase and fumarate reductase. This study forms a physiological basis for understanding the significance of elevated oxygen stress as an additional method for inhibition of microbial growth in relation to food preservation.

## 4.1 Introduction

Oxidative stress refers to a condition in which the production of reactive oxygen species results in adverse effects upon cell fitness. The cellular injury produced during hyperoxia involves generation of oxygen radicals, namely, superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical (HO·). $O_2^-$ is moderately reactive, but it can diffuse a considerable distance to reach potential targets and it is more selective in its reactivity with intracellular proteins than $H_2O_2$ or HO· (Fridovich, 1999). Intracellular $O_2^-$ might exert deleterious effects in biological systems with or without the participation of $H_2O_2$ (Fridovich, 1986; Gort and Imlay, 1998).

Lactic acid bacteria are fermentative, aerotolerant micro-organisms and they do not employ a proton translocating electron-transport chain; rather they use $O_2$ as external electron acceptor (Higuchi et al., 1984; Condon, 1987). Small changes in environmental $O_2$ can provoke alterations in energy transduction, biomass yield and fermentation products, and excess $O_2$ might induce toxicity in these bacteria. During aerobic growth under ambient conditions, lactic acid bacteria can accumulate significant amounts of $O_2^-$ and/or $H_2O_2$ either via oxidative reactions of sugars or via the involvement of NADH oxidase/NADH peroxidase (Murphey and Condon, 1984; Kot et al., 1996). In contrast to *Escherichia coli*, lactic acid bacteria do not contain many of the membrane-bound, respiratory enzymes which are primary sources of $O_2^-$ or $H_2O_2$ (Imlay, 1995).

The ability to cope with oxidative stress varies among different groups of lactic acid bacteria, and protection towards reactive oxygen species involves mainly the coupled NADH oxidase/NADH peroxidase (Warriner and Morris, 1995). The presence of a superoxide dismutase (SOD), the enzyme that catalyses the dismutation of $O_2^-$ to $H_2O_2$ and $O_2$ has been demonstrated in cell-free extracts of *Lactobacillus* spp. and *Lactococcus* spp., but the physiological function of this enzyme is not totally elucidated (Gonzalez et al., 1991; Sanders et al., 1995; Chang and Hassan, 1997). Recently, de Angelis and Gobbetti, (1999) highlighted the significance of SOD in increased growth rates of *Lactobacillus*

*sanfranciscensis* at high $O_2$ levels. Imlay and Fridovich (1991) have shown that intracellular generation of 6 μM $O_2^-$ $sec^{-1}$ is sufficient to impose significant oxidative stress in *E. coli* in the absence of defensive enzymes, under oxic conditions. Gort and Imlay (1998) presented strong evidence that *E. coli* synthesiszes just enough SOD to defend biomolecules against endogenous $O_2^-$**.**
*L. sake* is involved in numerous food fermentation and spoilage processes. Significant differences in $O_2$ tolerance have been demonstrated between *L. sake* strains from different sources. In the current study, the mechanism of $O_2$ toxicity was investigated in two *L. sake* strains with different responses to elevated (90%) $O_2$ conditions at 8°C. The use of high $O_2$ concentrations (>40%) at low temperatures can lead to generation of oxidative stress and can be used as an additional hurdle to microbial growth to increase the effectiveness of other preservation techniques (Amanatidou et al., 1999). We determined the rate of intracellular $O_2^-$ production in an SOD-deficient strain compared to SOD-proficient strains. The presence of sub-cellular enzymes, which affect the ability of the cells to eliminate reactive oxygen species, was investigated in an $O_2$-insensitive strain and an $O_2$-sensitive strain of *L. sake*

## 4.2 Material and Methods

### Strains and growth conditions

*L. sake* DSM 6333 (*L. sake*$^{sens}$) and *L. sake* NCFB 2813 (DSM 20497; *L. sake*$^{ins}$) were characteristed by the differences in their $O_2$ sensitivity. Cultures were routinely grown overnight in 150 ml MRS broth (CM359, Oxoid, Hampshire, UK,) to an $OD_{660}$ of about 0.6 at 8°C. Aliquots of the cultures were transferred into fresh medium that had been pre-incubated for 48 h at 8°C with 10% $CO_2$ + 90% $N_2$ or 20% $O_2$ + 80% $N_2$ or 90% $O_2$ + 10% $N_2$. Cultures were purged under a constant stream of gases to maintain stable gas conditions. The gas composition was checked daily with a gas analyser (Servomex Series 1400, ADC 7000, ThIS Analytical B.V., Breda, The Netherlands). All the experiments were performed at 8°C in a temperature-controlled environment. Prior to enumeration, no significant differences were observed in the plating efficiencies of cultures incubated under 10% $CO_2$ + 90% $N_2$, compared to those incubated under 20% $O_2$ + 80% $N_2$. Therefore, all plates used for viable counts were incubated at ambient conditions.

### UV mutagenesis

UV mutagenesis was carried out to enhance the selection of the $O_2$ resistant sub-populations of the *L. sake*$^{sens}$ strain. In order to assess resistance to high $O_2$, plates inoculated with 1 ml of a 24 h culture of *L. sake*$^{sens}$ at a population density of $10^9$ cells were exposed to UV for 1 min using an ultraviolet lamp (UV, 252 nm, fluency rate of light 0.75 $mW.cm^{-2}$) in a dark room and subsequently incubated at 25°C for 48 h. About 85 of the colonies that developed after UV exposure were selected, routinely grown overnight in 150 ml MRS broth and subsequently diluted to an initial population of about $10^7$ cells $ml^{-1}$ in the same medium equilibrated to 90% $O_2$ + 10% $N_2$ for 48 h. Three $O_2$-insensitive colonies of the strain *L. sake*$^{sens}$ were the isolated and one mutant, named strain LSUV4, was selected for additional experiments. The effect of the UV is two-fold: it stimulates mutation and partially selects those mutans with an increased ability to withstand the sub-lethal free radical stress induced by the UV. Because of this dual action, no conclusion can be drawn regarding the frequency of mutation alone.

### Growth and survival upon transfer from 10% $CO_2$ + 90% $N_2$ to 90% $O_2$ + 10% $N_2$

To study the behaviour of micro-organisms upon transfer from 10% $CO_2$ + 90% $N_2$ to 90% $O_2$ + 10% $N_2$, cultures were first diluted in MRS broth and grown under 10% $CO_2$ + 90% $N_2$ at 8°C for five generations to a cell density of $10^7$ -$10^8$ $cfu.ml^{-1}$. Cells were subsequently washed twice in potassium phospate buffer ($K_2HPO_4/KH_2PO_4$, $KP_i$), 50 $mmol.l^{-1}$, pH 7.0; half of the washed cell suspension was diluted in fresh medium and incubated at 90% $O_2$ + 10% $N_2$. The other half was transferred into new medium and incubated at 10% $CO_2$ + 90% $N_2$. To study the effect of high $O_2$ concentrations on cells

during the exponential phase of growth, cultures of *L. sake*$^{sens}$ or *L. sake*$^{ins}$ were grown to about $10^8$ cfu.ml$^{-1}$ under 10% $CO_2$ + 90% $N_2$, washed, aliquoted in two portions and diluted in fresh MRS medium that had been equilibrated for 2 days under a stream of 90% $O_2$ + 10% $N_2$ or 10% $CO_2$ + 90% $N_2$. Growth rates under different gas conditions were determined by plating 1 ml of the culture at regular time intervals.

### Enzyme activities

All enzyme activities were determined in cell-free extracts of cells harvested in the exponential phase ($10^6$ cfu.ml$^{-1}$) or late exponential phase ($10^7$ or $10^8$ cfu.ml$^{-1}$) of growth. Six hundred ml of cells in exponential phase or 300 ml of cells in late-exponential phase were harvested by centrifugation (5500 x *g*, 10 min, 4°C) and washed twice in $KP_i$ buffer (30 mmol.l$^{-1}$, pH 7.4) containing chloramphenicol (170 μg.ml$^{-1}$), which was added to all samples to prevent further protein synthesis during preparation of cell-free extracts. The cells were resuspended in lysis buffer (30 mmol.l$^{-1}$ potassium phosphate, 0.4 mol.l$^{-1}$ sucrose, pH 7.4) containing mutanolysin (25 U.ml$^{-1}$) and lysozyme (500 μg.ml$^{-1}$) and incubated at 37°C for 45 min, whereupon protoplasts were collected after centrifugation (1300 *x g*, 15 min, 4°C). Protoplasts were resuspended in $KP_i$ buffer and then disrupted with a probe sonicator in an ice bath (Brandson Sonic Power Co., Plainview, N.Y., U.S.A.) for three cycles of 0.5 min each, with 1-min intervals. Cell debris was removed by centrifugation (40000 *x g*, 20 min, 4°C) and the resulting cell-free extract was then used to determine enzyme activities. SOD was measured by a modification of the xanthine/ xanthine oxidase/ cytochrome *c* method (McCord & Fridovich, 1969). One SOD unit is defined as the amount that inhibits 50% of the cytochrome *c* reduction. Enzymatic and non-enzymatic SOD activities were distinguished by assaying boiled and non-boiled, dialysed, cell-free extracts. Cyanide sensitive (Cu-Zn-dependent enzymes) and cyanide-insensitive SOD activities (Fe- and Mn-dependent enzymes) were distinguished by the addition of 30 mmol.l$^{-1}$ KCN to the reaction mixture. NADH oxidase activities were measured spectrophotometrically at 340 nm with 0.133 mmol.l$^{-1}$ NADH and 8.3 μmol.l$^{-1}$ FAD in 37 mmol.l$^{-1}$ sodium phosphate buffer ($Na_2HPO_4/NaH_2PO_4$, NaPi), pH 7.0, as described by Anders et al., (1970). NADH peroxidase was measured in 19.7 mmol.l$^{-1}$ sodium phosphate buffer, pH 7.0, and 14 mmol.l$^{-1}$ sodium citrate buffer, pH 5.5, with 1.33 mmol.l$^{-1}$ $H_2O_2$ under strictly anoxic conditions using a Thunberg-type silica cuvette with a 1-cm light path. One NADH-enzyme unit was defined as the amount of enzyme that converted 1 μmol of NADH min$^{-1}$ in cell-free extract.

Fumarate reductase activity was assayed under anoxic conditions at 340 nm by measuring the oxidation of reduced hydrosulfite by fumarate and methylviologen as electron carrier (Morishita and Yajima, 1995). Fumarase activity was assayed at 240 nm in $KP_i$ buffer, pH 7.4, by monitoring the conversion of L-malate to fumarate with a molar extinction coefficient $\varepsilon$=24410.M$^{-1}$.cm$^{-1}$. The activities of the enzymes were expressed as the rate of the absorbance-decrease $\Delta$A min$^{-1}$.mg protein$^{-1}$.

### $O_2^-$ detection in membrane vesicles and cytosolic fraction

To determine the $O_2^-$ concentration in membrane vesicles and cytosolic fractions, cells were grown in MRS medium continuously flushed with 10% $CO_2$ + 90% $N_2$ to late-exponential phase ($10^7$ -$10^8$ cfu.ml$^{-1}$), transferred into new medium equilibrated with 90% $O_2$ + 10% $N_2$ and incubated for 48h at 8°C. The cells were lysed and membrane vesicles were prepared based on the method described by Imlay and Fridovich (1991) modified for lactic acid bacteria. Cultures were centrifuged and washed twice in 50 mmol.l$^{-1}$ $KP_i$ buffer, pH 7.8, re-suspended to an $OD_{660}$ of 10 (2.8 mg protein.ml$^{-1}$), lysed with ultrasonication as described above. Cultures were centrifuged (40000 *x g*, 20 min) to remove cell debris, and the supernatant was fractionated by centrifugation (3 h at 85000 *x g*). The supernatant (cytosolic fraction) was removed and the pellet (membrane vesicles) was resuspended in 30 mmol.l$^{-1}$ $KP_i$ buffer, pH 7.4, to 0.1% of the original volume. Vesicles and cytosolic fractions (0.9±0.05 and 2.6±0.1 mg protein.ml$^{-1}$, respectively) were stored at 0°C prior to their use in experiments. $O_2^-$ concentration was determined with the cytochrome *c* method as described by Boveris (1984). The

reduction of cytochrome *c* by $O_2^-$ was spectrophotometrically monitored over time. Since the reduction reaction is fast compared to $O_2^-$ production, the rate of cytochrome *c* reduction is equal and determined by the $O_2^-$ production rate. The reaction mixture (3 ml) consisted of 200 μmol.l$^{-1}$ NADH, 100 μmol.l$^{-1}$ cytochrome *c* and 50 mmol.l$^{-1}$ $KP_i$ buffer at pH 7.8 and was air saturated prior to addition of 10 μl vesicle suspension or 50 μl cytosolic fraction, resulting in $10^7$ -$10^8$ bacteria cells. In some experiments, 10 mmol.l$^{-1}$ lactate or 10 mmol.l$^{-1}$ of the flavin inhibitor diphenyleneidonium (DPI) was added. The rate of cytochrome *c* reduction was followed at 550 nm in a cuvette maintained at 8$^o$C and the results expressed as nmol cytochrome *c* reduced cell$^{-1}$.s$^{-1}$. To obtain the $O_2^-$ production rate, the $O_2^-$-independent reduction of cytochrome *c* was subtracted from the measured values. The $O_2^-$-independent reduction was measured under the same environmental conditions with the addition of 30 U SOD and thus a diminished $O_2^-$ concentration. Every assay was repeated at least three times. $O_2$ uptake rates by whole cells were assayed prior to isolating vesicles and cytosolic fraction. A biological $O_2$ monitor electrode (YSI Model 5300, Yellow Springs Instruments Co. Inc., Yellow Springs, Ohio, U.S.A.) was used for the measurements. A number of experiments were carried out to ensure that the $O_2$ available to the vesicles during the measurements did not differ from that present in intact cells (e.g. due to an $O_2$ gradient). This was done by measuring the $O_2$ uptake of about $10^8$ cells and subsequently of the vesicles obtained from an equal number of cells.
The assumed steady-state concentration in SOD containing cells can be derived from the equality of the rate of production and the rate of dismutation. The rate constant *k* for the dismutation reaction is 7.8.s$^{-1}$ (unit SOD.ml$^{-1}$)(Fridovich, 1985).

$$\left(\frac{d[O_2^-]}{dt}\right)_{prod} = \left(\frac{d[O_2^-]}{dt}\right)_{dismut} = k \cdot [SOD] \cdot [O_2^-]_{ss} \qquad (1)$$

Given the rate of production and the SOD concentration, the steady-state concentration of $O_2^-$ can be solved from equation 1.

### Protein carbonyl assay

Oxidative damage to proteins results in the formation of carbonyl groups. Carbonyl groups (aldehydes and ketones) are introduced into proteins by metal-catalysed oxidation or by oxidation of amino acid residues (lysine, arginine, proline and histidine). The presence of carbonyl groups was measured using the method described by Reznick and Packer (1994). In this assay, reactive oxygen species that attack amino acid residues in proteins to produce carbonyl groups that can be measured after reaction with 2,4-dinitrophenylhydrazine. The carbonyl content was estimated in bacterial cultures that were grown under 10% $CO_2$ + 90% $N_2$ up to exponential phase and were subsequently transferred to MRS medium under 90% $O_2$ + 10% $N_2$. Samples were withdrawn for analysis after 0, 24, 72 and 144 h of incubation under 90% $O_2$ + 10% $N_2$. For this assay, cell- free extracts were obtained according to the procedure described in the section “Enzymatic activity”. Instead of sonication, glass beads were used to obtain complete lysis of the cells by rapid vortexing in three cycles of 2 min each. The extracted proteins were concentrated to at least 2 mg protein.ml$^{-1}$. The carbonyl content was calculated by obtaining the spectra at 355-390 nm after reaction with dinitrophenyl-hydrazine, using a molar extinction coefficient ε=22000.M$^{-1}$.cm$^{-1}$.

### Other methods

Protein concentrations in whole-cell suspensions and cell-free extracts were measured by the method of Lowry et al., (1951). NADH was determined with the luminometric method described by Wulff (1988) at an emission wavelength 493 nm in a thermperature-controlled cuvette at 37$^o$C.

### Reagents

Horseradish peroxidase and diaphorase were obtained from Boehringer GmbH (Mannheim, Germany). Glutathione, hydroxylammonium chloride and hydrogen peroxide were obtained from Merck

(Darmstadt, Germany), Diphenyleneiodonium chloride (DPI) from Aldrich Chemical, Co (Milwaukee, Wi, USA.) and all other chemicals were purchased from Sigma Chemical Co (St. Louis, Mo., U.S.A.).

## 4.3 Results

### Influence of high $O_2$ concentration on the growth of *L. sake*

The strains under investigation were selected after a broad screening of *L. sake* strains for their ability to grow in MRS at 8°C and their different responses upon $O_2$ exposure, (A. Amanatidou, unpublished data). The highest specific growth rates ($\mu_{max}$) were observed for all three strains under 10% $CO_2$ + 90% $N_2$ (Table 1). Typically, the specific growth rate of *L. sake*$^{sens}$ declined upon $O_2$ exposure and a rapid reduction of the viable-cell counts in MRS was observed after 216 h of incubation at 90% $O_2$ + 10% $N_2$ and upon reaching mid- to late-exponential phase of growth ($10^7$ -$10^8$ cfu.ml$^{-1}$) (Figure 1A). The growth pattern of strain LSUV4, a mutant derived from *L. sake*$^{sens}$ after UV exposure to 90% $O_2$ + 10% $N_2$, was not significantly different from that of the wild-type strain. However, in contrast to the parent, *L. sake*$^{sens}$, strain LSUV4 retained viability even after 288 h of incubation under 90% $O_2$ + 10% $N_2$. The $\mu_{max}$ of *L. sake*$^{ins}$ was lower in the presence of 20% $O_2$ + 80% $N_2$ or 90% $O_2$ + 10% $N_2$ than in the presence 10% $CO_2$ + 90% $N_2$, but no decrease in the maximal viable-cell counts was observed during growth. Next, the effect of high $O_2$ concentrations on the growth and survival of *L. sake* cells upon transfer from 10% $CO_2$ + 90% $N_2$ to 90% $O_2$ + 10% $N_2$ was studied. The $\mu_{max}$ and maximum population density of *L. sake*$^{ins}$ were not affected upon transfer to 90% $O_2$ + 10% $N_2$ compared to growth under 10% $CO_2$ + 90% $N_2$ (Figure 1B). A decline of about 1 log in viable numbers of strain *L. sake*$^{sens}$ was observed during the first 72 h, followed by a rapid decline of 4 log units in the viable-cell numbers of strain *L. sake*$^{sens}$ was observed during the first 72 h, followed by a rapid decline of 4 log units in the viable-cell numbers during the next 24 h. Addition of catalase and SOD in the medium did not affect the rate of the decline in cell viability (Figure 1B). The rapid decline was not prevented when $10^{-3}$ M of the Fe(II) chelator ferrozine was added in the incubation medium (data not shown).

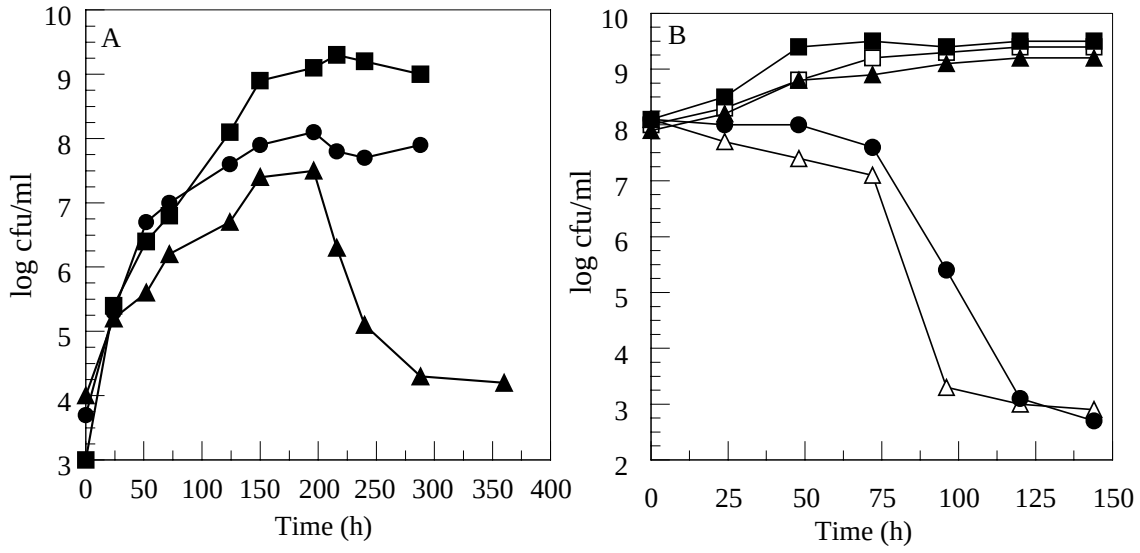


***Figure 1**. Panel A: Growth of strains* L. sake$^{ins}$ (■), L. sake$^{sens}$ (▲) *and LSUV4 (●) during exposure to 90% $O_2$ + 10% $N_2$ at 8°C in MRS broth. Cultures in late-exponential phase of growth were inoculated in medium equilibrated for 48 h with 90% $O_2$ + 10% $N_2$. Panel B: Viability of* L. sake$^{ins}$ *and* L. sake$^{sens}$ *grown under 10% $CO_2$ + 90% $N_2$ until the exponential phase of growth was reached upon transfer to the same medium 10 $CO_2$ + 90 $N_2$ or to medium equilibrated with 90% $O_2$ + 10% $N_2$ . Symbols: ■:* L. sake$^{ins}$ *under 10% $CO_2$ + 90% $N_2$; ▲:* L. sake$^{ins}$ under *90% $O_2$ + 10% $N_2$); □:* L. sake$^{sens}$ *under 10% $CO_2$+ + 90% $N_2$; Δ:* L. sake$^{sens}$ *in 90% $O_2$ + 10% $N_2$; ●:* L. sake$^{sens}$ *in 90% $O_2$ + 10% $N_2$; + 20 U.ml$^{-1}$ SOD+ 10 μg.ml$^{-1}$ catalase added to the medium every 12 hours*

***Table 1.*** *Maximum specific growth rate ($\mu_{max}$) of cultures of* L. sake$^{sens}$ *and* L. sake$^{ins}$ *in MRS broth at 8$^{o}$C during growth under different gas conditions*

| | $\mu_{max}$ ($h^{-1}$) | | |
|---|---|---|---|
| Strain | 10% $CO_2$+90% $N_2$ | 20% $O_2$+80% $N_2$ | 90%$O_2$+10% $N_2$ |
| *L. sake* $^{sens}$ | 0.062 ± 0.004 | 0.025 ± 0.004 | 0.022 ± 0.004 |
| *L. sake* $^{ins}$ | 0.078 ± 0.003 | 0.066 ± 0.005 | 0.043 ± 0.009 |

## Enzyme activities in relation to $O_2$ sensitivity

The activity of antioxidative enzymes extracted from cultures that were grown to the exponential and late-exponential growth phase in MRS broth at 8$^{o}$C at all three gas conditions was investigated. Catalase and pyruvate oxidase activities were not detected in the cell-free extracts of the parental strain *L. sake*$^{sens}$ or the mutant strain LSUV4, which was $O_2$-tolerant. Glutathione reductase activity was detected up to levels of 5.2 mU.mg protein$^{-1}$ under all gas conditions in strain *L. sake*$^{ins}$. In strain *L. sake*$^{sens}$, the activity did not exceed 2.1 mU.mg protein$^{-1}$ in the late-exponential phase (data not shown).

NADH oxidase activities were detected in all cell-free extracts under the different gas conditions (Table 2). NADH oxidase activities were higher in late-exponential phase cells than in mid-exponential phase cells for all three strains. Activities were increased four-fold in crude extracts of late-exponential cells of *L. sake*$^{sens}$ that were grown under 90% $O_2$ + 10% $N_2$ compared to 10% $CO_2$ + 90% $N_2$. Exposure of cells to ambient $O_2$ levels was correlated with an increase in the observed NADH oxidase activity in both *L. sake*$^{sens}$ and *L. sake*$^{ins}$, indicating the importance of this enzyme for aerobic survival. NADH oxidase activities in *L. sake*$^{sens}$ were higher than in *L. sake*$^{ins}$. This observation is in agreement with the lower $O_2$ consumption observed in *L. sake*$^{ins}$ (Table 3). NADH oxidase levels of LSUV4 were similar to those measured in the wild-type strain *L. sake*$^{sens}$. NADH oxidase activity was enhanced by the addition of FAD as coenzyme and increased to the maximum level within the first few hours upon transfer of *L. sake*$^{sens}$ to 90% $O_2$ + 10% $N_2$ (data not shown).

NADH peroxidase was detected in cultures of all strains growing in the presence of $O_2$. The higher peroxidase activities detected in extracts of late–exponential phase *L. sake*$^{sens}$ cultures grown under 20% $O_2$ + 80% $N_2$ compared to 90% $O_2$ + 10% $N_2$, could be attributed to experimental artefacts due to the inability to totally exclude NADH oxidase activity upon peroxide determination.

SOD activity was determined in dialysed, cell-free extracts of strains *L. sake*$^{sens}$, *L. sake*$^{ins}$ and LSUV4. The activity detected in these extracts upon exposure to $O_2$ was cyanide-insensitive (Mn and FeSOD). SOD-simulating activity that could interfere with the assay was detected in heat-denatured centrifuged extracts of both strains. This activity was below 5% in crude extracts of *L. sake*$^{ins}$ and about 50% of the total SOD activity in crude extracts of *L. sake*$^{sens}$. The SOD-simulating activity is most likely caused by compounds that act as radical scavengers, and was subtracted from the levels determined in the dialysed cell-free extracts. The corrected SOD activities are presented in Table 2. SOD activities were higher in late-exponential than in mid-exponential phase cells in both strains. SOD levels were 10- to 40-fold higher in *L. sake*$^{ins}$ than in *L. sake*$^{sens}$, depending on the growth phase and the gas conditions. Induction of SOD activity in cells of the $O_2$-insensitive strain LSUV4 was three to four times higher than in the wild-type *L. sake*$^{sens}$. The low levels of SOD activity in exponential phase cells of *L. sake*$^{sens}$ coincided with impaired growth under 90% $O_2$ + 10% $N_2$ (Figure 1; Table 2). A two-fold increase in the level of SOD in *L. sake*$^{ins}$ was observed in late-exponential phase cells grown under 20% $O_2$ + 80% $N_2$, compared 10% $CO_2$ + 90% $N_2$. Further increase of the $O_2$ level to 90% $O_2$ did not induce an increase of the SOD levels.

***Table 2.*** *Antioxidative enzymatic activities in cell free extracts of* L. sake$^{sens}$*, and* L. sake$^{ins}$ *and of the LSUV4 mutant ($O_2^-$ -insensitive mutant derived from* L. sake$^{sens}$*) cells in mid-exponential ($10^6$ cfu.ml$^{-1}$) and late-exponential ($10^7$-$10^8$ cfu.ml$^{-1}$) phase of growth in MRS broth at 8°C and under different gas conditions*

| Strain | Gas concentrations | Growth phase | NADH oxidase [mU.mg protein$^{-1}$] | NADH peroxidase [mU.mg protein$^{-1}$] | SOD [U.mg protein$^{-1}$] |
|---|---|---|---|---|---|
| *L. sake*$^{sens}$ | 10%$CO_2$+90%$N_2$ | Mid exp.[a] | 10±2 | <5 | 0.85±0.5 |
| | | Late exp.[b] | 30±3 | <5 | 1.3± 0.3 |
| | 20%$O_2$+80%$N_2$ | Mid exp. | 21±3 | 11±1 | 0.95± 0.2 |
| | | Late exp. | 90±10 | 61±6 | 0.4±0.3 |
| | 90%$O_2$+10%$N_2$ | Mid exp. | 95±5 | 14±3 | 0.5±0.2 |
| | | Late exp. | 122±18 | 29±6 | 0.6±0.1 |
| *LSUV4* | 10%$CO_2$+90%$N_2$ | Mid exp. | 7±3 | <5 | 1.1±0.6 |
| | | Late exp. | 27±5 | <5 | 1.9± 0.5 |
| | 20%$O_2$+80%$N_2$ | Mid exp. | 16±2 | 7±1 | 3.9± 0.1 |
| | | Late exp. | 95±8 | 64±5 | 4.6±0.1 |
| | 90%$O_2$+10%$N_2$ | Mid exp. | 96±6 | 9±4 | 5.2±0.2 |
| | | Late exp. | 115±10 | 21±3 | 5.4±0.1 |
| *L. sake*$^{ins}$ | 10%$CO_2$+90%$N_2$ | Mid exp. | 5.2±0.3 | <5 | 4.9±0.3 |
| | | Late exp. | <5 | <5 | 9.5±0.9 |
| | 20%$O_2$+80%$N_2$ | Mid exp. | 16 ±1.5 | 11±4 | 9.5±2 |
| | | Late exp. | 47±3 | 29±3 | 17.1±4 |
| | 90%$O_2$+10%$N_2$ | Mid exp. | ND | 19±3 | 11.0±2 |
| | | Late exp. | 55±7 | 44±6 | 18.1±6 |

*ND: not determined; a: mid-exponential phase of growth; b: late-exponential phase of growth*

***Table 3.*** *Growth parameters of exponential cells of strains* L. sake$^{ins}$, L. sake$^{sens}$ *and LSUV4 exposed to $O_2$+$N_2$ (90+10, v/v) for 72 h in MRS broth, at 8°C. The respiration rate of $10^7$ cfu.ml$^{-1}$ was measured as described in the "Material & Methods"*

| Growth parameters | *L.sake*$^{sens}$ | LSUV4 | *L.sake*$^{ins}$ |
|---|---|---|---|
| NADH (µmol.l$^{-1}$) | 49±15 | ND | 64±22 |
| Specific SOD activity (U.cell$^{-1}$) | $10^{-11}$ | $1.04.10^{-10}$ | $2.2.10^{-9}$ |
| (U.ml$^{-1}$ intracellular volume) | (6.68) | (69.3) | (1466) |
| Respiration rate (molecules $O_2$ .cell $^{-1}$.s$^{-1}$) | $3.2x10^4$ | $3.2x10^4$ | $1.58x10^4$ |

*ND: not determined*

### $O_2^-$ generation in membrane and cytosolic fractions of *L. sake*$^{sens}$ and *L. sake*$^{ins}$

To obtain more insight into the physiological role of SOD, the intracellular generation of $O_2^-$ in membrane vesicles and in cell-free extracts of *L. sake*$^{sens}$ cultures that were grown under 90% $O_2$ + 10% $N_2$ at 8°C was determined. The cytochrome *c* method could not be applied to measure the level of $O_2^-$ in *L. sake*$^{ins}$ and LSUV4 due to the interference by the high levels of SOD present in the two strains. However, considering that LSUV4 had the same growth characteristics as *L. sake*$^{sens}$ it was possible to calculate the steady-state $O_2^-$ concentration in this strain, after assuming the same rate of $O_2^-$ formation and correcting for the presence of SOD (Table 3). The steady-state internal $O_2^-$ concentration of *L. sake*$^{sens}$ was 0.134 μM and that of strain LSUV4 was 0.013 μM. The specific SOD activity was 6.68 and 69.3 U $ml^{-1}$ intracellular volume, respectively.

In *L. sake*$^{sens}$, 92% of the intracellular $O_2^-$ was generated in the cell-free extracts rather than in membrane vesicles (Table 4). The rate of $O_2^-$ generation was enhanced by addition of NADH (but not NADPH) and FAD and inhibited by addition of NAD+. The dependence of $O_2^-$ generation in cell-free extracts on NADH up to 120 μM is clearly shown in Figure 2A; possibly, the addition of NADH acts as a trigger for $O_2^-$ production. As shown in Figure 2A, a plateau in the generation of $O_2^-$ appears at concentrations between 120 and 140 μM NADH or higher. No evidence for NADH-depended $O_2^-$ generation by the membrane fraction of *L. sake*$^{sens}$ was demonstrated (Figure 2B). It was calculated that about 22% of the total $O_2$ consumed was reduced to $O_2^-$ from the cell-free extracts and membrane fraction of the *L. sake*$^{sens}$. The level calculated for strain LSUV4 was lower due to the catalytic action of SOD.

It can be concluded that the cytosolic fraction of the *L. sake*$^{sens}$ contributes to most of the $O_2^-$ generation in this strain. $O_2^-$ formation was dependent on the levels of NADH present in the cytosolic cell-free extract and was inhibited by 10 μM of the flavin inhibitor DPI (Figure 2A). The data point to the involvement of enzymes with a role in cytosolic NADH redox equilibrium as probable sources of $O_2^-$.

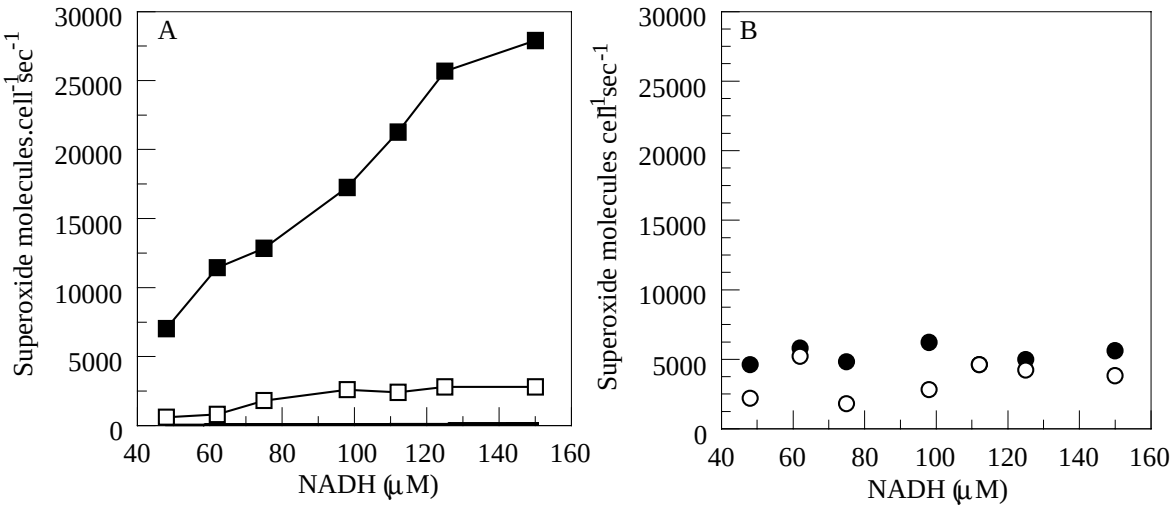


***Figure 2.*** *NADH-dependent $O_2^-$ production in (A) the cytosolic fraction of strain of the SOD-deficient strain* L. sake$^{sens}$ *and in (B) the membrane fraction of* L. sake$^{sens}$ *grown to the exponential phase of growth at 8°C. Cell-free extracts (2.1 ± 0.3 mg protein.ml$^{-1}$) or membrane vesicles (0.010 ml) were pre-incubated for 10 min after addition of NADH at different concentrations, under a continuous stream of 90% $O_2$ + 10% $N_2$, 200 ml.min$^{-1}$. Cytochrome c reduction was measured as described in the "Material & Methods" section. Symbols: □, presence of 10 mmol.l$^{-1}$ of the flavin inhibitor DPI; ■, absence of DPI; ○, presence of 10 mmol.l$^{-1}$ lactate; ●, absence of lactate.*

***Table 4.*** *Calculated $O_2^-$ generation rates in the cytosolic fraction of exponentially growing cells of* L. sake$^{sens}$*, after addition of NADH/NADPH, NAD+ or FAD to cell-free extracts and membrane vesicles. Cells were pregrown up to exponential phase at 90% $O_2$ + 10% $N_2$.*

| | $O_2^-$ production rates (molecules.cell$^{-1}$.s$^{-1}$) | |
|---|---|---|
| **Treatment** | In cell-free extracts | In membrane vesicles |
| None | 6310 | 520 |
| FAD (13 μmol.l$^{-1}$) | 8600 | ND |
| NADH (40 μmol.l$^{-1}$) | 13500 | ND |
| NADH (40 μmol.l$^{-1}$) + NADPH (10 μmol.l$^{-1}$) | 13960 | 570 |
| NAD+ (40 μmol.l$^{-1}$) | 3860 | 600 |

*ND: not determined*

## Detection of protein damage in the cytosolic fraction

Carbonyl formation is an early marker for protein oxidation. Increased protein damage upon exposure of exponential phase cultures to 90% $O_2$ + 10% $N_2$ was evident after 72 and 144 h for *L. sake*$^{sens}$ compared to *L. sake*$^{ins}$ (Figure 3). The high carbonyl level evident for the *L. sake*$^{sens}$ strain indicates extensive damage to proteins and enzymes.

The modulation of biosynthesis of enzymes containing [4Fe-4S] clusters upon exposure to oxidative stress imposed by elevated $O_2$ is demonstrated in Table 5. Fumarate reductase and fumarase were used as markers. These enzymes participate in the reductive succinate synthesis via a tricarboxylate pathway that is partly active in *Lactobacillus* spp. The activity of fumarate reductase and fumarase decreased upon exposure to high $O_2$ compared to the levels measured under 10% $CO_2$ + 90% $N_2$. The effect was more pronounced for strain *L. sake*$^{sens}$ than for *L. sake*$^{ins}$. No fumarate reductase was detected in the cell-free extracts of *L. sake*$^{sens}$ after 1- h exposure to 90% $O_2$ + 10% $N_2$. Deoxygenation resulted in restoration of 80% of the total fumarate reductase activity that was lost upon oxygenation. About 60% of the fumarase activity was recovered upon deoxygenation. For *L. sake*$^{ins}$, deoxygenation resulted in a 80-100% recovery of the damaged enzymes. Fumarase recovery was not affected when deoxygenated cells were incubated in the presence of 170 μg chloramphenicol. ml$^{-1}$, which indicated that this process is not dependent on *de novo* protein synthesis. Bycontrast, 40% of the fumarate reductase acitivity was recovered upon deoxygenating in the presence of chloramphenicol (data not shown).

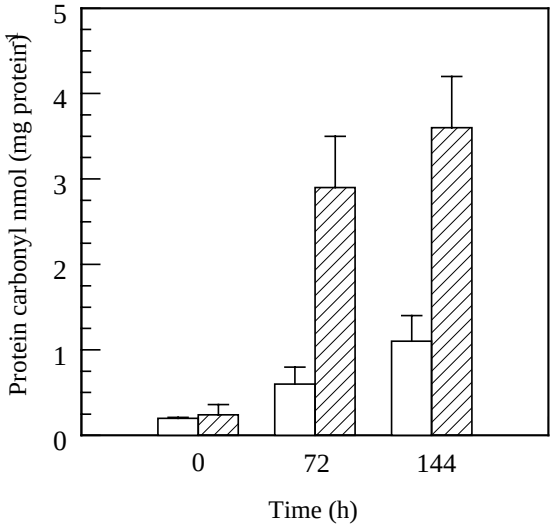


***Figure 3****. Protein carbonyl content in L. sake $^{ins}$ (open bars) and L. sake$^{sens}$ (hatched bars) after 0, 72 and 144 hours of exposure to 90% $O_2$ +10% $N_2$.*

***Table 5.*** *Activities of fumarate reductase and fumarase A in cell-free extracts of* L. sake$^{sens}$ *and of* L. sake$^{ins}$ *prepared from cells grown up to late-exponential phase under 10% $CO_2$ + 90% $N_2$ and 90% $O_2$ +10% $N_2$ conditions. Extracts were incubated at 10% $CO_2$ + 90% $N_2$ (anaerobic); or under 10% $CO_2$ + 90% $N_2$ and then under 90% $O_2$ + 10% $N_2$ (oxygenated); or first under 10% $CO_2$ + 90% $N_2$, then under 90% $O_2$ + 10% $N_2$ and then under 10% $CO_2$ + 90% $N_2$ (deoxygenated). After 1 h of incubation at each condition, samples of the cell-free extracts were removed and the fumarase and fumarate reductase activity in the extracts were measured. The protein concentration of the extracts was 1.5 mg.ml$^{-1}$.*

| Strain | Fumarate reductase activity [$\Delta A_{340}$ (mg protein)$^{-1}$.min$^{-1}$] | | | Fumarase activity [$\Delta A_{240}$ (mg protein)$^{-1}$.min$^{-1}$] | | |
|---|---|---|---|---|---|---|
| | Oxygenated | Anaerobic | Deoxy-genated | Oxygenated | Anaerobic | Deoxy-genated |
| *L sake*$^{sens}$ | <0.05 | 1.32±0.07 | 0.78±0.1 | 0.35±0.05 | 0.67±0.05 | 0.54±0.1 |
| *L sake*$^{ins}$ | 0.38±0.05 | 0.47±0.09 | 0.51±0.05 | 0.44±0.05 | 0.49±0.05 | 0.60±0.05 |

## 4.4 Discussion

In this study, the responses to elevated $O_2$ concentrations of two *L. sake* strains, *L. sake*$^{ins}$ and *L. sake*$^{sens}$, and of the $O_2$-insensitive variant of *L. sake*$^{sens}$ strain LSUV4 were investigated. The sensitivity of *L. sake*$^{sens}$ to 90% $O_2$ was reflected by low growth rates compared to growth in the absence of $O_2$ and early entry into stationary phase, followed by rapid cell death. Extended studies on the response of lactobacilli to increased $O_2$ levels reported significant differences among several species (Gotz et al., 1980; de Angelis and Gobbetti, 1999). In *L. delbruckii*, aeration is reported to cause early entry into stationary phase and to reduce the biomass without modifying the $\mu_{max}$ (Marty-Teysset et al., 2000). On the other hand, *L. plantarum* showed significant tolerance to 100% $O_2$ during exponential growth on glucose (Archibald and Fridovich, 1981a).

Addition of the antioxidative enzymes catalase and SOD, or of the Fe(II) chelator ferrozine to the culture medium did not protect cells of *L. sake* from the detrimental effects of high $O_2$ concentrations. An inability to chelate iron in order to reverse oxygen toxicity is not in agreement with previous findings from Duwat et al., (1995a). They suggested that $O_2$ toxicity could be attributed to the high extracellular generation of iron-dependent $HO^{\cdot}$ radicals due to Fenton reaction. The inability of these compounds to protect *L. sake*$^{sens}$ suggests that $O_2$ toxicity is the result of intracellular rather than extracellular generation of radicals. $O_2$ toxicity can possibly be prevented if excessive one-electron reduction of dioxygen is inhibited, e.g. by keeping the radicals produced during $O_2$ consumption at low levels.

### Enzyme activities in relation to $O_2$ sensitivity

Survival and growth of lactic acid bacteria under oxic conditions require activation of the NADH oxidase/peroxidase system (Sakamoto et al., 1996; Yi et al., 1998). NADH oxidase is thought to control the intracellular redox balance and protect cells from $H_2O_2$ toxicity. $H_2O_2$ is generated in *L. sake*$^{sens}$ at levels that are not directly harmful to the cells (<200μM), but its removal is necessary to minimize the detrimental effects of oxidative stress (A. Amanatidou, unpublished data). The specific production rate of $H_2O_2$ in the presence of 90% $O_2$ was 0.77±0.1 pmol.s$^{-1}$.mg bacterial protein$^{-1}$ in *L. sake*$^{ins}$ and 3.4±0.2 pmol.s$^{-1}$.mg bacterial protein$^{-1}$ in *L. sake*$^{sens}$ (A. Amanatidou, unpublished data). The presence of an NADH oxidase system that can generate $H_2O_2$ in *L. sake*$^{sens}$ is supported by the absence of other $H_2O_2$-generating enzymes (lactate and pyruvate oxidase) in this strain. The low levels of $H_2O_2$ employed in the cell-free extracts of *L. sake*$^{ins}$ suggests that an NADH:$H_2O$ oxidase rather than an NADH:$H_2O_2$ oxidase is active in this strain. The levels of NADH oxidase and SOD exceeded those of NADH peroxidase and glutathion peroxidase in *L. sake*$^{ins}$, which would lead to excess $H_2O_2$ in the presence of an NADH:$H_2O_2$ oxidase. A higher level of NADH oxidase was observed upon

exposure to 90% than to 20% $O_2$, and the levels were higher in the late-exponential than in the mid-exponential phase of growth. In contrast to our observation for *L. sake*, expression of NADH oxidase in *L. delbruckii* is not regulated upon switching from exponential to stationary phase or by the substrate $O_2$ and product $H_2O_2$ (Marty-Teysset et al., 2000).

NADH peroxidase catalyses the reduction of $H_2O_2$ to $H_2O$ and potentially fulfils the role of heme-containing catalases by reducing intracellular levels of $H_2O_2$ while also re-generating NAD+ (Condon, 1987; Ross and Claiborne, 1997). The high levels of NADH peroxidase observed in *L. sake*$^{ins}$ pinpoints the significance of this enzyme in redox balance in *L. sake*, as has been shown for other lactic acid bacteria *(*Sakamoto et al., 1996). The presence of abundant SOD in *L. sake*$^{ins}$ could be attributed to an increased generation of $H_2O_2$ despite the total absence of NADH oxidase. However, the presence of NADH peroxidase ensures $H_2O_2$ detoxificaton. Recently, Niimura et al., (2000) reported that the $H_2O_2$-forming NADH oxidase of *Amphibacillus xylanus* and *Sporolactobacillus inulinus* show extremely high peroxide reductase activity for both $H_2O_2$ and alkyl-hydroperoxide in the presence of the 22-KDa protein component AhpC. Although Nishiyama et al., (2001) demonstrated the presence of the system in several bacterial species that can grow aerobically, it is not known if it functions in the *L. sake* strains tested in here. Considering that an NADH:$H_2O_2$ oxidase was not present in *L. sake*$^{ins}$ as this strain could generate very low levels of $H_2O_2$, it is unlikely that the NADH oxidase/AhpC system is functioning in this strain.

The significance of SOD in relation to elevated $O_2$ resistance in *L. sake* is demonstrated by the two- to three-fold induction of SOD in the $O_2$-insensitive mutant LSUV4. Strain LSUV4 grew in the presence of 90% $O_2$., and SOD activity in this strain was lower than in *L. sake*$^{ins}$, albeit sufficient to compensate for the toxic effect of $O_2$ in this strain. Gonzalez et al., (1989) suggested that SOD is present in several lactic acid bacteria speciesand they were the first to report true SOD enzyme activity in *Lactobacillus* spp. was presented by Gonzalez et al., (1989). Two-fold higher levels of SOD were demonstrated in aerated compared to static cultures of *Lactococcus lactis* (Hansson and Haggstrom, 1984; Sanders et al., 1995).

Increased SOD activity in the presence of $O_2$ is an indication that $O_2^-$ is an intermediate product of $O_2$ metabolism of exponentially growing cells, and a significant source of oxidative stress. Previous reports questioned the formation of $O_2^-$ even as an intermediate product of $O_2$ metabolism (Thomas and Pera 1983). Archibald and Fridovich (1981a) stressed the importance of $O_2^-$ in relation to $O_2$ sensitivity and of the active dismutation system of SOD in lactic acid bacteria. Resistance of the *L. sake* strains to oxidative stress induced by elevated $O_2$ should not be attributed solely to the presence or absence of SOD; Rather, this complex response also involves the induction of regulons expressing both protective and repair enzymes. For *L. sake*$^{sens}$, we found that six proteins are induced within 4 h of exposure in *L. sake*$^{sens}$ upon exposure to elevated $O_2$ at 8$^oC$, while at least 16 proteins are induced in *L. sake*$^{ins}$ under the same conditions (A. Amanatidou and J. Wouters, unpublished data). A set of 40 proteins is induced in *E. coli* upon exposure to 60 μM $H_2O_2$. An increase in the levels of about 30 proteins has been observed in the same strain upon exposure to $O_2^-$-generating agents (Demple, 1991; Stortz et al., 1990; Eisenstark et al., 1996).

### SOD and $O_2^-$ formation in *L. sake*

A basic conclusion arising from this study is that in *L.sake*, $O_2^-$ radicals are generated upon the reaction of $O_2$ with components of the cytoplasmic fraction. In an earlier work, Imlay and Fridovich (1991) demonstrated that the respiratory chain is the apparent source of most endogenous $O_2^-$ in *E. coli* and that this micro-organism lacks the enzymes that deliberately generate $O_2^-$. By contrast, in *L. sake*$^{sens}$ only 8% of the generated $O_2^-$ was due to components of the membrane fraction. Despite the fact that total $O_2^-$ production in *L. sake*$^{sens}$ did not exceed 10% of the total $O_2$ consumption, the presence of elevated SOD activity in the $O_2$-insensitive variants suggests that the ability to cope with $O_2$ is strongly linked to the ability to reduce $O_2^-$ radical. The 10-fold lower SOD level for the wild-type *L. sake*$^{sens}$ than for the $O_2$-insensitive LSUV4 was accompanied by a 9- to 10-fold decrease in the rate

of $O_2^-$ elimination. Gort and Imlay (1998) proved that the relation between $O_2^-$ and SOD concentration varies inversely in *E. coli*. No other enzymes with $O_2^-$-scavenging capacity have been identified in lactic acid bacteria (Condon 1987). The absence of a significant SOD-independent scavenging mechanism is supported by the fact that $O_2^-$ toxicity worsens when very low SOD activities are present, as with the wild-type strain *L. sake*$^{sens}$.

### Possible sites of $O_2^-$ formation in *L. sake*

We demonstrated that the cytosolic fraction of *L. sake*$^{sens}$ contributes to most of the $O_2^-$ generation in this strain. An earlier study in *E. coli* indicated that intracellular $O_2^-$ is formed primarily by autoxidation of components of the respiratory chain (Imlay 1995). Many of the components of the respiratory chain that have been identified as primary sources of $O_2^-$ in *E. coli* (such as the NADH dehydrogenase) are not operative in lactic acid bacteria (Messner and Imlay 1999). As shown in this study, a small percentage of the total $O_2^-$ generation is linked to membrane activity.. Unlike in respiratory pathways, most cytosolic redox enzymes are not prone to univalent autoxidation while reduced flavoenzymes tend to generate $O_2^-$ (Massey et al., 1969; Imlay 1995). We suspect that enzymes related to cytosolic NADH oxidation (at least up to 120 or 140 μM) are probable important sources of $O_2^-$ in *L. sake*. Sanders et al., (1997) suggested also that lowering the level of the NAD/NADH ratio by increasing NADH would serve to induce an increase in reactive oxygen species.
There is some evidence that the NADH oxidase of *L. sake* is a main source of $O_2^-$ at elevated $O_2$ conditions. Halliwell and Gutteridge (1989) proposed that bacterial NADH oxidase is useful because it allows the cell to survive exposure to limited amounts of $O_2$, but if this enzyme is overwhelmed by too much $O_2$, irreversible damage can occur. Claiborne et al., (1993) have shown that the presence of cysteinyl side chains in NADH oxidase and peroxidase might contribute to $O_2^-$ production due to incorrect side reduction of oxygen by one electron. Not surprisingly, addition of FAD to the cell free extracts resulted in a 36% increase in the rate of $O_2^-$ production. Addition of FAD to the assay mixture of NADH oxidase increased the rate of NADH oxidation. This suggests that the NADH oxidase of *L. sake* is a flavoprotein, as has been demonstrated for other NADH oxidases from lactic acid bacteria (Schmidt et al., 1986). Flavins are recognised as the predominant sources of $O_2^-$ and $H_2O_2$ of the respiratory chain because they are relatively accessible to $O_2$ and less hydrophobic than e.g. iron-sulphur clusters or quinones. Free reduced flavins react rapidly with $O_2^-$ and may be themselves sources of intracellular $O_2^-$ (Gaudu et al., 1994). The hypothesis that flavoproteins and probably NADH oxidase are main sources of $O_2^-$ is supported by the observation that $O_2^-$ formation is inhibited in the presence of DPI. DPI is an inhibitor of NAD(P)H oxidase or other flavoprotein-containing oxidases and represents an alternative to an anaerobic environment for eliminating oxidative damage or killing (Hampton and Winterbourn, 1995). Although the effect of DPI on NADH oxidase activity was not determined, previous reports demonstrated that the flavoprotein NADH oxidase from *L. delbruckii* was inhibited by DPI (Marty-Teysset et al., 2000).

### Detection of protein damage in the cytosolic fraction

The role envisioned for $O_2^-$ is transferring electrons and increasing the pool of free iron available to catalyse the production of oxidative DNA damage. Increasing the pool of free iron can result from the release of iron from the [Fe-S] clusters of a subset of enzyme hydratases, or directly from bacterial iron-storage proteins (Keyer and Imlay, 1996). Oxidative modifications can also cause inactivation of enzymes due to introduction of carbonyl groups into amino acid side chains of the protein. Therefore, formation of carbonyl groups (aldehydes and ketones) is presumptive evidence of unspecific oxidative modifications. We observed higher levels of carbonyl groups in *L. sake*$^{sens}$ than in *L. sake*$^{ins}$ upon exposure of exponential cells to 90% $O_2$ + 10% $N_2$. Protein denaturation by reactive oxygen species increases proteolytic susceptibility and, furthermore, oxidative modifications of enzymes with

catalytic centres might convert them into catalytically inactive or less active forms (Davis and Lin, 1988).

Fumarase and fumarate reductase are two redox enzymes that contain catalytically essential [Fe-S] clusters. They both play a central role in respiration and they are both active in several lactic acid bacteria (Morishita and Yajima, 1995). Fumarases contain [4Fe-4S] clusters, which are susceptible to oxidative modifications and therefore appear to be sensitive to $O_2$, $O_2^-$ and $H_2O_2$ (Liochev and Fridovich, 1993). The recovery of fumarase upon $O_2$ removal and subsequent incubation under 10% $CO_2$ + 90% $N_2$ is in agreement with previous findings from Flint et al., (1993b), who demonstrated that inactivation of enzymes is not due to chemical oxidation of amino acid residues, since this type of reactions is not reversible. In contrast to fumarase, dihydroxy-acid dehydratase or other enzymes with [4Fe-4S] clusters which are fully recovered after removal of the excess $O_2$, the partial reactivation of fumarate reductase upon $O_2$ removal indicates a somewhat different mechanism of inactivation of this enzyme. Fumarate reductase can generate $O_2^-$ at very high levels and there is evidence that this enzyme does not lose activity at ambient $O_2$ (Kargalioglou and Imlay, 1995). The structure of fumarate reductase of *E. coli* has been recently described by Iverson et al., (2000); it consists of four subunits that contain FAD, [3Fe-4S] clusters and at least two quinones. However, Cecchini et al., (1995) reported that single amino acid substitution in fumarase reductase near the [3Fe-4S] cluster results in an enzyme that is either more sensitive to HO· radicals or produces more of them. The authors emphasised the importance of the proper protein environment near the quinone and [3Fe-4S] site for normal function of this enzyme. Possibly, the inactivation of fumarase reductase is due to degradation of enzymic protein as well as of the cluster.

A by-product of [Fe-S] cluster damage is the copious iron released into the cytosol, where it catalyses the oxidation of DNA in conjunction with $H_2O_2$ (Keyer and Imlay, 1996). This mechanism has been identified in *E. coli*. For other bacteria, such as the Gram(+) *L. sake*, further investigation is needed.

It is well known that cumulative oxidative cell damage generated by elevated O2 can cause cell stasis or death. In this report, we studied the consequences of exposure to elevated O2 and the responses of the typical lactic acid bacterium *L. sake* to the imposed endogenous stress. Basal superoxide fluxes were determined in the sub-cellular fraction and it was concluded that cytosolic enzymes are involved in generation and elimination of the imposed stress. Understanding microbial responses to oxidative stress can play a pivotal role in food preservation, in which reactive oxygen species are used to reduce bacterial contamination either directly or indirectly.

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# Chapter 5
## High oxygen and high carbon dioxide modified atmospheres for shelf life extension of minimally processed carrots

A. Amanatidou, R.A.Slump, L.G.M. Gorris & E.J. Smid
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## Abstract

The impact of high $O_2$ + high $CO_2$ modified atmospheres (MA), on the preservation of minimally processed carrots was studied. A combination of 50% $O_2$ + 30% $CO_2$ prolonged the shelf life of sliced carrots compared to storage in air by 2 to 3 d. When the carrots received a pre-treatment with a 0.1% citric acid dip and a sodium alginate edible coating prior to packaging, shelf life was extended for 5 to 7 d. Advantages and disadvantages of the proposed MA over previously recommended MA (1% $O_2$ + 10% $CO_2$) related to a range of physicochemical and micro-biological characteristics of carrots are discussed.

## 5.1 Introduction

Minimally processed (MP) carrots (washed, sliced, trimmed, cut or peeled) are used as ready-to-eat snacks or salad vegetables. MP carrots are sold within 7 to 8 days after preparation but poor quality may limit shelf life to 4 to 5 days (Carlin et al., 1990a). Deterioration occurs due to the development of off flavors, acidification, loss of firmness, and discoloration (Andersson, 1984; Carlin et. al., 1989; Bolin and Huxsoll, 1991; Howard and Griffin, 1993).

In general, the use of CA/MA (controlled/modified atmospheres) is beneficial for minimally processed products. Typically, a concentration of 5 to 10% $CO_2$ and 2 to 5% $O_2$ is applied to extend shelf life of these products (Kader et al., 1989). For carrots, the reports on MA storage are contradictory. Bruemmer (1988) claimed that harvested carrots are physiologically too mature for senescence control, and thus MA storage is not beneficial. At 2 to 10% $O_2$ and 10 to 40% $CO_2$, sugar content may be retained to a greater extent than in air-stored samples, but spoilage can occur due to excessive growth of lactic acid bacteria (Carlin et al., 1990b). Kato-Noguchi and Watada (1996, 1997a) reported that glycolysis is accelerated and that the ethanol and acetaldehyde levels are increased at $O_2$ concentration below 2% as compared to air storage. In a few cases, MP carrots responded well under anoxic conditions (i.e. 0.5% $O_2$; Izumi et al., 1996; Kakiomenou et al., 1996), although the risk due to growth of anaerobic pathogenic micro-organisms may be increased under these conditions.

Oxygen enriched atmospheres (>30%) have been tested for packaging of iceberg lettuce, oranges, and potato tubers (Aharoni and Houck, 1982; Heimdal et al., 1995). Sprouting of whole carrots was increased and mold growth was inhibited at 40% $O_2$ (Abdel-Rahman and Isenberg; 1974). Day (1996a) suggested that high $O_2$ concentrations inhibit enzymatic activity, prevent moisture losses and microbial contamination during wet handling of carrots. Combinations of elevated $O_2$ and $CO_2$ may however delay growth of aerobic and anaerobic micro-organisms, as was observed in an *in vitro* study, previously (Amanatidou et al., 1999).

The objective of this study was to investigate a range of quality indices (color, texture, sugar, phenols, thiobarbituric acid values, off-odor, and microbial spoilage) in order to identify possible benefits of the storage of MP carrots under high levels of $O_2$ + $CO_2$ in comparison to air and previously used MA storage. Carrots were exposed to controlled gas mixtures at 8$^{o}$C, a temperature generally used at retail

storage. Since disinfection is commonly applied to slow down deterioration processes (Eytan et al., 1992; Sapers et al., 1995), the effect of dipping in chlorine and alternative disinfectants (citric acid, $H_2O_2$, and so forth) in combination with MA storage was studied. A sodium alginate edible coating was used as a barrier to white discoloration (Nussinovitch and Hershko; 1996; Cisneros-Zevallos et al., 1997).

## 5.2 Material and methods

### Product preparation and processing

Carrots (cultivar "Amsterdamse bak") were obtained from the Dutch Greenery (Utrecht, The Netherlands), within 2 wk of harvest. Each experiment was repeated three times in the period of September 1997 to February 1998. Roots of medium size were selected and washed, and all heavily contaminated parts were removed. Carrots were sliced using a Sammic CA300 (Azpeitia, Spain) food processor in discs with an average size of 1 x 3 cm. The parts of the processor were regularly disinfected with 70% ethanol during preparation.

Sliced carrots were washed twice in sterile, distilled water (untreated carrots), a solution of NaOCl containing 200 $mg.l^{-1}$ active chlorine or 5% $H_2O_2$ (v/v) for 2 min. The effect of a citric acid treatment was studied by dipping the sliced carrots twice in a solution of 0.1% or 0.5% (w/v) citric acid. Untreated and treated carrots were dried in air for 15 min at room temperature. Aseptic conditions were kept during preparation, and the processor and surfaces were at time intervals disinfected with 70% ethanol.

In order to study the effect of the edible coating, the carrots were dipped in a solution of 2% $CaCl_2$ (w/v) or 2% $CaCl_2$ + 0.1% citric acid. After drying, an alginate based coating Satialginate S170 (System Bio Industries Benelux, Badhoevendorp, The Netherlands), pH 7.8 was sprayed on the surface. Carrots (70 g) from each treatment were transferred to plastic boxes that were disinfected with ethanol prior to use. Boxes with the carrots were placed in a temperature-controlled room maintained at 8°C, 92% RH in hermetically closed containers connected to a flow-through system and continuously flushed with the desired combinations of gases. Pure $N_2$, $O_2$, and $CO_2$ were mixed using mass flow controllers (5850 TR series Brooks Instruments B.V., Veenendaal, The Netherlands) at a flow rate 200 $ml.h^{-1}$. The following combinations of gases were used (a) 90% $O_2$ + 10% $CO_2$, (b) 80% $O_2$ + 20% $CO_2$, (c) 50% $O_2$ + 30% $CO_2$, (d) 70% $O_2$ + 30% $CO_2$, (e) 1% $O_2$ + 10% $CO_2$, (f) air control. Equilibrium conditions in the chamber were reached after 2h. At certain time intervals, 2 boxes from each treatment were removed from the containers and used for microbiological and physicochemical analysis.

### Microbiological analysis

Total aerobic mesophiles were enumerated on plate count agar (PCA, Oxoid, Hampshire, UK) after 3 d of incubation at 25°C. Pseudomonads were enumerated on Pseudomonas Agar Base supplemented with Cetrimide-Fucidin-Cephaloridine (CFC agar, Oxoid, Hampshire, UK) after 3 d at 25 °C. Counts of lactic acid bacteria (LAB) were performed on de-Man-Rogosa-Sharpe agar (MRS agar, Oxoid, Hampshire, UK) after incubation for 4 d at 25°C and *Enterobacteriaceae* were enumerated on Violet-Red-Bile-Glucose agar (VRBGA, Oxoid, Hampshire, UK) after 24 h at 37°C. The pour plate technique was used for the enumeration of LAB and *Enterobacteriaceae*. Duplicate samples were examined on each day of analysis.

### Quality analysis

*Color*

*C*olor measurements were made using a Minolta chromameter model CR200 (Minolta Camera Benelux BV, Utrecht, the Netherlands). The L*, a*, and b* data were transformed to a Whiteness

Index score using the equation 100 - $[(100\text{-}L^*)^2 + a^{*2} + b^{*2}]^{0.5}$ (Bolin and Huxsoll, 1991). Each datapoint is presented as the mean of measurements on both sides of 20 different carrot disks.

*Texture*
In preliminary studies, Texture Profile Analysis of carrots showed that the most comparative parameter between samples was firmness. It was measured with a texture analyzer (TA.XT2I, Texture Technologies, New York, USA), equipped with a 10 mm cylindrical ebonite probe. A speed of 1 $mm.sec^{-1}$ and a penetration distance 10 mm were used and firmness was expressed as maximum compression force (N). The data are presented as means of 10 independent measurements.

*pH*
Aliquots of 25 g of carrots were homogenized with an equal volume of distilled water. The pH of the homogenate was determined at each sampling time with a glass electrode (Metrohm model 691).

*Sugars*
Sugars (sucrose/D-glucose/D-Fructose) were quantified by using a test combination kit (Boehringer, Mannheim, Germany). Prior to the determination, samples (25 g) were homogenized with equal amount of water and clarified with 2.5 ml Carrez I and 2.5 ml Carrez II solution. pH was adjusted to 7.2 with 0.1-$mole.l^{-1}$ sodium hydroxide). Homogenates were transferred quantitatively into a 100-ml volumetric flask and rinsed with water. Next, n-Octanol (0.1 ml) was added and the flask was shaken until the foam had disappeared. Finally, the extracts were filtered and immediately used for the assay.

*Total phenols*
Total soluble phenols were extracted in 80% ethanol and measured using the Folin-Ciocalteu reagent (Swain and Hillis, 1959).

*Thiobarbituric acid values*
Samples (50 g) were homogenized in 100 ml distilled water. Aliquots of 25 ml of the homogenate were mixed with equal volume of 10% trichloroacetic acid (TCA) and filtered. After extraction the thiobarbituric acid method described by Barry-Ryan and O'Beirne (1998) was used to measure the degree of lipid oxidation. TBA value is defined as the increase in the absorbance due to formation of condensation products after the reaction of the equivalent of 1 mg of $sample.ml^{-1}$ volume with 2-thiobarbituric acid. Absorbance was read at 532 nm. All values were reported as percentages of the highest absorbance obtained. Three independent measurements were performed for each condition.

*Ethylene, acetaldehyde, and ethanol production*
A portion of 120 g of carrots from each treatment was placed in a glass jar (vol. 1Lt) fitted with a rubber septum, flushed with the desired combination of gases, sealed, and kept at $8^oC$. The ethylene concentration inside the jar was measured with a gas chromatograph (GC) equipped with a flame ionization detector (CHROMPACK Model 437A, Varian, Inc. Walnut Creek, USA) using an external standard. Acetaldehyde and ethanol were measured with a GC (CHROMPACK Model CP9001, Varian Inc., Walnut Creek, USA), using Helium as a carrier gas. The ethylene, ethanol and acetaldehyde concentrations were expressed as μmole of $volatile.g^{-1}$ fresh weight.

*Statistical analysis*
Data were subjected to analysis of variance and the Duncan's Multiple Range test. Each experiment was performed 3 times with 2 repetitions. Standard errors of the measurements are presented in the tables.

## 5.3 Results and discussion

### Disinfection of carrots

*Effect on total quality*

Washing with destilled water resulted in spoilage of MP carrots after 4 d at 8°C (data not shown) After 8 d, softening and browning of the surface was clearly observed. Browning is probably related to oxidation of phenols (Chubey and Nylund, 1969). Disinfection with chlorine or 5% (v/v) $H_2O_2$ enhanced off-flavors or bitter taste, respectively. Sapers and Simmons (1998) recommended removal of residual $H_2O_2$ after treatment but such action was not taken in our study, and this might explain the adverse effect of $H_2O_2$ treatment on the quality attributes. Washing in 0.1% and especially 0.5% (w/v) citric acid successfully kept the original appearance of the carrots for 8 d at 8°C (Table 1). At the highest citric acid concentration (0.5%) a harsh taste was noted, probably as the result of acidification. The use of a protective edible coating retarded moisture losses and bleaching but did not extend shelf life. However, the quality characteristics of MP carrots were maintained for 10 d when 0.1% citric acid was incorporated in the edible coating (data not shown).

*Effect on color*

Increased Whiteness Index (WI) values are related to the visual development of white discoloration (Table 1). White discoloration is an enzyme stimulated reaction related to dehydration of surfaces or formation of the wound barrier lignin (Tatsumi et al., 1991; Cisneros-Zevallos et al., 1995). For water-treated samples, increased WI values were recorded between d 1 and 4 of storage. Sapers et al., (1995) reported rapid discoloration immediately after treatment with 5 or 10% $H_2O_2$, related to the browning of lettuce and carrots and bleaching of strawberries and raspberries.

A comparative study between several commercial waxes and coatings identified the hydrocolloid coating sodium alginate S170, with $Ca^{++}$ as gelling agent, as a suitable coating for carrot slices (data not shown). Orange/red color of carrots was maintained for at least 8 d (Table 1). Alginate coatings allow the control of surface discoloration of MP carrots (Li and Barth, 1998). Sodium alginate reacts with polyvalent cations such as $CaCl_2$ to form a gel (Kester and Fennema, 1986). Nussinovitch and Hershko (1996) reported several applications of alginate coatings on vegetable products as barriers to moisture losses have. Citric acid alone or incorporated in the coating allowed color retention and inhibited white discoloration (Table 1). Reyes et al., (1996) used an edible coating incorporating an acidulant to inhibit white discoloration for up to 4 wk at 4°C. Combined application of calcium and citric acid delayed browning of MP Chinese cabbage (Byeong and Klieber, 1997).

*Effect on firmness*

Chlorine treatment did not affect firmness of carrots as compared to the water-dipped samples. On the other hand, dipping in 5% $H_2O_2$ significantly increased firmness immediately after treatment and during storage (Table 1). The alginate coating gave a glossy appearance to the product and textural characteristics were retained for at least 8 d. Incorporation of 0.1 or 0.5% (w/v) citric acid in the edible coating had a slight effect on firmness even after 8 d (Table 1). Calcium and citric acid have been used to improve firmness of cooked carrots as well as shredded carrots (Stanley et al., 1995). Calcium is thought to preserve membrane integrity of carrot shreds by delaying senescence-related membrane lipid changes, but also by augmenting membrane restructuring processes (Picchioni et al., 1996).

*Effect on pH*

All treatments resulted in a lowering of the pH from 6.1 (water control) to approx. 5.7 to 5.9 (data not shown). Chlorine treatment did not affect initial pH. Acidification was observed to some extent after 8 d of storage at samples dipped in 0.5% citric acid (pH 5.4).

**Table 1**. Changes in firmness, Whiteness Index, and spoilage symptoms after several disinfection treatments of carrots carrots stored at 8°C in air, immediately after treatment (day 0) and after 8 d

| Disinfection method/ treatment | Day | Firmness (N) | Whiteness Index | Spoilage symptoms |
|---|---|---|---|---|
| Distilled water | 0 | 828[a] | 30.4 [a] | |
| | 8 | 690 [b] | 37.2[c,d] | White blush, browning, soft rot |
| Chlorine 200ppm | 0 | 835 [a,c] | 31.6 [a] | |
| | 8 | 790[a,b,c] | 38.7[d] | White blush, off-flavor |
| $H_2O_2$ 5% | 0 | 1189 [d] | 34.4 [b] | |
| | 8 | 1274 [d] | >42[d] | White blush, slime, texture, bitter taste |
| Citric acid 0.1% | 0 | 870 [a,b] | 32.5 [a,b] | |
| | 8 | 855 [a,b,c] | 35.4 [b,c] | ND[2] |
| Citric acid 0.5% | 0 | 901 [a,c] | 30.4 [a] | |
| | 8 | 795 [a] | ND[1] | Harsh (acid) taste |
| Coating (S170 + 2% $CaCl_2$) | 0 | 890 [a,c] | 32.3 [a,b] | |
| | 8 | 774 [a,b] | 35.9[b,c] | Slime |
| Citric acid 0.1% + coat. | 0 | 903[a,c] | 29.8 [a] | |
| | 8 | 828[a,c] | 34.9 [b] | ND[2] |
| Citric acid 0.5% + coat. | 0 | 923[b] | 30.6 [a] | |
| | 8 | 880[a,b] | 35.8 [b] | ND[2] |

[1] *Not measured*

[2] *No spoilage was detected after 8 d of storage*

[abcd] *Means with different letters are significantly different ($p < 0.05$)*

***Table 2**. Dynamics of microbial populations (log cfu.g$^{-1}$) as affected by disinfection treatments*

| Microbial group | Population (log cfu.g$^{-1}$) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | HOCl | 5% $H_2O_2$ | Coating (S170+ 2% $CaCl_2$) | 0.1% Citric acid | 0.1% Citric acid +coating | 0.5% Citric acid | 0.5% Citric acid +coating |
| Total viable counts | 6.4 | 6.0 | 6.0 | 6.1 | 5.9 | 5.3 | 4.9 | 4.5 |
| *Pseudomonas* spp. | 6.3 | 6.1 | 5.9 | 6.2 | 5.6 | 5.1 | 4.6 | 4.3 |
| Lactic acid bacteria | 5.6 | 5.0 | 5.0 | 5.3 | 5.0 | 4.1 | 4.0 | 4.2 |
| Enterobactericeae | 5.4 | 4.8 | 4.7 | 5.0 | 4.9 | 4.0 | 3.6 | 3.5 |

*Effect on microbial flora*

Microbial growth on MP carrots is favored by the high moisture and numerous cut surfaces (Brocklehurst et al., 1987). The initial population of untreated carrots was high 6.4 log cfu.g$^{-1}$). Spoilage of MP carrots under air is the result mainly of the action of pectolytic Pseudomonads. Other groups such as lactic acid bacteria and *Enterobacteriaceae* are also present on the surface of carrots after cutting. Chlorine or $H_2O_2$ dipping as well as coating treatment did not affect substantially the initial microbial load but reduced somewhat the level of Enterobacteria present (Table 2). By contrast, combinations of 0.1% or 0.5% citric acid and 2% $CaCl_2$ significantly reduced initial total flora for at

least 1 or 2 log cfu.$g^{-1}$ respectively. The combination treatment affected the development of the microbial flora up to 4 d of storage but after 8 d, no differences in the total viable counts were recorded (data not shown). Our results on the effect of citric acid or $CaCl_2$ alone on the microbial flora of minimally processed carrots are in agreement with those obtained by other researchers (Eytan et al., 1992; Izumi and Watada, 1994).

## High $O_2$ and High $CO_2$ controlled atmospheres

*General quality*

The poor quality of carrots observed after storage in air for 12 d (Table 3) is related to changes in texture, color, and increased decay incidence. Samples stored under 1% $O_2$ + 10% $CO_2$ had a minimum shelf life of 12 d, which was further extended to 15 d after a dipping in citric acid and coating (data not shown). Good quality was observed for carrots stored under 50% $O_2$ + 30% $CO_2$ for at least 12 d. Although concentrations of $CO_2$ higher than 20% are not generally recommended for storage of respiring products like carrots, Carlin et al., (1989) reported $CO_2$ concentrations as high as 30 to 40% in equilibrium packs. After 12 d of storage, the quality of carrots was poor when the $O_2$ concentration was further increased (70% to 90%) combined with 10 to 30% $CO_2$ regardless of the treatment.

*Effect on color*

Increased WI values were apparent after 8 d in untreated (water dipped) samples independent of gas condition applied during storage. Treated (coated and dipped in 0.1% citric acid) carrots kept their characteristics for at least 12 d under 50% $O_2$ + 30% $CO_2$ or 1% $O_2$ + 10% $CO_2$ (Table 3). No obvious correlation existed between the gas concentration and the WI, although the values for carrots stored under 90% or 80% $O_2$ combined with 10% or 20% $CO_2$ were high. Good retention of the orange color has been previously reported at high $CO_2$ levels in acidified carrots (Juliot et al., 1989). Surface browning occurred due to oxidation of carrot phenols of samples stored in air and occasionally in 1% $O_2$ + 10% $CO_2$ but not when more than 50% $O_2$ was used. According to Day (1996a), enzymatic discoloration should not be expected at high $O_2$ MAP due to a substrate inhibition. Heimdal et al., (1995) did not find any correlation between browning and high oxygen on packed iceberg lettuce.

*Effect on firmness*

Increased firmness was typically noted with carrots exposed to 70% to 90% $O_2$ for 12 d. On the contrary, carrots stored in air were signifcantly softer after 12 d of storage (Table 3). Loss of firmness under these conditions may be related to an increased proliferation of pectolytic pseudomonads. Retention of firmness was quite satisfactory for treated samples stored under 1% or 50% $O_2$.

*Effect on total phenols*

A high concentration of total soluble phenols was observed in carrots stored under air (Figure 1). Most likely, this increase is related to polymerization of phenols catalyzed by microbial oxidases (Howard et al., 1994). Accumulation of phenols is a physiological response to infections or injuries. At 10% $CO_2$, total phenol-content remained at low level in the presence of 1% $O_2$, whereas an increase was observed in the presence of 90% $O_2$, especially for untreated samples. Storage in the presence of 50% $O_2$ + 30% $CO_2$ strongly reduced the accumulation of total phenols as compared to air. Under all gas atmosphere conditions, the phenolic content of treated samples was lower than that of untreated samples. Howard and Dewi (1996) found that treatment with citric acid, but not coating, slightly reduced the amount of total phenols of peeled carrots. The bitter taste, observed in air-stored samples, is related to increased concentrations of isocoumarin, chlorogenic, and hydrobenzoic acid (Sarkar and Phan, 1979; Babic et al., 1993), as a response of carrots to severe stress.

*Effect on sugars*

D-Sucrose is the main sugar contributing to the taste of carrots (61 mg.g$^{-1}$ fresh wt). After 12 d at 8$^{o}$C, the sucrose content of carrots stored in air or 80% $O_2$ + 20% $CO_2$ was as low as 21 mg/g fresh wt. In contrast, samples stored under 50% or 70% $O_2$ + 30% $CO_2$ or 1% $O_2$ + 10% $CO_2$ and retained more than 60% of the initial sucrose content (Figure 2). Sensory analysis for bitterness showed that unpeeled carrots stored in 1% $O_2$ were consistently sweeter than those stored in air (data not shown). Carlin et al., (1990b) found good retention of sucrose in the presence of 10% to 40% $CO_2$ with 2% or 10% $O_2$ and Howard and Dewi (1996) did not find any differences on the sugar content of coated and uncoated carrots stored in air. In this study, D-sucrose content was significantly retarded when coating and citric acid treatment, was combined with 20% or 30% $CO_2$.

*Effect on ethylene production*

Ethylene as high as 1.25 μmole/g$^{-1}$fw was measured in the headspace of sliced carrots 30 minutes after cutting. Preliminary experiments indicated a rapid increase in the level of ethylene of cut carrots during the first hours of storage (data not shown). Although carrot is a non-climacteric crop, ethylene may reduce postharvest quality by promoting senescence, low temperature injuries and microbial decay. In carrots exposed to ethylene level >0.125 μM on the headspace, the synthesis of so-called "stress metabolites", such as the bitter compounds isocoumarin and eugenin, was stimulated (Lafuente et al., 1996).

Surprisingly, increased accumulation of ethylene (1.63 μmole.g$^{-1}$ fw by the treated and 1.15 μmole.g$^{-1}$ fw by the untreated carrots) was observed at 50% $O_2$ + 30% $CO_2$ but not at 90% $O_2$ + 10% $CO_2$ ( Table 4). $CO_2$ is a well known inhibitor of ethylene synthesis but Pal and Buescher (1993) found that exposure to 30% $CO_2$ indeed accelerated ethylene evolution in carrots possibly due to an early injury response. Li and Barth (1998) found excessively high concentrations of ethylene on MP carrots after use of an edible coating with very low pH (2.7). The pH of the coating used in our study was 7.8 and thus phytotoxicity due to low pH is not likely. After 12 d of storage, traces of ethylene (<0.06 μmole.g$^{-1}$ fw) were measured under all conditions (data not shown).

***Table 3.*** *Effect of $O_2$ and $CO_2$ concentrations and dipping on firmness, Whiteness Index, and % of rotten discs of treated and untreated carrots stored for up to 12 d at 8$^{o}$C*

| | Firmness (N) | | Whiteness Index | | %Rot |
|---|---|---|---|---|---|
| Treatment | Day 0 | Day 12 | Day 0 | Day 12 | Day 12 |
| *Untreated carrots* | | | | | |
| 1% $O_2$+10% $CO_2$ | 813 $^{a}$ | 832 $^{a}$ | 30.3 $^{a}$ | 36.1 $^{c}$ | 30 |
| 50% $O_2$+30%$CO_2$ | | 910 $^{b,c}$ | | 37.5 $^{c,d}$ | 0 |
| 70% $O_2$+30%$CO_2$ | | 922 $^{b,c}$ | | 39.6 $^{d}$ | 0 |
| 80% $O_2$+20% $CO_2$ | | 903 $^{b,c}$ | | 38.6 $^{c,d}$ | 0 |
| 90% $O_2$+10%$CO_2$ | | 950 $^{b,c}$ | | 40.9 $^{d}$ | 0 |
| Air | | 755 $^{d}$ | | 38.1 $^{d}$ | 80 |
| *Coated and dipped* | | | | | |
| 1% $O_2$+10% $CO_2$ | 865 $^{a,b}$ | 890 $^{a,b}$ | 29.4 $^{a}$ | 32.4 $^{a,b}$ | 0 |
| 50% $O_2$+30%$CO_2$ | | 886 $^{b}$ | | 31.6 $^{a,b}$ | 0 |
| 70% $O_2$+30%$CO_2$ | | 893 $^{b}$ | | 33.6 $^{b}$ | 0 |
| 80% $O_2$+20% $CO_2$ | | 950 $^{c}$ | | 35.8 $^{b}$ | 0 |
| 90% $O_2$+10%$CO_2$ | | 910 $^{b,c}$ | | 36.8 $^{b,c}$ | 0 |
| Air | | 730 $^{d}$ | | 36.2 $^{c}$ | 50 |

$^{a,b,c,d}$ *Means with different letters are significantly different ($p < 0.05$)*

***Table 4***. *Ethanol, acetaldehyde and ethylene accumulation of treated (dipped in 0.1% citric acid and coated) and untreated (water dipped) carrots stored at 8°C under 3 atmospheres, for 48 h*

| Volatiles | Air | | 90% $O_2$ + 10% $CO_2$ | | 1% $O_2$ + 10% $CO_2$ | | 50% $O_2$ + 30% $CO_2$ | |
|---|---|---|---|---|---|---|---|---|
| | Untr. | Treated | Untr. | Treated | Untr. | Treated | Untr. | Treated |
| Ethanol ($\mu$mole.g$^{-1}$ fw) | 5.0 [b] | 0.75 [a] | 0.5 [a] | 1.0 [a] | 1416 [e] | 2712 [f] | 38 [d] | 20 [c] |
| Acetaldehyde ($\mu$mole.g$^{-1}$ fw) | 0.75 [a] | 1.125 [a] | 0.5 [a] | 1.25 [a] | 56 [d] | 95 [e] | 7.5 [c] | 3.8 [b] |
| Ethylene ($\mu$mole.g$^{-1}$ fw) | 0.92 [b] | 0.32 [a] | 0.41 [a] | 0.54 [a] | 0.30 [a] | 0.26 [a] | 1.15 [c] | 1.63 [c] |

[a,b,c,d,e,f] *Means with different letters are significantly different ($p < 0.05$)*

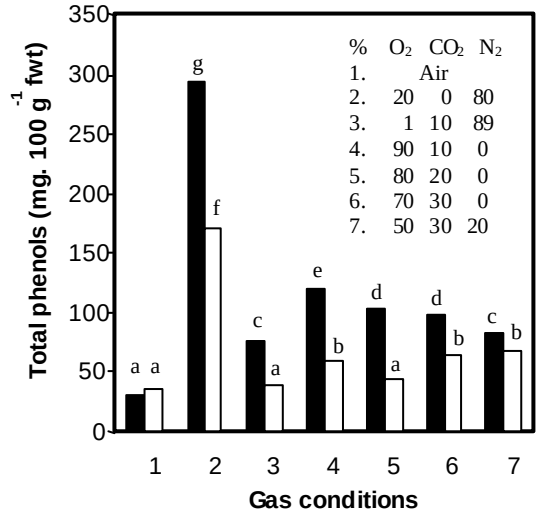


***Figure 1.*** *Changes in total phenol content of untreated (water dipped) carrot discs (black bars) or treated (washed in 0.1% citric acid and coated) carrot discs (white bars) under 6 controlled atmospheres for 12 d at 8°C*

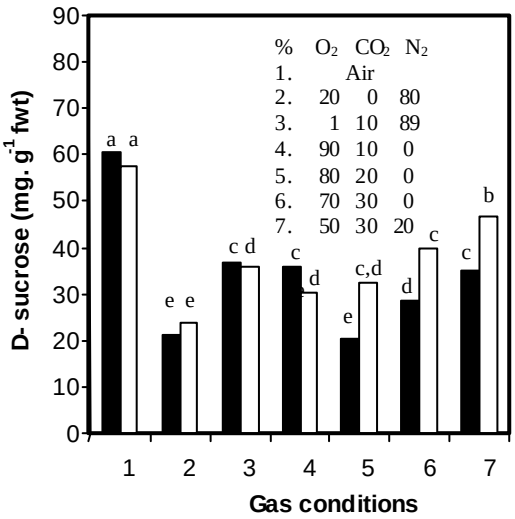


***Figure 2***. *Changes in D-sucrose content content of untreated (water dipped) carrot discs (black bars) or treated (washed in 0.1% citric acid and coated) carrot discs (white bars) under 6 controlled atmospheres for 12 d at 8°C*

***Table 5:*** *Thiobarbituric acid (TBAR) values of treated and untreated carrots stored for 12 d at 8°C, in 6 atmospheres. TBARS are expressed as a percentage of the higher absorbance recorded at 532 nm*

| | TBARS (%) | | | | | |
|---|---|---|---|---|---|---|
| Gas conditions | 1% $O_2$ + 10% $CO_2$ | 70% $O_2$ + 30% $CO_2$ | 50% $O_2$ + 30%$CO_2$ | 80% $O_2$ + 20%$CO_2$ | 90% $O_2$ + 10%$CO_2$ | Air |
| Untreated carrots | 100 [a] | 91 [a,b] | 86 [b] | 69 [d] | 56 [e] | 58.2 [d,e] |
| Treated carrots | 93 [a] | 82 [b,c] | 85 [b] | 65 [d] | 48 [f] | 50.8 [f] |

[a,b,c,d,e,f] *Means with different letters are significantly different ($p < 0.05$)*

*Effect on volatile accumulation*

After 4 d of storage, increased ethanol and acetaldehyde production was detected for samples stored at 1% $O_2$ + 10% $CO_2$ compared to air stored samples both for untreated and treated carrots (Table 4). High ethanol concentrations affected the taste of carrots stored under these conditions. Accumulation of acetaldehyde and ethanol was suppressed in the presence of 50% to 90% $O_2$ despite of the $CO_2$

levels. At low $O_2$ levels, glycolytic flux in carrots is accelerated; ethanol and acetaldehyde levels increase in response to hypoxia (Kato-Noguchi and Watada, 1996; 1997b). Increased or decreased response of carrots tissue to low or high $O_2$ respectively is sustained by the concept of the dual role of $O_2$ in regulating respiration (Leshuk and Saltveit, 1991). Citric acid treatments slow down respiration and glycolytic metabolism of carrot discs (Kato-Noguchi, 1997a).

*TBA values*

Lipid oxidation (measured as percentage of the highest TBA value observed) appeared to increase during storage especially for water treated samples. Lipid oxidation was not observed at air stored samples (Table 5). This may indicate that the development of off-odors under air is mainly caused by the spoilage microflora, which produces off-flavor volatiles. It is unlikely that the increased TBA values recorded at 1% $O_2$ + 10% $CO_2$ after 12 d of storage are due to lipid oxidation. The method used for the determination is not specific for malonaldehyde and interference by other aldehydes is possible. Carrots kept in 90% $O_2$ + 10% $CO_2$ had low TBA values. High $O_2$ did not retard formation of secondary oxidation products in the presence of 30% $CO_2$.

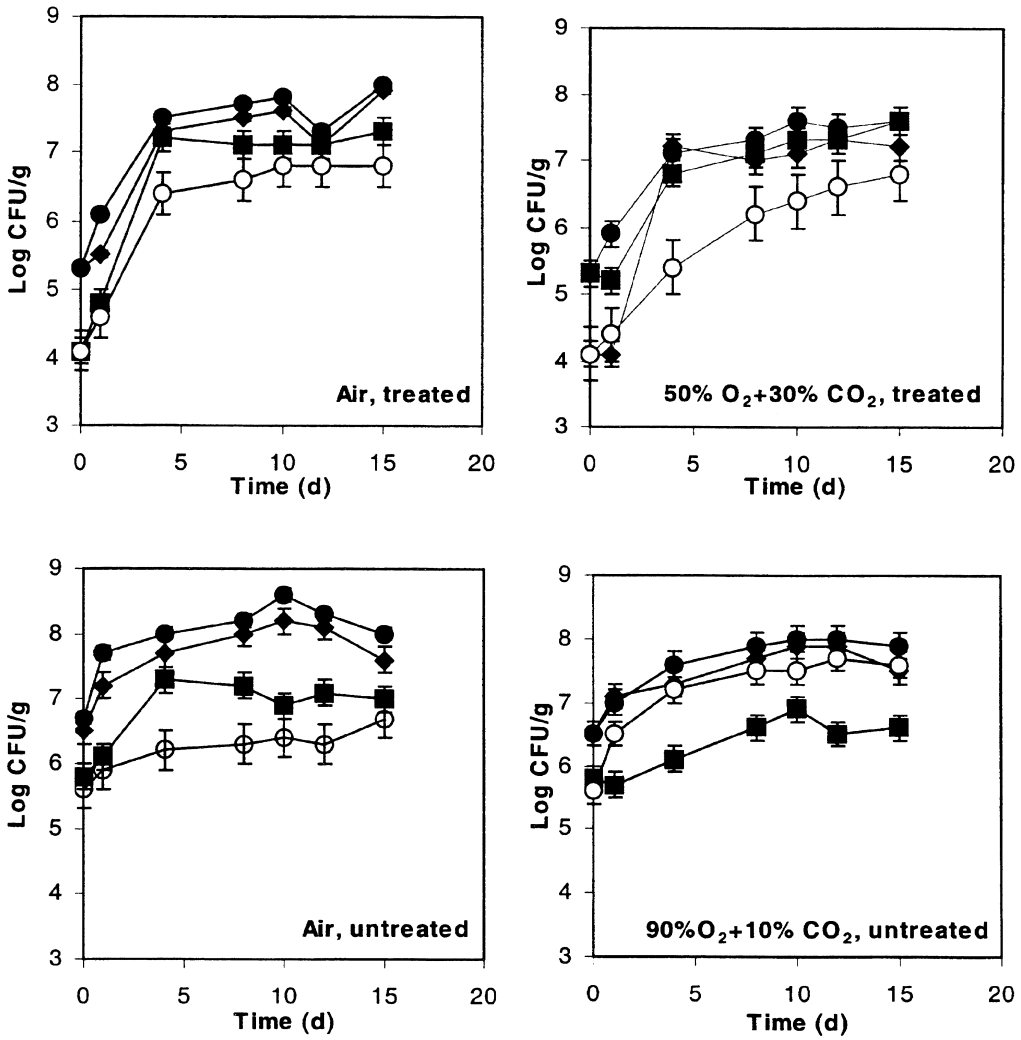


***Figure 3.*** *Changes in the microbial populations of treated carrots (dipped in 0.1% citric acid and coated) during storage at 8°C in air, treated (A) or 50% $O_2$ + 30% $CO_2$, treated (B) or air, untreated (C) or 90% $O_2$ + 10% $CO_2$, untreated (D). Symbols: ●=Total viable counts. ♦=Pseudomonas. ■=Lactic acid bacteria.* ○ = Enterobacteriaceae. *Standard errors are presented on the graphs.*

*Effect on microbial flora*

The maximum level of bacterial growth in air was reached for all samples at d 8 to 10, and very little changes occurred after that (data not shown). For untreated carrots stored in air, total counts were 8.8 log cfu.$g^{-1}$ after 10 d of storage. Excessive growth and, thus, spoilage due to lactic acid bacteria was never observed in untreated carrots, stored under air. This might be related to accumulation of phenols with antimicrobial properties under these conditions. After 12 d of storage at 8˚C, total viable counts of the water dipped samples exceeded 8.0 log cfu.$g^{-1}$ with pseudomonads being dominant under all gas atmospheres. For treated samples, the total viable count was as high as 7.5 log cfu.$g^{-1}$, mainly due to partial inhibition of pseudomonads (Figure 3). Lactic acid bacteria were dominant in the presence of high $O_2$+high $CO_2$ after acid coating treatment. Enterobacteria were inhibited under 50% $O_2$ + 30% $CO_2$ but stimulated under 80% or 90% $O_2$. Microbial spoilage due to extended growth of lactic acid bacteria was observed after 12 d in samples stored under 80% or 90% $O_2$ with 20% or 10% $CO_2$, respectively.

## 5.4 Conclusions

On the basis of the evaluation of a range of quality indices, it is concluded that minimally processed carrots washed with 0.1% citric acid retained fresh product characteristics for at least 8 d, especially when treated with $CaCl_2$ and an alginate coating. The quality of MP carrots stored under 50% $O_2$+30% $CO_2$ was similar or better than those stored at 1% $O_2$ + 10% $CO_2$ after 8 to 12 d at 8$^o$C. Shelf life was further extended from 12 to 15 d, but only when products were disinfected with 0.1% citric acid and coated prior to storage under modified atmospheres. Oxygen levels over 70% resulted in poor product quality when combined with 10% to 30% $CO_2$. However, carrots could tolerate $CO_2$ up to 30% in the presence of 50% $O_2$. Overall, high-oxygen MA storage can be used as an alternative to low-oxygen MA storage for minimally processed carrots, since it allows the product to retain fresh, natural characteristics and retards microbial growth during prolonged storage.

**Acknowledgements**

This work was funded by the Commission of the European Union through contract FAIR-CT96-5038 to author A.A. The authors thank S. Fervel and J. Verschoor for assistance with the measurements of the ethylene and respiration metabolites. H.J. Neerhof is acknowledged for providing the edible coating and for helpful discussions. Finally, the authors are indebted to Prof. D. Knorr for critical reading of the manuscript.

# Chapter 6
## Effect of combined application of high pressure treatment and modified atmospheres on the shelf life of fresh atlantic salmon

A. Amanatidou, O. Schlüter, K. Lemkau, L.G.M Gorris, E.J. Smid & D. Knorr
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## Abstract

High Pressure (HP) processing at low temperatures combined with modified atmosphere packaging (MA) was used for the preservation of salmon. A shelf life extension of 2 days was obtained after a HP treatment of 150 MPa for 10 min at 5°C compared to unpressurised, vacuum-packed salmon. MA storage (50% $O_2$ + 50% $CO_2$) alone extended the shelf life of salmon for 4 days at 5°C. When salmon had been subjected to HP treatment in the presence of 50% $O_2$ + 50% $CO_2$, the threshold value for microbial spoilage of salmon (7.0-7.2 log cfu.$g^{-1}$) was not reached for at least 18 days at 5°C. Spoilage micro-organisms (lactic acid bacteria, *Shewanella putrefaciens*) as well as pathogens (*Listeria monocytogenes* Scott A, *Salmonella typhimurium*) spiked on salmon prior to the treatment, were more susceptible to HP in the presence of 50% $O_2$ + 50% $CO_2$. The mode of action of compressed gases is probably related to intracellular formation of reactive oxygen species as well as to phase transition phenomena. Although microbial growth on salmon was retarded, the combined HP and MA treatments, at the settings used in this study, promoted a detrimental effect on colour and changes in the balance of oxidative rancidity.

## 6.1 Introduction

Fresh salmon is a high quality product with considerable economic importance. It is usually sold vacuum-packed and chill-stored without further treatment. The shelf life of salmon is difficult to estimate due to the lack of standardised criteria for the determination of freshness but a commercial shelf life is usually limited to only 1 week when stored between 2 and 8°C. Lowering of quality is manifested through changes in appearance, odour, colour or texture (Declerck, 1976). New preservation strategies that prolong the shelf life while ensuring microbial safety would enable the distribution of salmon outside current markets.

Modified atmosphere (MA) packaging has been used to extend the shelf life and maintain high quality of salmon (Stier et al., 1981, Pastoriza et al., 1996). Despite the advantages of MA packaging, caution should be taken since improper handling and/or extended storage might pose a public health hazard due to the survival of micro-organisms, such as *Listeria* spp. and *Salmonella* spp., or production of toxin by infectious *Clostridium botulinum* (Stammen et al., 1990; Reddy et al., 1997).

High pressure (HP) processing has been used for the preservation of chicken, pork, surimi gels as well as salmon products, with considerable positive effects on protease activity, textural properties, taste and flavour (Knorr, 1993; Ohshima et al., 1993; O'Brien and Marshall, 1996). HP for microbial decontamination has been extensively reviewed but complete microbial inactivation is currently not possible (Knorr, 1995; Smelt, 1998). In order to obtain effective sterilisation, the combination of pressure with other treatments, such as mild heat, is required.

A combined application of high pressure and gases (krypton, xenon, $N_2O$ and $CO_2$) is effective in inactivating vegetative cells of bacteria *in vitro* (Thom and Marquis, 1984; Debs-Louka et al., 1999). ZoBell and Hittle (1967) reported that hydrostatic pressure increased the sensitivity of a range of micro-organisms to $O_2$. $CO_2$ in supercritical state provides a promising technique for manufacturing heat sensitive foods, although the prolonged pressure treatment required (2-4 h) is a restraining factor for industrial application (Kühne and Knorr, 1990; Patterson et al., 1995).

At present there is no literature data available documenting the effect of combined application of high pressure and gases on the microbial flora of real food systems. The objective of this study was, therefore, to investigate such effects with salmon by testing the feasibility of using HP (up to 200 MPa) at low temperatures (0-5$^{o}$C) combined with MA (50% $O_2$ + 50% $CO_2$) to inhibit the natural microbial spoilage of salmon. The effect on inactivation of two foodborne pathogens i.e. *List. monocytogenes* and *Salm. typhimurium* spiked on salmon was also examined. In order to identify the effect of the combined treatment on the quality of salmon several physico-chemical characteristics (texture, colour and thiobarbituric acid) were examined. Advantages and disadvantages of the combined preservation technique for the preservation of salmon are discussed.

## 6.2 Material and methods

### Product

Fresh Atlantic salmon fillets were obtained from a local fish company in Berlin and transferred to the lab within 24 h of de-boning. Salmon was stored under melting ice during transportation. Immediately after transportation, the skin was removed aseptically. The salmon was cut in pieces (average weight 20 g), which were immediately sealed in UV sterilised bags consisting of polyester/polyethylene (Combithen PM5/15K1240, Wolff Walsrode AG, Walsrode, Germany) using a Webomatic sealer (Werner Bonk, Bochum, Germany). Experiments were carried out on three different batches of fish. Aseptic conditions were kept during preparation and handling.

### Experimental design

HP treatment was performed in a high hydrostatic pressure unit supplied by Nova (Switzerland). The vessel had a volume of 880 ml (internal diameter 45 mm). The maximum design pressure was 420 MPa at an operating temperature of -10 to 50°C. A thermocouple type K was fitted through the upper plug to measure the inner temperature of the vessel during the pressure treatment. The vessel was filled with a pressure-transmitting medium consisting of a mixture of distilled water and 5% oil (Mobil Hydrosol 78). The temperature in the vessel was controlled externally by flexible tubes coiled around the vessel and connected to a cryostat (D1-GH, HAAKE, Karlsruhe, Germany). The cooling medium in the cryostat was a mixture of glycol and pure water. A high pressure reciprocating pump (DSXHW, Haskel Ltd., California, USA) pumped the pressure medium into the vessel from a reservoir. The pressure in the vessel was measured using a pressure-transducer (HP28, Intersonde Ltd., Watford, England) connected near to the inlet of the vessel.

Optimal conditions for pressure and treatment time were determined in a broad screening session. All combinations of four treatment times (0, 10, 30, 60 min) with four HP conditions 0, 100, 150, 200 MPa were evaluated. Vacuum–sealed bags were pressurised in the pre-cooled vessel. During pressurisation the temperature was kept between 1 and 5$^{o}$C. The pressurised samples were stored in a temperature control room at 5 ± 1 $^{o}$C. At time intervals up to 18 days of storage, samples were withdrawn for microbial and quality analysis.

For modified atmosphere packaging experiments, bags were flushed under a stream of gases at a constant rate of 500 ml.min$^{-1}$ for 10 s. A gas-mixing unit (Witt Gasetechnik GMbH, co. Witten, Germany) was used to create a mixture consisting of 50% $O_2$ + 50% $CO_2$ (± 1%). The mixture used was selected after performing a number of preliminary experiments (data not shown). For the combined treatment, samples were sub-divided and some samples were first flushed with gas 50% $O_2$ + 50% $CO_2$ and then pressurised at 150 MPa at 5$^{o}$C for 10 min within 5 hours after MA packaging. The remaining samples were first pressurised and then MA-packed. Unpressurised vacuum or MA-packed samples were used as controls. After packaging, samples were stored at 5 ± 1 $^{o}$C prior to microbiological and physicochemical analysis.

### Quality analysis

Colour measurements were made using a Minolta chromameter model CR200 (Minolta GmbH, Ahrensburg, Germany). The L* and a* -values describe the intensity of white and red colour, respectively. In order to overcome the problem of the heterogeneity of the colour of salmon samples, 3 pieces were blended and the colour was expressed as the mean of 9 replicated measurements of the homogenised salmon. Texture differences were measured as cutting strength of salmon with a texture analyser (TA.XT2i, Texture Technologies, New York, USA) equipped with a knife blade operating at a speed of 2 $mm.s^{-1}$ and a cutting distance of 5 mm. Mean cutting strength was calculated and the respective coefficients of variation were expressed as the force *(g)* required for cutting 1 mm into the salmon. Cutting strength was taken as the mean of 6 measurements on different pieces of similar size. Lipid oxidation was expressed as thiobarbituric acid reactive substances (TBARs). As TBARs we defined the increase in the absorbance due to the formation of condensation products after the reaction of the equivalent of 1 mg of sample/ml volume with 2-thiobarbituric acid. Samples were prepared and measured as previously described (Amanatidou et al., 2000). TBARs were expressed as the absorbance recorded at 532 nm. Three independent measurements were performed for each condition. Oxygen concentration in the head space of the package was measured in triplicate for each condition at days 0, 8, 14 and 18 using an oxygen analyser (Model LC 700F, Toray Engineering Co. Ltd., Osaka, Japan).

### Microbiological analysis

At regular time intervals, 3 samples from each condition were analysed for microbial growth. Each sample was removed aseptically and homogenised for 1 min in a stomacher bag containing 50 ml physiological peptone salt (PFZ, 0.1% peptone + 0.85% NaCl). Aliquots (1 ml) of each homogenised sample were diluted in 9 ml PFZ and plated in proper microbiological medium. Total aerobic counts were enumerated on amodified Long and Hammer's agar (mLHA; van Spreekens, 1974) and incubated at 10°C for 7 days. Pseudomonads were enumerated on Pseudomonas Agar Base supplemented with Cetrimide-Fucidin-Cephaloridine (CFC agar, Oxoid, Hampshire, UK) after 2 days at 20 °C. Counts of lactic acid bacteria (LAB) were determined on MRS agar after incubation for 4 days at 25°C and *Enterobacteriaceae* were enumerated on Violet Red Bile Glucose Agar (VRBGA, Oxoid, Hampshire, UK) after 24 h at 37°C. Finally hydrogen sulfide producing bacteria (mainly *Shewanella putrefaciens*) were enumerated on Lyngby Iron Agar (Lyngby, Denmark) after 4 days at 20°C. The pour plate technique was used for the enumeration of LAB, *Enterobacteriaceae* and *Sh. putrefaciens*. All counts were expressed as $\log_{10}$ colony forming units $cfu.g^{-1}$ salmon sample.

### Inoculation experiments

Where appropriate, salmon samples were inoculated with an 18-h culture of *List. monocytogenes* Scott A or *Salm. typhimurium* DSM 4780. Cells were centrifuged (6000 $rev.min^{-1}$, for 10 min at 5°C), washed with distilled water (x2), subsequently diluted into ACES buffer (*N*-[2-acetamido]-2-aminoethane sulfonic acid; Sigma Chemical Co., St. Louis, Mo., USA) and finally inoculated on the salmon up to a final population of $10^8$ $cfu.g^{-1}$. Samples were MA-packed, pressurised at 150 MPa for 5, 15, and 30 min at 3 ± 2°C and plated to determine microbial counts. Untreated samples were used as control. Between the moment of the treatment and analysis, samples were stored at 5 ± 1°C.

*List. monocytogenes* was surface plated on Palcam Listeria Selective medium (PLSM, Oxoid, Hampshire, UK) and enumerated after incubation at 25°C for 48 h. *Salm. typhimurium* was plated on Xylose Lysine Desoxycholate (XLDA, Oxoid, Hampshire, UK) and enumerated after incubation at 37°C for 24 h.

### Statistical analysis

The growth data for the enumeration of bacterial groups from eight sampling times were used to estimate the maximum specific growth rate $\mu_{max}$ ($h^{-1}$). The modified Gompertz function was used and

parameters were estimated with PSI PLOT version 5.52 software (Poly Sofware Int., Salt Lake City, USA).

## 6.3 Results

### Quality analysis

*Colour*

*L** and *a** values coincide well with the visual changes in lightness and red colour, respectively, of salmon filets. A product with lightness above the threshold value of 70 or red colour below 13 is unacceptable. *L** values of salmon increased with increasing intensity and time of pressure treatment (Figure 1a). A high pressure treatment of 150 MPa for 60 min or 200 MPa for 10 min resulted in an opaque product (*L** value > 70). Treatment with compressed gases (50%$O_2$ + 50% $CO_2$ 150 MPa, 10 min) resulted in a product with acceptable lightness even after 14 days of storage at 5$^o$C (Figure 2a). *a** values (which indicate the red colour intensity of the salmon) declined with increasing pressure and time of treatment but in acceptable levels (Figure 1b). The detrimental effect of pressure (150 MPa, 10 min) on *a** value was enhanced when salmon was treated with compressed 50% $O_2$ + 50% $CO_2$ as shown in Figure 2b. After 14 days of storage, *a** values were below the threshold value of acceptance (*a** value < 13) for all gas-packed samples (both pressure and not pressure-treated), while all the vacuum-packed samples were at acceptable levels. This indicates a detrimental effect of MA on colour. In Table 1, the time course of the changes in colour values of the gas-packed samples is represented. As shown on the table, unacceptable *a** values were recorded for the gas-packed salmon after 12 days of storage.

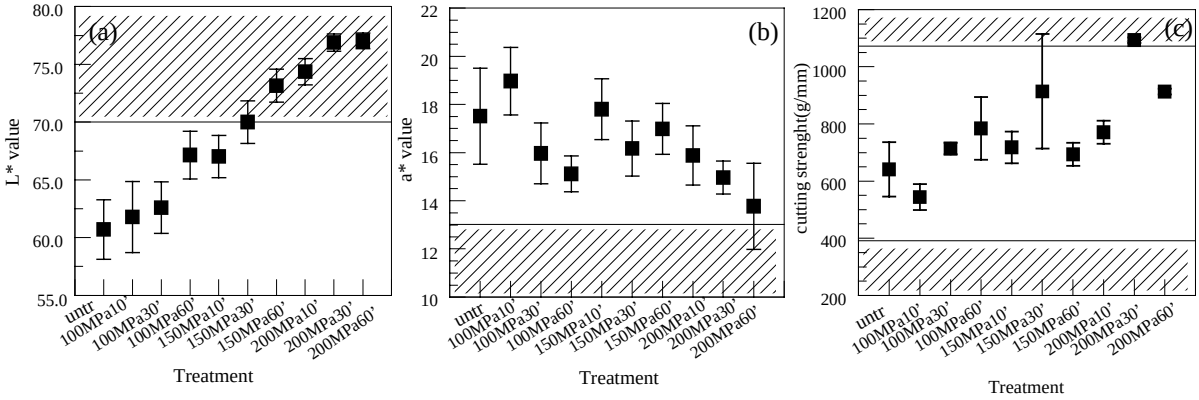


***Figure 1***. *Effect of different combinations of high-pressure / time treatment on: (a)* L* *values; (b)* a* *values; and (c) cutting strength of salmon pieces immediately after treatment. The hatched area indicates the level at which the product is unacceptable*

*Cutting strength*

Prolonged HP treatment (30 min at 150 or 200 MPa) increased cutting strength compared to untreated salmon but a significant effect was only observed after a HP treatment of 200 MPa for 60 min (Figure 1c). Pressurised vacuum-packed samples, had significantly higher cutting strength compared to unpressurised vacuum-packed samples stored at 5$^o$C for 14 days and therefore were unacceptable due to increased hardness (Figure 2c). On the contrary, cutting strength of MA-packed samples was lower, indicating an increase in the softness upon storage. In Table 1, the time course of the changes in cutting strength of the gas-packed samples is represented. The textural characteristics were retarded when the stored samples were pressurised prior to MA packaging. However, the variation between replicate samples was significant (Figure 2c).

*TBARs*

Fig. 2d illustrates the effect of high pressure (150 MPa for 10 min) on the TBARs of salmon samples treated and / or stored in the presence of 50% $O_2$ + 50% $CO_2$. Initial TBARs were not significantly affected by any of the treatments compared to fresh samples. TBARs increased significantly after 14 days of storage at 5°C for all samples and especially for MA-packed samples as well as those treated with compressed gases. Salmon samples with TBARs above 1.9 were characterised by unacceptable organoleptic characteristics (rancid taste).

*Gas composition*

The initial gas concentration in the packages was 50% $O_2$ + 50% $CO_2$ (± 1%). The solubility of $CO_2$ was increased when MA-packed salmon was pressurised at 150 MPa for 10 min and, thus, the $O_2$ concentration in the gas phase was increased (57%) as measured a few hours after treatment. After 18 days, the $O_2$ concentration in all of the samples had decreased. The lowest concentration measured was 37% for salmon treated in the presence of gases (Table 2).

***Table 1**. Effect of modified atmospheres on* L* *values,* a* *values, and cutting strength of salmon pieces stored under 50% $O_2$ + 50% $CO_2$ at 5°C*

| Conditions and storage time (h) | Physicochemical characteristics | | |
|---|---|---|---|
| | Cutting strength ($g.mm^{-1}$) | *L** value | *a** value |
| Vacuum | | | |
| 0 | 640 ± 82 | 58 ± 1 | 18 ± 0.8 |
| 4 | 710 ± 73 | 57 ± 1 | 16 ± 0.6 |
| 8 | 820 ± 69 | 60 ± 2 | 17 ± 0.3 |
| 12 | 766 ± 47 | 61 ± 1 | 20 ± 0.5 |
| MAP | | | |
| 0 | 590 ± 56 | 58 ± 1 | 18 ± 0.7 |
| 4 | 620 ± 58 | 57 ± 1 | 18 ± 0.7 |
| 8 | 820 ± 50 | 68 ± 1 | 16 ± 0.3 |
| 12 | 766 ± 37 | 66 ± 1 | 13 ± 0.4 |

***Table 2**. Changes in the $O_2$ concentration of MA-packed salmon during storage at 5°C*[a]

| Days | $O_2$ (%) | | |
|---|---|---|---|
| | A | B | C |
| 0 | 52 | 51 | 57 |
| 8 | 52 | 54 | 57 |
| 14 | 43 | 44 | 43 |
| 18 | 40 | 40 | 37 |

[a] *Abbreviations: A, storage in MAP 50%$O_2$ + 50% $CO_2$; B, 150 MPa, 10 min, followed by storage in MAP; C, MAP followed by HP treatment*

## Microbial analysis

*Initial spoilage flora*

The initial flora present on fresh salmon was high ($10^4$ cfu.g$^{-1}$ or higher) and consisted mainly of pseudomonads. Yeasts, lactic acid bacteria (LAB), hydrogen sulfide-producing bacteria (presumably *Sh. putrefaciens*) and *Enterobacteriaceae* were also present. Pressure treatment at 200 MPa for 30-60 min resulted in a product with counts of < 100 cfu.g$^{-1}$ (Figure 3a). The initial flora of salmon was not reduced significantly (< 1 log) by pressure treatment at 150 MPa for 10 min compared to unpressurised salmon. A significant reduction of the initial level of pseudomonads and *Sh. putrefaciens* was recorded even at pressure levels of 100 MPa for 30 min (Figure 3b, d) although the total flora was not significantly reduced by this treatment (Figure 3a). LAB were totally inactivated after a HP treatment at 200 MPa independent of the duration of the treatment (< 10 cfu.g$^{-1}$, Figure 3c).

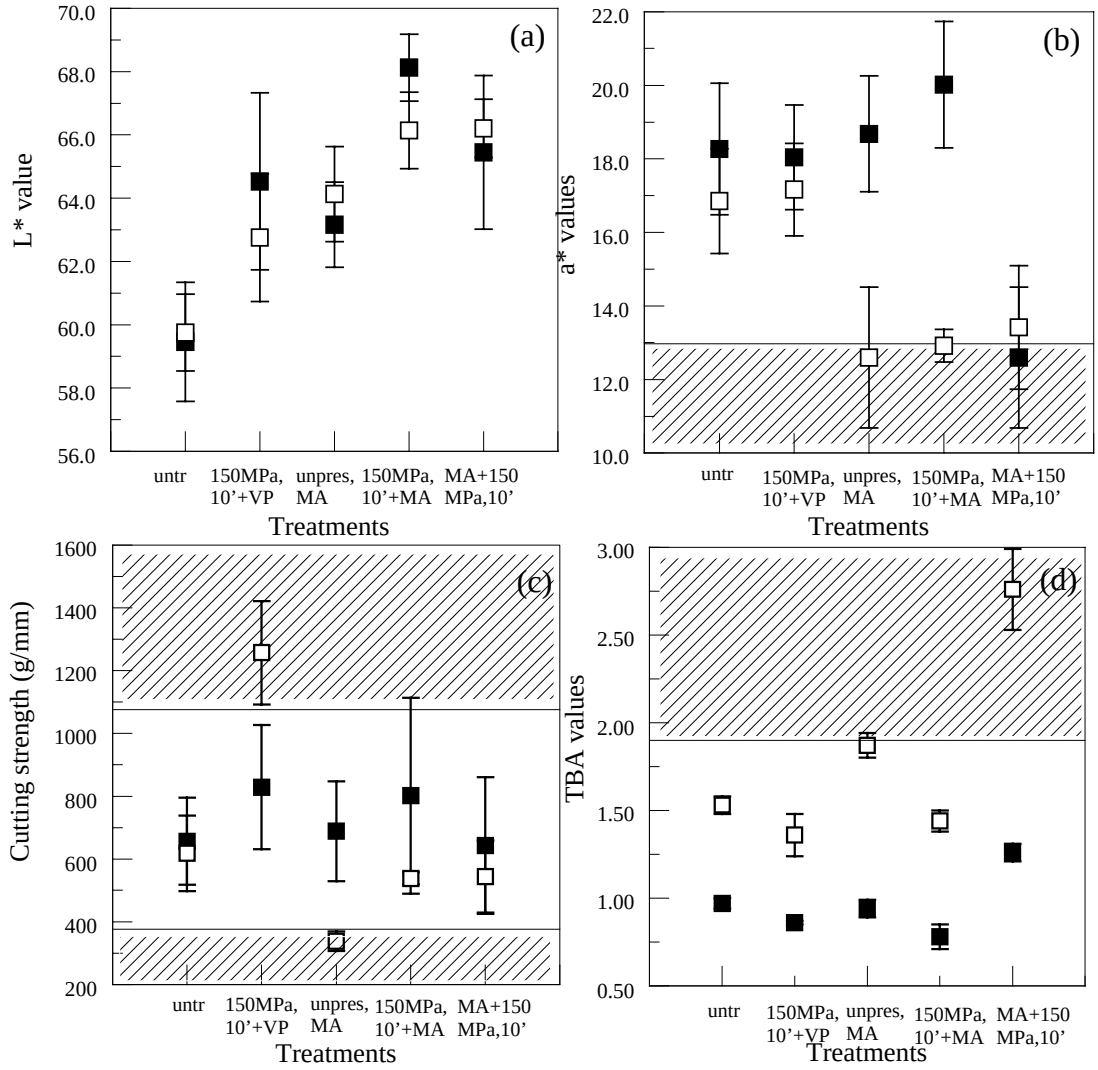


***Figure 2**. Effect of combined high-pressure treatment and modified atmospheres on: (a)* L* *values; (b)* a* *values; (c) cutting strength of salmon; and (d) TBA values. ■: at day 0; □: after 14 days of storage at 5°C. Abbreviations: untr: unpressurised, vacuum-packed samples; 150 MPa, 10' + VP, pressure treated and vacuum-packed; unpres + MA, unpressurised and packed at 50% $O_2$ + 50% $CO_2$; 150 MPa, 10' + MA, pressurised and subsequently packed at 50% $O_2$ + 50% $CO_2$; MA + 150 MPa, 10', samples packed at 50% $O_2$ + 50% $CO_2$ prior to pressure treatment. The hatched area indicates the level at which the product is unacceptable*

***Table 3**. Changes in total viable counts,* Sh. putrefaciens *and lactic acid bacteria on salmon after different treatments and during storage at 5°C*

| Storage treatment and time (days) | Mean viable counts (± S.D.) | | |
|---|---|---|---|
| | Total counts | *Sh. putrefaciens* | LAB |
| A: vacuum-packed, untreated | | | |
| 1 | 5.20 ± 0.02 | 4.32 ± 0.03 | 4.03 ± 0.07 |
| 2 | 5.91 ± 0.15 | 4.76 ± 0.45 | 5.31 ± 0.45 |
| 4 | 6.70 ± 0.24 | 5.98 ± 0.35 | 6.67 ± 0.41 |
| 6 | 7.04 ± 0.51 | 6.23 ± 0.61 | 7.03 ± 0.34 |
| 8 | 7.41 ± 0.37 | 7.22 ± 0.24 | 7.38 ± 0.39 |
| 12 | 7.54 ± 0.26 | 7.06 ± 0.39 | 7.35 ± 0.28 |
| 18 | 7.57 ± 0.09 | 5.72 ± 0.10 | 7.21 ± 0.07 |
| B: vacuum-packed and pressurised at 150 MPa/10 min | | | |
| 1 | 4.72 ± 0.08 | 3.17 ± 0.10 | 3.67 ± 0.42 |
| 2 | 5.33 ± 0.34 | 3.34 ± 0.16 | 4.92 ± 0.44 |
| 4 | 6.45 ± 0.28 | 3.98 ± 0.17 | 6.25 ± 0.51 |
| 6 | 6.99 ± 0.56 | 4.11 ± 0.24 | 6.73 ± 0.37 |
| 8 | 7.46 ± 0.37 | 4.63 ± 0.04 | 7.37 ± 0.64 |
| 12 | 7.55 ± 0.33 | 5.45 ± 0.08 | 7.58 ± 0.28 |
| 18 | 7.54 ± 0.08 | 5.72 ± 0.10 | 7.48 ± 0.13 |
| C: MAP | | | |
| 1 | 5.21 ± 0.24 | 4.32 ± 0.03 | 4.03 ± 0.57 |
| 2 | 5.09 ± 0.25 | 4.31 ± 0.15 | 4.81 ± 0.51 |
| 4 | 5.82 ± 0.56 | 4.25 ± 0.24 | 5.24 ± 0.38 |
| 6 | 6.25 ± 0.49 | 5.61 ± 0.45 | 6.02 ± 0.37 |
| 8 | 6.76 ± 0.04 | 3.95 ± 0.56 | 6.53 ± 0.27 |
| 12 | 7.34 ± 0.06 | 4.24 ± 0.47 | 7.14 ± 0.43 |
| 14 | 7.40 ± 0.10 | 5.20 ± 0.14 | 7.50 ± 0.10 |
| 18 | 7.51 ± 0.33 | 5.92 ± 0.32 | 7.35 ± 0.18 |
| D: treated at 150 MPa, 10 min and packed at 50% $O_2$ + 50% $CO_2$ | | | |
| 1 | 4.73 ± 0.40 | 3.47 ± 0.32 | 3.92 ± 0.32 |
| 2 | 5.00 ± 0.36 | 3.65 ± 0.37 | 4.45 ± 0.27 |
| 4 | 5.61 ± 0.57 | 4.12 ± 0.43 | 5.29 ± 0.33 |
| 6 | 6.08 ± 0.15 | 4.87 ± 0.37 | 5.33 ± 0.42 |
| 8 | 6.91 ± 0.23 | 5.36 ± 0.61 | 6.88 ± 0.25 |
| 12 | 7.55 ± 0.11 | 6.12 ± 0.52 | 7.23 ± 0.04 |
| 14 | 7.50 ± 0.23 | 6.34 ± 0.37 | 7.41 ± 0.21 |
| 18 | 7.75 ± 0.20 | 6.92 ± 0.13 | 7.53 ± 0.31 |
| E: Packed at 50% $O_2$ + 50% $CO_2$, and treated at 150 MPa, 10 min | | | |
| 1 | 4.43 ± 0.06 | 2.13 ± 0.05 | 3.22 ± 0.27 |
| 2 | 4.64 ± 0.15 | 2.78 ± 0.07 | 4.11 ± 0.36 |
| 4 | 5.19 ± 0.38 | 4.05 ± 0.10 | 4.89 ± 0.39 |
| 6 | 5.67 ± 0.29 | 3.19 ± 0.11 | 5.26 ± 0.48 |
| 8 | 6.37 ± 0.50 | 3.65 ± 0.18 | 6.33 ± 0.17 |
| 12 | 6.96 ± 0.45 | 4.00 ± 0.33 | 6.67 ± 0.36 |
| 14 | 7.10 ± 0.15 | 4.21 ± 0.17 | 6.45 ± 0.11 |
| 18 | 7.05 ± 0.07 | 3.71 ± 0.20 | 6.78 ± 0.14 |

*Storage experiments*

Microbial growth is the main spoilage cause of salmon. The critical level of microbial contami-nation (7.0-7.2 log cfu.g$^{-1}$) is a threshold value, which correlates with the development of fishy, ammoniacal odours (microbial spoilage odours), on the basis of organoleptic tests. Spoilage of vacuum stored samples was observed already after 6-7 days of storage at 5$^{o}$C and was probably due to the high counts of *Sh. putrefaciens*. With MA-packed samples, the growth rate of total microbial count was slower and LAB were dominant. Pressure treatment of vacuum-packed salmon at 100 MPa for 10 or 30 min and storage at 5$^{o}$C stimulated the growth rate of the total flora (Figure 4). The growth rate was significantly reduced during storage following pressure treatment of 150 MPa for 30 and 60 min or 200 MPa.

The critical level for microbial growth was reached after 7-8 days for vacuum-packed salmon treated at 150 MPa for 10 min and after 10-12 days of storage for MA-packed samples. Microbial shelf life was not extended further when HP treatment was followed by storage in 50% $O_2$ + 50% $CO_2$, although the growth of all bacterial groups was retarded for 18 days when samples were MA-packed prior to the HP treatment (Table 3). The specific growth rate ($\mu_{max}$) of *Sh. putrefaciens* was significantly reduced by pressure treatment, whereas the growth of LAB was more affected by the MA treatment (Table 4).

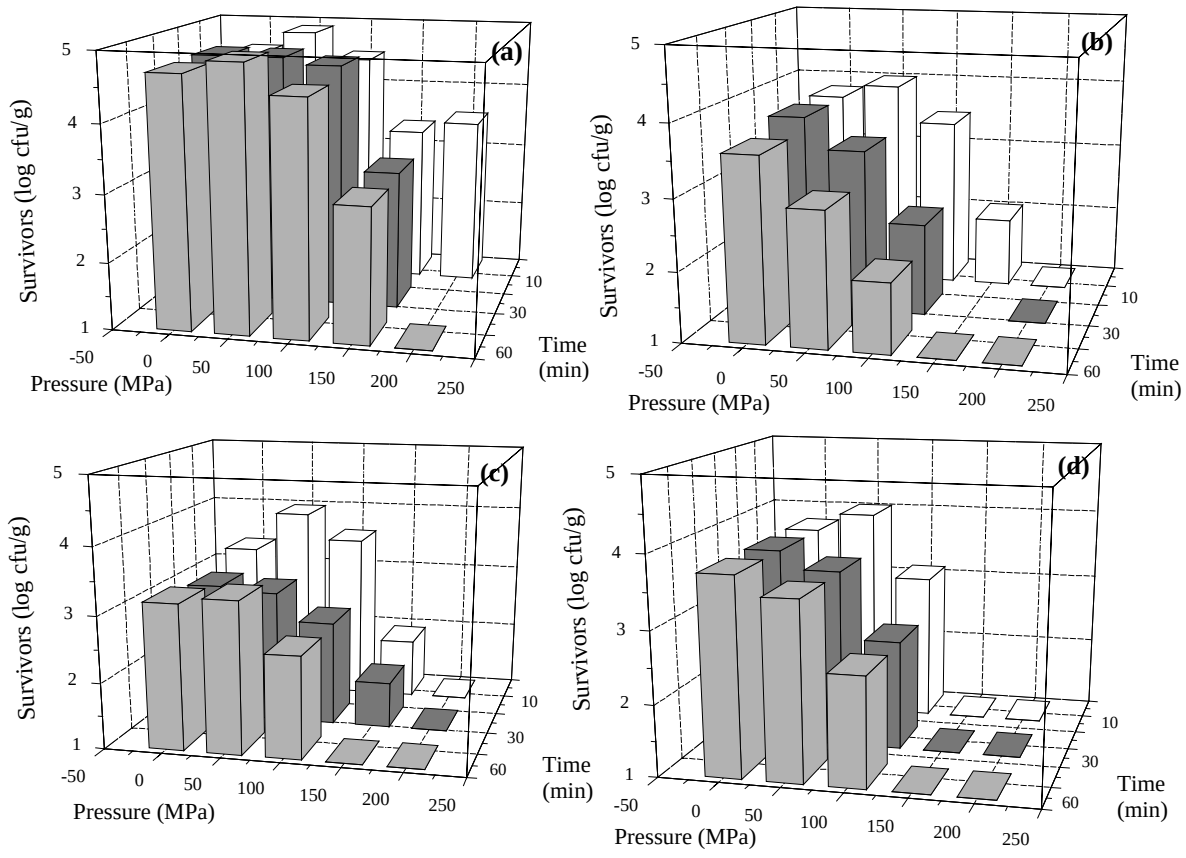


***Figure 3**. Logarithmic reduction of (a) total counts, (b) pseudomonads, (c) lactic acid bacteria and (d)* Sh. putrefaciens *of vacuum-packed salmon treated at different levels of pressure and time at 5$^{o}$C*

***Table 4**. Maximum specific growth rate $\mu_{max}$ ($h^{-1}$) of the total flora,* Sh. putrefaciens *and lactic acid bacteria grown on packed salmon after storage at 5°C*

| Bacterial group | $\mu_{max}$ ($h^{-1}$) ± S.D. | | | | |
|---|---|---|---|---|---|
| | A | B | C | D | E |
| Total viable count | 0.020 ± 0.001 | 0.016 ± 0.001 | 0.012 ± 0.003 | 0.012 ± 0.001 | 0.019 ± 0.011 |
| *Sh. putrefaciens* | 0.023 ± 0.002 | 0.009 ± 0.001 | 0.027 ± 0.008 | 0.011 ± 0.001 | 0.001 ± 0.001 |
| Lactic acid bacteria | 0.024 ± 0.002 | 0.023 ± 0.002 | 0.017 ± 0.001 | 0.015 ± 0.003 | 0.015 ± 0.001 |

*A, vacuum-packed; B, vacuum-packed, pressurised at 150 MPa / 10 min; C, MAP 50% $O_2$ + 50% $CO_2$; D, pressure treatment 150 MPa / 10 min followed by MAP; E, MAP and pressurised at 150 MPa / 10 min*

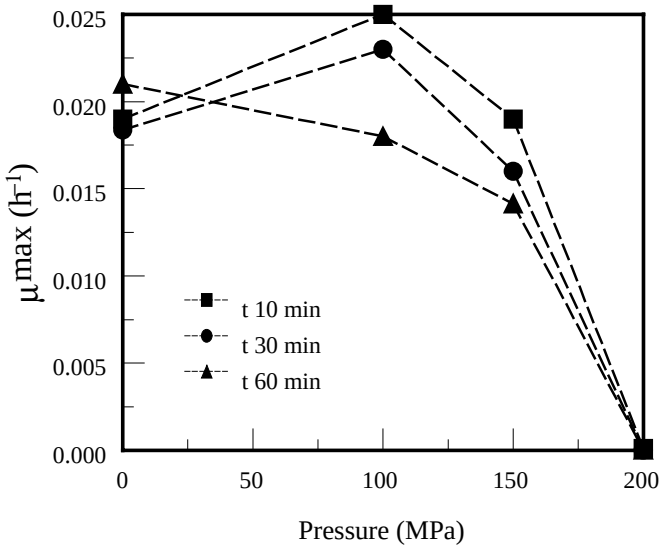


***Figure 4**. Plot of the maximum specific growth rate ($\mu_{max}$, $h^{-1}$) of the total counts of vacuum-packed salmon during storage at 5°C vs. pressure for three different treatment times*

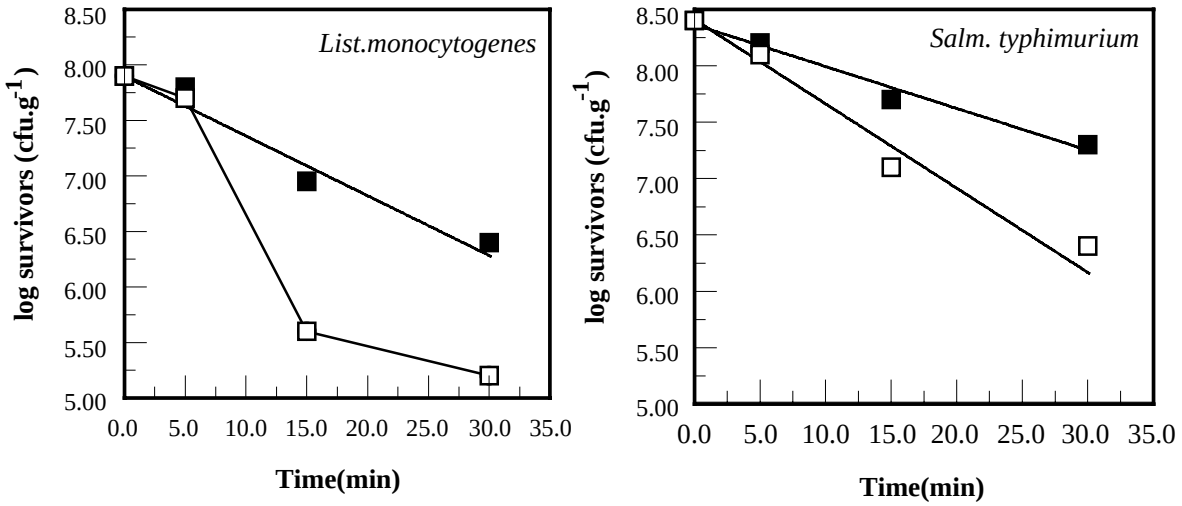


***Figure 5**. Survivor curves of* Listeria monocytogenes *Scott A and* Salmonella typhimurium *inoculated on salmon before HP treatment (150 MPa) at 1°C, in the presence of (■) vacuum or (□) 50% $O_2$ + 50% $CO_2$*

### Inoculation experiment

Compressed gases were effective towards *List. monocytogenes* and *Salm. typhimurium* inoculated on salmon. Compared to untreated samples, a reduction of 1.5 log cfu was observed for *List. monocytogenes* after a treatment of vacuum-packed samples at 150 MPa for 30 min, while a 3.5 log reduction was achieved when samples were packed with 50% $O_2$ + 50% $CO_2$ prior to HP treatment (Figure 5). Under the same conditions, viable counts of *Salm. typhimurium* were reduced for 1 and 2 log, respectively.

### Effect of the combined treatment on the shelf life of salmon

This study describes the effect of single and combined application of HP treatment and MA packaging on the preservation of fresh salmon. The gas conditions used were selected on the basis of a preliminary examination, not discussed here. The effect on microbial growth and several physicochemical characteristics (colour, texture, oxidation, and pH) was evaluated. Based on this evaluation, the different lengths of shelf life of salmon under the conditions studied are presented in Figure 6.

The shelf life of vacuum-packed salmon was limited to only 6-7 days due to extended microbial growth, although natural fresh-like, physicochemical characteristics were retained satisfactorily even after prolonged storage at 5$^{o}$C (18 days). MA storage extended growth to approximately 12 days. Further extension of shelf life was limited due to the detrimental effect on the colour of salmon upon extended storage under MA conditions. Based on the current observation we could suggest that the best results related to the extension of shelf life and retention of quality characteristics of salmon were achieved in samples which where pressurised at 150 MPa for 10 min and subsequently packed under MA conditions before storage at 5$^{o}$C. Treatment with compressed gases delayed microbial growth for at least 18 days but poor colour and extensive oxidation limited the shelf life to about 10 days under these conditions.

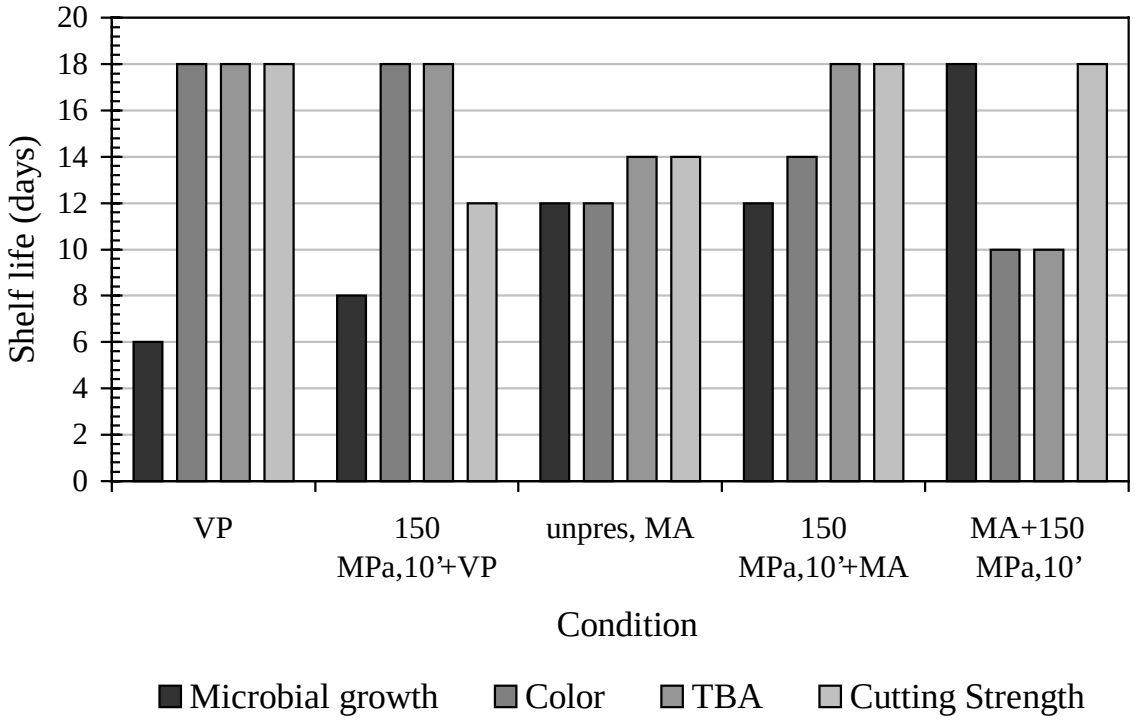


***Figure 6**. Shelf life of salmon as determined by microbial growth, colour, TBA values and cutting strength at five different experimental conditions (abbreviations as in Fig. 2)*

## 6.4 Discussion

A problem with the assessment of the shelf life of fish is that there are no objective standards for rejection or acceptance. In this study, organoleptic characteristics were taken into consideration for determining shelf life based on expert's opinion and microbial numbers of total viable counts, counts of hydrogen-sulphide producing bacteria and lactic acid bacteria. These spoilage micro-organisms were present at high numbers at the end of storage of salmon under the conditions tested. Various reports in literature have shown that LAB and *Sh. putrefaciens*, but also pseudomonads or other micro-organisms, represent the main groups of spoilage micro-organisms found on fish during storage under vacuum or MA at low temperature (Gram, 1992; Camell et al., 1997; Koutsoumanis and Nychas, 1999). *List. monocytogenes* Scott A and several *Salmonella* species are also associated to the processing and preparation of salmon (Farber, 1991b).

Several literature reports indicate the use of MA for preservation of salmon. Pastoriza et al., (1996) suggested that salmon slices stored under an atmosphere enriched with $CO_2$ are of high quality after 18 days at chilled storage, although Haard and Lee (1982) observed bland taste and powdery texture when fresh salmon was stored under pure $CO_2$. An atmosphere of 40% $CO_2$ inhibited microbial growth and extended sensory acceptance of salmon fillets (Brown et al., 1980). Gill and Molin (1991) attributed the high growth rates of lactic acid bacteria and *Sh. putrefaciens* under 30% $O_2$ + 40% $CO_2$ + 30% $N_2$ to the tolerance of these micro-organisms to $CO_2$. $O_2$ serves as a protection against botulism, which is an important safety hazard of salmon (Fey and Regenstein, 1982).

On the other hand, little information is available on the efficacy of pressure treatment on the inactivation of micro-organisms on real food matrixes, like that of salmon. A treatment at 340 MPa for 60 min at 23°C was required for a 6 log reduction in viable counts of *List. monocytogenes* Scott A and a 2 log reduction of *Salm. typhimurium* inoculated on chicken food (Metrick et al., 1989; Styles et al., 1991). Although the current study indicated that the antimicrobial effect of high pressure is enhanced at low temperatures, it is generally accepted that efficient sterilisation can only be achieved when HP treatment is combined with other preservation techniques.

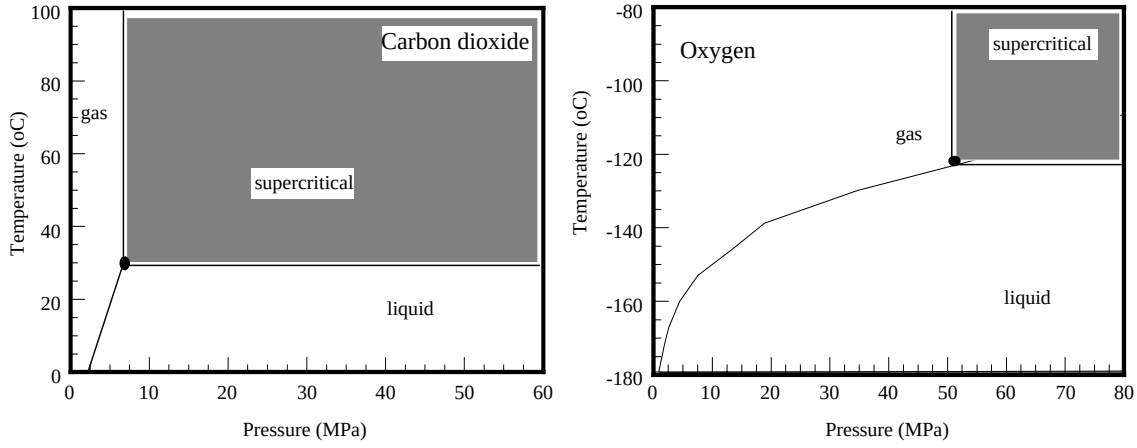


***Figure 7****. Solid, liquid and supercritical state shown in the phase diagram for carbon dioxide and oxygen (data from VDI-Wärmeatlas, Anonymous, 1994)*

The mechanism underlying the antimicrobial activity of compressed gases is complex. Most likely, the sublethal injury of pressure-treated micro-organisms increased their sensitivity to gases. As shown in the gas phase diagram (Figure 7) at a pressure treatment of 100-200 MPa and at 5°C, $CO_2$ is in liquid state. According to Lin et al., (1992b), compressed $CO_2$ dissolves in the aqueous phase, then expands upon sudden pressure release to cause cell damage and disruption of intracellular enzymes and organelles. Antimicrobial activity is also related to the decrease in intracellular pH, disturbance of homeostasis as well as the extraction of microbial constituents (Ballestra et al., 1996). Susceptibility

should increase at low temperatures due to higher $CO_2$ solubility (Ogrydziak & Brown, 1982). The sites of $CO_2$ inhibition in the presence of $O_2$ should be different from those during anaerobic conditions (Dixon and Kell, 1989). During pressure treatment at 100-200 MPa at 5$^o$C, $O_2$ is falling to the supercritical state as shown in the phase diagram (Figure 7). Although Marquis and Thom (1992) failed to demonstrate a substantial antimicrobial action by $O_2$ under pressure up to 40 MPa (a pressure at which $O_2$ in the gas phase is employed), they concluded that compressed gases are potent enhancers for intracellular oxidative damage to vegetative cells.

Apart from the antimicrobial or bacteriostatic effect of the MA, HP or combined treatments the effect on physichochemical characteristics on salmon in relation to shelf life extension should not be ignored. The detrimental effect on the colour of salmon was the hindering factor for application of compressed gases for the preservation of salmon. No clear conclusions can be drawn from the present study concerning the weakening of the red colour of MA stored salmon. The major functions of $O_2$ in modified atmospheres for the preservation of muscle foods are colour retention, inhibition of odour development and drip loss (as $O_2$ increases up to 50%). $CO_2$ enriched atmospheres may have an adverse effect on colour as reported by Silva and White (1994). This observation might be of importance for the current study considering that $CO_2$ was present at 50% in the MA conditions employed here. Most of the carotenoids of salmon (astaxanthin, β-carotene, astacene) can react rapidly with oxidising agents or free radicals (Woodall et al., 1997). Myoglobin, also present in pigmented fish may act as a catalyst of oxidation (Deng et al., 1977).

Goto et al., (1993) recommended a treatment between 0 and 500 MPA at 5$^o$C for 20 min for salmon meat. In the current study, a pressure treatment of 150 MPa has proven suitable for fresh salmon. Weakening of the red colour resulted in an unacceptable product at 200 MPa. The effect on the colour of salmon depends not only on the pressure level, but also on the holding time in the range of 10-60 min. The colour of fish is dictated largely by several pressure-stimulated or inhibited factors such as the pH, the pigments, the chemical state and the degree of denaturation, but the exact mechanisms underlying colour changes are not elucidated (Ledward, 1998). Loss of the translucency of the fish flesh is due to denaturation of different myosins between 100-200 MPa (Angupanich and Ledward, 1998). The colour repression, which followed compressed gases treatment, is a complex phenomenon, as pressure treatment might have a catalytic effect on the chemical reaction between $O_2$, $CO_2$ and components of the tissue.

Changes in protein structure and function may affect the texture of salmon and can be related to the increased hardness observed at increased pressure and holding time (Simpson, 1997). The increased softness of salmon, in the presence of gases, might be attributed to the increase in the solubility of gases in the salmon tissue during prolonged storage.

Oxidative rancidity is an important organoleptic characteristic for rejection or approval of fish after prolonged storage. Wang and Brown (1983) showed that neither $CO_2$ prevents, nor $O_2$ promotes rancidity of muscle foods. In the current study it was found that, although TBA values give a good indication for lipid oxidation, there is no evidence that increased oxidation is exclusively due to the gases present in the atmosphere. Cheah and Ledward (1997) suggested that catalytic oxidation is not depending on the presence of oxygen during pressure treatment. Parry, (1993) recommended a mixture of 60% $CO_2$ + 20% $O_2$ + 20 % $N_2$ for storage of salmon, as this gas composition did not lead to rancidity (thiobarbituric acid value) problems. On the contrary, salmon treated with compressed gases at 100 MPa for 60 min had very low TBA values after 14 days of storage at 5$^o$C (A. Amanatidou, unpublished data).

From the results of this study it can be concluded that the combined application of high pressure (150 MPa, 10 min) and modified atmospheres (50% $CO_2$ + 50% $O_2$) is a feasible means for preservation of fresh salmon, due to a shelf life extension of at least 5 days compared to vacuum packaging. The combined application was superior to MA packaging or pressure treatment alone, as it allowed a better control of microbiological characteristics, colour, texture and lipid oxidation. On the other hand, the use of compressed gases resulted in a significant inhibition of microbial spoilage of salmon (more than

10 days) compared to vacuum packaging, but the modifications of the physicochemical characteristics and functionality attributed to this novel technology, do not warrent its application for shelf life extension.

**Acknowledgements**

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# Chapter 7
# General Discussion

## 7.1 Free radicals and oxygen toxicity

The effect of oxidative stresses on certain bacteria has been well documented. Lushckak (2001) has described a general theory of oxidative stress that applies to both eukaryotes and prokaryotes. Oxidative stress in microbial cells shares many similarities with other types of cells. However, features such as the action of reactive oxygen species (ROS) on cell constituents, the identity and properties of oxidative stress intermediates of oxidative stress and the role of transition metals significantly between different cell types (Sigler et al., 1999). Several recent studies have confirmed the involvement of oxidative stress in several aspects of food preservation as discussed in Chapter 1. Oxygen toxicity in living organisms has been observed since the beginning of this century, but only in 1954 Gerschman et al., proposed that most of the damaging effect of $O_2$ could be attributed to the formation of ROS. The toxic effects of ROS have been overlooked for many years. The reason is that the rates of enzyme inactivation by $O_2$ in aerobic microbial cells under ambient oxygen conditions are too low to explain the rate at which toxic effects of $O_2$ develop. Additionally, ROS are subject to non-enzymatic decomposition depending on the availability of reactants or may be removed by enzymatic reduction to less reactive, non-radical species. The rate at which free radicals accumulate upon exposure to elevated oxygen levels and the mechanisms of high oxygen toxicity have not been studied in details. Recent findings that exposure to high oxygen concentrations may cause substrate inhibition of several enzymes, has given a new drive to the high oxygen research for food preservation (Day, 1996b). However, on a cellular level, the nature and activity of biochemical processes involved in high oxygen toxicity in biological systems have been practically ignored. Understanding the mechanisms underlying oxygen toxicity at low temperature in micro-organisms is of great relevance for food preservation.

The objective of the work described in this thesis was to demonstrate the application of high oxygen as an new preservation factor, The initial hypothesis was that high oxygen in addition to factors such as $CO_2$, low temperature and high pressure will possibly increase microbial shelf-life and retard physicochemical spoilage of minimally processed products. This hypothesis was verified for two food products, namely minimally processed carrots and fresh salmon. Particular focus was on the mechanisms of oxygen toxicity in the lactic acid bacterium *Lactobacillus sake*. This bacterium was chosen because of its importance for the spoilage of minimally processed food products.

## 7.2 Oxygen metabolism and toxicity in LAB

$O_2$ may act as an external electron acceptor in reactions with NADH oxidase(s) and thus affect cellular metabolism. Recent studies have shown that even for facultative anaerobic lactic acid bacteria oxygen can be beneficial if heme is present during aerated growth and if the bacteria are able to shift to respiration (Duwat et al., 2001). In some LAB several substrates (mannitol, lactate etc.) can only be fermented when $O_2$ is present (Axelsson, 1993). In homofermentative LAB, lactate dehydrogenase competes with the NADH oxidase to generate energy rich intermediates and to maintain cellular homeostasis (Murphey et al., 1985). Exposure of heterofermentative LAB to $O_2$ results in an altered redox state and greater NADH oxidase activities; as a consequence, sugar fermentation is shifted towards mixed fermentation and formate, lactate, acetate, acetoin, diacetyl, carbon dioxide, ethanol are produced. $H_2O_2$ is another main product of oxygen metabolism in LAB. Accumulation of $H_2O_2$ can be due to several factors such as inability to destroy $H_2O_2$, the presence of $H_2O_2$ -producing enzymes (such as pyruvate oxidase, $H_2O_2$-producing NADH oxidase, lactate oxidase and a-glycerophosphate oxidase) or both. Growth in the presence of ambient $O_2$ facilitates acetate formation and higher energy

conversion and two instead of one mole ATP is produced per mole glucose inverted (Cogan et al., 1989). The results are higher rates of glucose consumption and final growth yields. The main consequences of NADH oxidase overproduction are the total absence of lactate and regulation of NADH /NAD+ levels (Lopez de Felipe et al., 1997). Lopez de Felipe and Hugenholtz (1999) have shown that at increased dilution rates NADH oxidase was overproduced in a heterofermentative *L. lactis* strain, pyruvate flux was accommodated via pyruvate dehydrogenase and pyruvate was converted to acetoin and diacetyl via α-acetolactate. The end product formed from glucose depends on $O_2$ tension, sugar availability, pH and the absence or presence of specific enzymes (pyruvate oxidase, pyruvate dehydrogenase, acetolactate synthase, etc.) (Condon, 1987; Tseng and Montville, 1990). In Figure 1, metabolic pathways in which $O_2$ is actively involved in LAB are presented schematically.

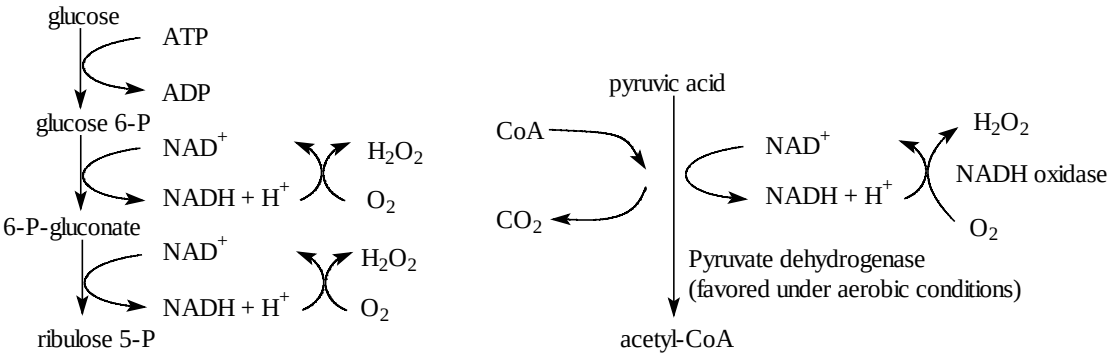


***Figure 1.*** *Metabolic pathways in heterofermentative Lactic acid bacteria in which $O_2$ is actively involved: (left) glucose metabolism (right) pyruvate metabolism*

### Mechanisms of high oxygen toxicity in LAB

The ability of LAB to tolerate high $O_2$ varies significantly between different genera. The results presented in Chapter 2 and 3 of this thesis have shown that the response to high $O_2$ can even differ significantly within a species, i.e. between strains of *L. sake* originating from different food products. Moreover, the same strain can exhibit different stress responses over time. In Figure 2, growth patterns recorded during exposure to high $O_2$ are shown of the oxygen-sensitive strain *L. sake* DSM 6333 and the oxygen-insensitive strain *L. sake* NCFB 2813, which were studied in detail in Chapter 3 and 4. Notably, *L. sake* DSM 6333 was able to recover after an initial log 4 reduction. A fraction of the population is somehow able to repair the radical damage when the rate of metabolism is slowed down. When switching to the exponential phase, both the anabolism and catabolism are no longer hampered. At this stage, due to the increasingly high metabolic activity, new radicals are formed and the stress-response cycle repeats itself. This “oscillatory” behaviour (or "suicide" response) has been reproduced several times and is typical for this strain. To confirm that the experimental results were not the consequence of contamination in the *L. sake* DSM 6333 culture, samples were taken at the initial maximum (A1) and the subsequent minimum (A2) of the population and processed for ribotype pattern determination. Identical ribotype patters were obtained for A1 and A2 (which were quite different from the patterns of two other, related strains tested), which confirms that contamination is not apparent. A possible explanation for the oscillatory behaviour is the uncoupling of growth and metabolism (Aldsworth et al., 1999; this thesis) due to a free radical burst. Recently, Dodd et al., (1997) suggested that upon exposure to a physical stress or to antimicrobial agents that cause sub-lethal injury to bacteria, an imbalance between anabolism and catabolism can develop which leads to generation of ROS.

The mechanism of high oxygen toxicity in *L. sake*, as proposed in this thesis, is significantly different from the mechanism proposed by Duwat et al., (1995a) for aerated cultures of *Lact. lactis*. Prolonged aeration of lactococcal cultures can lead to cell death and DNA degradation, mainly due to the

formation of extracellular radicals, whereas addition of exogenous catalase improved survival of aerated *Lact. lactis* at high temperatures. In contrast, addition of exogenous catalase did not affect growth and survival of *L. sake* DSM 6333 in any way (Chapter 4). More recently, Duwat et al., (2001) have shown that *Lact. lactis* can undergo respiratory growth if hemine is added to aerated cultures. *L. sake* can undergo respiratory growth even in the absence of hemine (A. Amanatidou, unpublished data). In both cases, under aerobic conditions, a switch can occur from homolactic to mixed-acid fermentation due to the low NADH/NAD+ ratios in cells growing under aerobic/high oxygen conditions. The ratio of lactate: acetate measured in *L. sake* DSM 6333 and *L. sake* NCFB 2813 under high oxygen conditions was 1:1 and 0.5:1.5, respectively (A. Amanatidou, unpublished data).

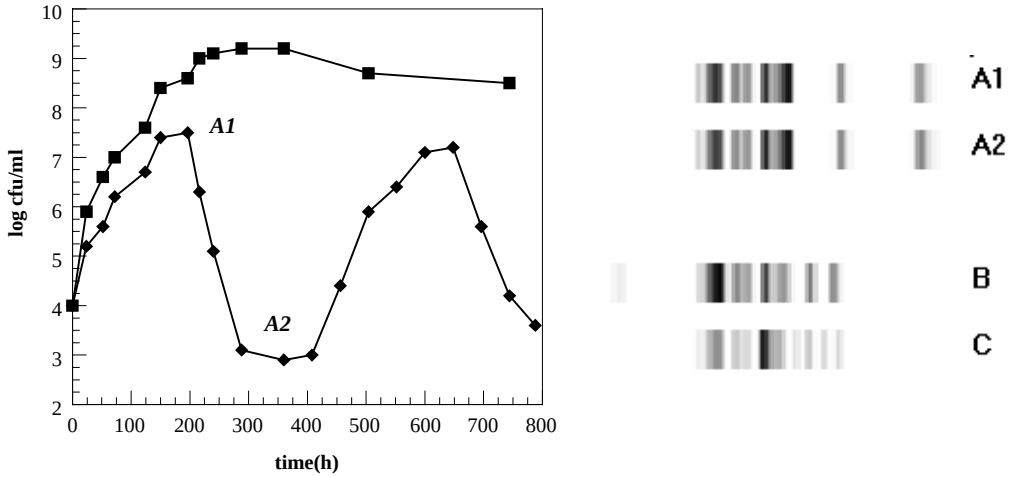


***Figure 2**. Left panel: growth patterns of* L. sake$^{ins}$ *(■) and* L. sake *DSM 6333 (♦) upon exposure to 90% $O_2$ + 10% $N_2$. Right panel: A1, A2 are ribotype patterns of a single colony bacterial population sampled at two different moments during growth; B and C are ribotype patterns of* L. sake *NCFB 2813 and L. sake OA1 (a* L. sake *type-strain)*

$O_2^-$ generation in *L. sake* occurs mainly in the cytosol rather than in the cytoplasmic membrane; intracellular sources of $O_2^-$ in *L. sake* are flavoproteins rather than respiratory enzymes, like in *E. coli* (Figure 2 and 3, Chapter 1). A model is proposed which describes a possible mechanism of oxygen-derived damage in *L. sake* and which may apply for other LAB (Figure 3). According to this model, NADH-oxidase is the main source of $O_2^-$ radical. At intermediate $O_2$ tensions, the role of NADH-oxidase in *L. sake* is to regulate the ratio of NADH/NAD+ and to reduce the level of $O_2$ toxic by-products. Under high $O_2$, when NADH-oxidase is exposed to high levels of radicals, its protective role is diminished. Chapters 3 as well as data from Messner and Imlay (1999) provide substantial evidence that complex redox moieties, mainly flavins, are potential electron donors for oxygen. Although the turnover number of autoxidised enzymes varies widely, it appears that most $O_2^-$ radicals may arise from a limited number of flavoenzymes. Conceivably, the abundant presence of NADH-oxidase in *L. sake* DSM 6333 (Chapter 4) is correlated to the formation of more $O_2^-$ and $H_2O_2$. The formation of oxygen reactive compounds in *L. sake* NCFB 2813 is restricted, because of the low level of activity of NADH: $H_2O_2$-oxidase.

Presumably, effective defence against ROS may require the balanced expression of general antioxidant enzymes, as demonstrated in the model. It cannot necessarily be achieved by an increase in the activity of a single enzyme such as NADH-oxidase or SOD. 2D-electrophoresis performed on cell-free extracts of *L. sake* DSM 6333 and *L. sake* NCFB 2813, from cells previously grown under high $O_2$, demonstrated that 7 proteins were explicitly expressed in *L. sake* DSM 6333 as compared to 15 proteins in *L. sake* NCFB 2813 (data not shown). In the case of *E. coli* and *Salm. typhimurium*, more than 30 proteins are involved in oxidative stress responses induced by exposure to low levels of $H_2O_2$, whereas 13 proteins can be induced upon exposure to $O_2^-$ generating conditions (Demple and

Halbrook, 1983; Walkup and Kogoma, 1989). Detailed characterisation of gene products required for $O_2^-$ and $H_2O_2$ survival is needed to better understand the mechanism of cellular defence towards elevated $O_2$ in LAB at the expression level.
Quite critical to cell physiology is whether autoxidation of flavoenzymes will generate either $O_2^-$ or/and $H_2O_2$, since these two oxidants have quite different impacts on cell fitness. When they are present in equimolar concentration, it seems that cells are more tolerant to $H_2O_2$ than to $O_2^-$. Exogenous addition of $H_2O_2$ at levels equivalent or higher to those excreted by cells in the exponential phase of growth under high $O_2$, was not sufficient to impose oxidative stress to *L. sake* DSM 6333. The observation that $H_2O_2$ is probably not a direct cause of oxidative damage does not exclude a role of this compound in other processes that generate certain toxic ROS, i.e. the hydroxyl radical HO·.

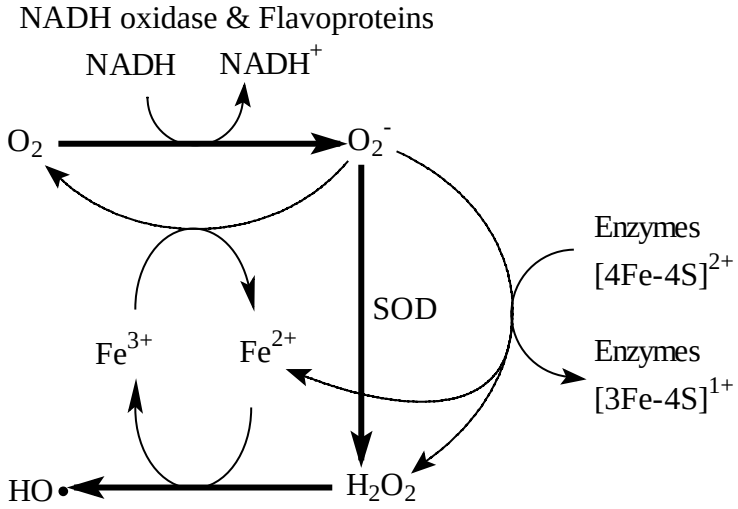


***Figure 3*** *Model to describe the process of cell damage by endogenous oxidants in* L. sake *upon exposure to elevated* $O_2$.

## 7.3 Elevated $O_2$ as an additional factor for food preservation

### Elevated $O_2$ as an inhibitor of microbial growth

The antimicrobial spectrum of oxygen is essentially limited to obligate anaerobes or some facultative anaerobic micro-organisms. Upon exposure to oxygen stress, those species that can overcome the inhibitory activity or consequences of the presence of oxygen have an advantage over those that cannot. The hypothesis investigated here is that high $O_2$ can be deployed as an additional preservation factor. High $O_2$ concentrations can enhance the inhibitory activity of other gases such as $CO_2$ on otherwise insensitive micro-organisms, as shown in Chapter 2. There it was found that most of the micro-organisms studied (i.e. *Pseudomonas fluorescens*, *Enterobacter agglomerans*, *Leuconostoc mesenteroides*, *Lactobacillus sake*) could tolerate $O_2$ (even up to 90%) or $CO_2$ (10 to 20%) *in vitro*, although growth was slowed down considerably. Studies by Jacxsens et al., (in press) have shown that certain strains of the facultative aerobe *Ps. fluorescens* are sensitive to high $O_2$; delayed growth or total growth inhibition were observed when high $O_2$ and $CO_2$ were used in combination. Although yeasts and moulds were not investigated in great detail in this thesis (except for two *Candida* spp.; Chapter 2), several recent and studies have shown that high $O_2$ levels alone and in combination with $CO_2$ result in lower growth of moulds compared to low or ambient $O_2$ concentrations (Wszelaki and Mitcham, 1999; Hoogerwerf et al., 2000).
While high $O_2$ levels apparently lead to intracellular generation of ROS, high $CO_2$ levels further disturb cellular homeostasis and membrane functions resulting in serious impairments that are

enhanced at low temperature. This approach confirms the concept of multitarget preservation, addressed by Leistner (2000), and confirmed previous observations from Gonzalez-Roncero and Day (1998). The concept could apply for instance to minimise microbial spoilage and prolong shelf-life of respiring products, such as fruits and vegetables, in which the respiration activity can rapidly deplete $O_2$, thus creating an atmosphere that supports growth of anaerobic pathogens. Application is feasible, if high $O_2$ does not have a detrimental effect on taste, colour or organoleptic properties.

The effects of the combined treatments on the natural flora of two minimally processed carrots and salmon were studied in Chapter 5 and 6. The complex microbial interactions on minimally processed carrots were a direct consequence of the levels of both gases applied. High $O_2$ delayed the development of spoilage symptoms by retarding growth of lactic acid bacteria and growth of pseudomonads, was significantly affected by high $CO_2$. The effect was enhanced at lower temperatures up to 4$^o$C (A. Amanatidou, unpublished data). The effects of high $O_2$ and $CO_2$ were further enhanced when used in combination with high hydrostatic pressure. The study described in Chapter 6 is actually the first published study describing the combined effect of supercritical $O_2$, liquid $CO_2$ and high pressure on the natural microbial flora of a food product. Even in that case, growth was enhanced at the low temperature used for treatment and subsequently storage of the product (5$^o$C). The lethal effects of high pressure gases such as $CO_2$, $N_2$, $N_2O$ or Ar on various micro-organisms are well documented (Nakamura et al., 1994; Kumagai et al., 1997; Shimoda et al., 1998). Application of supercritical $O_2$ has not been considered for food preservation previously, due to safety and technological reasons. The effectiveness of the treatment has been studied at temperatures above ambient (>40$^o$C). Enomoto et al., (1997) have demonstrated that upon explosive decompression, gas absorption by the microbial cells is increased especially when gases that are relative soluble in water are used. The method of explosive decompression has been used in the study presented in Chapter 6. This study is restricted in reporting the results of the application of high pressure $O_2$ and $CO_2$ at chill temperature, however the mechanism of inactivation is of outmost interest and should be clarified in the future. In conclusion, compressed gases enhanced bacterial killing at relatively low pressure, which might make their application in practice both feasible and attractive. Compressed gases are an example of mild treatment according to the concept of hurdle technology as the combined application enhances antimicrobial activity of the otherwise not inhibiting factors, due to a synergistic and occasionally additive effect.

### Effect of elevated oxygen on the quality of fresh products

Possible advantages effects of a combination treatment on the original/desirable physico-chemical and organoleptic characteristics of a product needs to be carefully considered when designing a combined preservation treatment. Considering a preservation concept with high $O_2$ - high $CO_2$ packaging at refrigeration storage temperature for perishable or processed products, the oxygen and carbon dioxide tolerance as well as the cold tolerance of the product at hand should be taken into account. In this thesis, it was observed that respiring products such as carrot slices do not tolerate $O_2$ concentrations higher than 70% due to noticeable detrimental effect on certain physicochemical characteristics such as colour and taste. It was found that 50% $O_2$ had a minimal detrimental effect (in cases even a positive effect) on colour and significantly reduced exudation; additionally, under 50% $O_2$, an increase in the tolerance of sliced carrots to levels of $CO_2$ (30%) was aparent. Such high concentrations of $CO_2$ would otherwise have a negative impact on organoleptic characteristics (e.g., acidity due to LAB growth). Jacxsens et al., (2000; in press) reported that the responses of several types of products to high $O_2$ is variable. Grated celery and carrots (root tissue) and shredded chicory endive (leafy tissues) appeared to be less sensitive to increased $O_2$ than sliced mushrooms. Enzymatic discoloration observed in some respiring food products (iceberg lettuce, oranges, lettuce) is reduced upon high $O_2$ storage (Heimdal et al., 1995; Day, 2000). In their recent review, also Kader and Shimshon (2000) noted the highly variable effects (stimulatory, neutral or inhibitory) of exposure to “superatmospheric” $O_2$ concentrations on the rate of respiration and ethylene production of a product. The effects were

depended on a range of factors, such as the commodity maturity and ripeness stage, the concentrations of $O_2$, $CO_2$ and ethylene in the atmosphere as well as the storage time and temperature. It seems that respiring products with a relatively low respiration rate and sufficient levels of antioxidants in their tissues would be among the minimally processed foods that are most suitable to apply the technology of high $O_2$/high $CO_2$ modified atmosphere packaging with.
Similar observations were made in the case of salmon treated with compressed gases. Whereas microbial spoilage was certainly retarded, enhanced lipid oxidation (related to the high fat content of salmon) and a rapid loss of the red colour indicated that application of the compressed gases was not favourable for product quality of salmon. Compressed gas technology is considered a simple, safe and inexpensive technique for treating heat sensitive materials. Supercritical fluids such as $CO_2$ have relatively high diffusion coefficients, favourable mass transfer properties and are non-toxic; thus, they do not give residual problems. They can be used in food processing, for instance to improve technological processes such as microencapsulation, particle formation, extraction of high additive compounds, etc. (Sihvonen et al., 1999). Supercritical $CO_2$ has been used to inactivate enzymes such as acid proteases, glucoamylases and lipases and has been used to improve the quality of animal products such as dried egg yolk and butter (Ishikawa et al., 1995). On the other hand, technological advantages of high pressure treatment of vacuum-packed products are the preservation or improvement of product structure, protection of colour and nutrients and minimal changes in taste (Cheftel, 1992; Knorr, 1999). As shown in this thesis, the combined high pressure and supercritical gases treatment had an interesting effect on texture, which could be used to render specific, desired physicochemical characteristics and enhance functionality of novel food products. The technology could be applied for processing of added-value products such as high value additives, functional foods and neutraceuticals, in which the natural and solvent free preparation mode may add to their market value.

## 7.4 Future prospects for food application

Application of high $O_2$ can be justified in those cases where the effect on microbial stability is beneficial and the effect on quality characteristics is favourable. High $O_2$, in combination with $CO_2$ and refrigerated storage, has up to now been used specifically for the preservation of red meats as it enhances colour retention. The work in this thesis indicates that there certainly are other food products for which high $O_2$/high $CO_2$ modified atmosphere packaging in combination with other technologies (chilling; hydrostatic pressure) offers quality. Benefits with regard to microbiological spoilage and product safety are the increased effect of antimicrobial compounds under high $O_2$ tensions. In addition there is very little chance for anaerobiosis to occur (thus very little risk of growth of highly hazardous anaerobic pathogens)
The following aspects should receive due attention when the successful application of the high $O_2$/high $CO_2$ modified atmosphere concept should be pursued further:

1. The spectrum of products for which this concept could be beneficial needs to be better defined. Some of the main characteristics are known but it would be helpful to make lists of products available for which high $O_2$ should be avoided or for which it would offer potential benefits. Suitable products would be those that are not very vulnerable to biochemical deterioration or those suffer a lot of water exudation. Products with, for instance, high fat contents are quite vulnerable to biochemical deterioration and would thus not be suitable. The concept thus would be better for lean fish such as sole, different type of shells and perch. Other minimally processed products for which high oxygen storage could have a beneficial effect are mushrooms or peeled and blanched potatoes.
2. The bacteriostatic or bactericidal effect of high $O_2$ depends on several environmental factors and further insight on the physiological and molecular level, of the mechanisms underlying the inhibition of micro-organisms by the combined treatments investigated in this thesis, should

provide. Better qualitative understanding might (in the future) enable quantification and prediction of the preservation effect; this would certainly help in the design of combination treatments and would improve the robustness of the concept in practice.

3. The work in this thesis has indicated that application of high $O_2$ may minimise the risk posed by the growth of hazardous anaerobic (or microaerophilic) pathogenic micro-organisms. However, it is not yet known whether in the case of high $O_2$ combination treatments, the phenomena of cross-tolerance and stress hardening in response to one or more of the treatments can become an issue. With certain treatments (e.g. acidification; mild heating), it is well known that sub-lethal exposure may build up resistance in the surviving microbial population to the same treatment ("stress-hardening") or even to different treatments ("cross-tolerance"). Both phenomena are related to the induction of protective factors and should be investigated in more detail.
4. Next to the efficacy of high $O_2$ combination treatments with respect to safety and stability of the product, the upscaling and safe deployment of the technology in practice needs to be assured before implementation in practice can be approved. A possible occupational safety issue is associated with the use of high concentrations of $O_2$ in a processing line, where there is a risk for fires or explosions to occur. Thus, for the benefit of large-scale application there need to be specific requirement with respect to the machinery and operational procedures that ensure the safety of the workforce. Some experience has been gained in the meat industry (where high $O_2$ packaging lines have been operational in several factories for many years) and in a few fruit processing factories (which have experimented with high $O_2$ packaging). The feasibility of tailoring the technology used in existing high $O_2$ packaging lines to application with respiring produce has been proven by Campden and Chorleywood Research Association (Day, 2001).

## References


**Abdel-Rahman, M. & Isenberg, F.M.R.** (1974) Effects of growth regulators and controlled atmospheres storage on stored carrots. *Journal of Agricultural Science*, 82: 245-249

**Aharoni, Y. & Houck, L.G.** (1982) Change in rind, flesh and juice color of blood oranges stored in air supplemented with ethylene or in oxygen enriched atmospheres. *Journal of Food Science*, 47: 2091-2092

**Ahvenainen, R.** (1996) New approaches in improving the shelf-life of minimally processed fruit and vegetables. *Trends in Food Science and Technology*, 7: 179-187

**Aldsworth, T.G., Sharman, R.L. & Dodd, C.E.R.** (1999) Bacterial suicide through stress. *Cellular and Molecular Life Sciences*, 56: 378-383

**Alzamora, S.M., Tapia, M.S. & Welti-Chanes, J.** (1998) New strategies for minimal processing of foods: the role of multitarget preservation. *Food Science & Technology International*, 4: 353-361

**Alzamora, S.M., Lopez-Mallo, A. & Tapia, M.S.** (2000) Overview In: *Minimally processed fruits and vegetables. Fundamental aspects and applications*. (ed. Alzamora, S.M., Lopez-Mallo, A. & Tapia M.S.), pp. 1-9, Aspen Publications Gaithersburg, Maryland, 2000

**Amanatidou A., Smid, E.J. & Gorris, L.G.M.** (1999) Effect of elevated oxygen and carbon dioxide on the surface growth of vegetable associated micro-organisms. *Journal of Applied Microbiology*, 86: 429-438

**Amanatidou, A., Slump, R.A., Gorris, L.G.M. & Smid, E.J.** (2000a) High oxygen and high carbon dioxide modified atmospheres for shelf-life extension of minimally processed carrots. *Journal of Food Science*, 65: 61-66

**Amanatidou, A., Schlüter, O., Lemkau, K., Gorris L.G.M., Smid, E.J. & Knorr, D.** (2000b) Effect of combined application of high pressure treatment and modified atmospheres on the shelf-life of fresh atlantic salmon. *Innovative Food Science & Emerging Technologies*, 1: 87-98

**Amanatidou, A., M.H.J. Bennik, Gorris, L.G.M. & Smid, E.J.** (2001) Superoxide dismutase plays an important role in the survival of *Lactobacillus sake* upon exposure to elevated oxygen. *Archives in Microbiology*, 176: 79-88

**Anders, R.F., Hogg, D.M. & Jago, G.R.** (1970) Formation of hydrogen peroxide by group N streptococci and its effect on their growth and metabolism. *Applied Microbiology*, 19: 608-612

**Andersson, R.** (1984) Characteristics of the bacterial flora isolated during spontaneous fermentation of carrots and red beets. *Lebensmittel Wissenschaft & Technologie*, 17: 282-286.

**Angelis de, M. & Gobbetti, M.** (1999) *Lactobacillus sanfranciscensis* CB1: manganese, oxygen, superoxide dismutase and metabolism. *Applied Microbiology & Biotechnology*, 51: 358-363

**Angupanich, K. & Ledward, D.A.** (1998) High pressure treatment effects on cod (*Gadus morhua*) muscle. *Food Chemistry*, 63: 39-50

**Anonymous** (1994) VDI-Wärmeatlas, 7. Auflage, VDI-Verlag GmbH, Düsseldorf, Germany

**Archibald, F.S. & Fridovich, I**. (1981a) Manganese and defences against oxygen toxicity in *Lactobacillus plantarum. Journal of Bacteriology*, 145: 442-451

**Archibald, F.S. & Fridovich, I.** (1981b) Manganese superoxide dismutase and oxygen tolerance in some lactic acid bacteria. *Journal of Bacteriology*, 146: 928-936

**Archibald, F.S. & Fridovich, I.** (1982) The scavenging of superoxide radical by manganous complexes in vitro. *Archives in Biochemistry & Biophysics*, 214: 452-563

**Aruoma, O.I.** (1997) Free Radicals: Diaitary advantages and disadvantages. In: *Oxidative stress and the molecular biology of antioxidant defences* (ed. Scandalios, J.G.) Cold Spring Harbor Laboratory Press pp. 841-860

**Axelsson, L.T**. (1993) Lactic acid bacteria: Classification and Physiology. In: *Lactic acid bacteria*, (ed. Salminen, S. & von Wright, A.) Marcel Dekker, Inc. New York

**Babic, I., Amiot, M.J. & Nguyen-The, C.** (1993) Changes in phenolic content in fresh ready-to-use shredded carrots during storage. *Acta Horticulturae*, 343: 123-128.

**Ballestra, P., Abreu da Silva, A. & Cuq, J.L.** (1996) Inactivation of *Escherichia coli* by carbon dioxide under pressure. *Journal of Food Science*, 61: 829-831,836.

**Barakat, R.K. & Harris, L.J.** (1999) Growth of *Listeria monocytogenes* and *Yersinia enterocolitica* on cooked modified atmospheres packaged poultry in the presence and absence of a naturally occurring microbiota. *Applied & Environmental Microbiology*, 65: 342-345

**Baranyi, J., Roberts, T.A. & McClure, P.** (1993) A non-autonomous differential equation to model bacterial growth. *Food Microbiology*, 10: 43-59

**Barnett, H.J., Stone, F.E., Roberts, G.C., Hunter, P.J., Nelson, R.W. & Kwok, J.** (1982) A study in the use of high concentration of $CO_2$ in a modified atmosphere to preserve salmon. *Marine Fishery Reviews*, 44: 3-28

**Baross, J.A. & Morita, R.Y.** (1978) Microbial life at low temperatures: ecological aspects. In: *Perspectives in Microbial Ecology* (ed. Megusar, F. & Gantar, M.). Microbial Society for Microbiology, Ljubljana pp. 185-190

**Barriga, M.I., Trachy, G., Willlemot C. & Simard, R.E.** (1991) Microbial changes in shredded Iceberg lettuce stored at controlled atmospheres. *Journal of Food Science*, 56: 1586-1588, 1599

**Barry-Ryan, C. & O'Beirne, D.** (1998) Quality and shelf-life of fresh cut carrot slices as affected by slicing method. *Journal of Food Science*, 63: 851-856.

**Bast, A., Haenev, G.R.M.M. & Doelman, C.J.A.** (1991) Oxidants and antioxidants: A state of art. *American Journal of medicine*, 91: 2S-13S

**Bennik, M.H.J., Smid, E.J., Rombouts, F.M. & Gorris, L.G.M.** (1995) Growth of psychrotrophic foodborne pathogens in solid surface model system under the influence of carbon dioxide and oxygen. *Food Microbiology*, 12: 509-519

**Bennik, M.H.J., Peppelenbos, H.J., Nguyen-The, C., Carlin, F., Smid, E.J. & Gorris, L.G.M.** (1996) Microbiology of minimally processed, modified atmosphere packaged chicory endive. *Postharvest Biology & Technology*, 9: 209-221

**Bennik, M.H.J., Vorstman, W., Smid, E.J. & Gorris, L.G.M**. (1998) The influence of oxygen and carbon dioxide on the growth of prevalent *Enterobacteriaceae* and *Pseudomonas* species isolated from fresh and modified atmosphere stored vegetables. *Food Microbiology*, 15: 459-469.

**Berrang, M.E., Brackett, R. E. & Beuchat, L.R.** (1989) Growth of *Listeria monocytogenes* on fresh vegetables stored at controlled atmosphere. *Journal of Food Protection*, 52: 702-705

**Bert, P.** (1878) Barometric pressure. Researches in experimental physiology. Paris. College Book Co, Columbus, OH translated by Hitchcock M.A., & Hitchcock F.A., 1943

**Beumer, R.R., te Giffel, M.C., Cox, L.J., Rombouts F.M. & Abee, T.** (1994) Effect of exogenous proline, betaine, and carnitine on growth of *Listeria monocytogenes* in a minimal medium. *Applied & Environmental Microbiology*, 60: 1359-1363

**Blackburn, C. & Davies A.R.** (1994) Effects of preconditioning on pathogenic bacteria in foods. In: *Food Technology International Europe* (ed. Turner, A.) Sterling Publ. Ltd. London, pp 37-40

**Bolin, H.R. & Huxsoll, C.C.** (1991) Control of minimally processed carrot (*Daucus carota*) surface discoloration caused by abrasion peeling. *Journal of Food Science*, 56: 416-418.

**Boveris, A.** (1984) Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. *Methods in Enzymology*, 105: 429-435

**Brackett, R.E.** (1994) Microbiological spoilage and pathogens in minimally processed refrigerated fruits and vegetables. In: *Minimally processed refrigerated fruits and vegetables* (ed. Wiley, R.C.) Chapman and Hall, New York, pp. 269-312

**Britigan, B.E., Ratcliffe, H.R., Buettner, G.R. & Rosen, G.M.** (1996) Binding of myeloperoxidase to bacteria: effect on hydroxyl radical formation and susceptibility to oxidant-mediated killing. *Biochimica Biophysica Acta*, 1290: 231-40.

**Brocklehurst, T.F., Zaman-Wong C.M. & Lund B.M.** (1987) A note on the microbiology of retail packs of prepared salad vegetables. *Journal of Appied Bacteriology*, 63: 409-415.

**Brown, C.M. & Johnson B.** (1971) Influence of oxygen tension on the physiology of *Saccharomyces cerevisiae* in continues cultures. *Antonie van Leeuvenhoek*, 37: 477-487

**Brown, W.D., Albright, M., Watts, D.A. Heyers, B., Spruce, B. & Price, R.J.** (1980) Modified atmosphere storage of rockfish (*Sebastes miniatur*) and silver salmon (*Oncorhynchus kisutch*). *Journal of Food Science*, 45: 93-98

**Bruemmer, J.H.** (1988) Quality changes of carrot sticks in storage. *Proceedings of the Florida State Horticultural Society*, 101: 207-210

**Byeong, S.K. & Klieber, A.** (1997) Quality maintenance of minimally processed Chinese cabbage with low temperature and citric acid dip. *Journal of Science Food Agriculture*, 75: 31-36

**Camell, C., Vaz-Pires, P. & Kirby, R.** (1997) Use of counts of hydrogen sulphide producing bacteria to estimate remaining shelf-life of fresh fish. In: *Methods to determine the freshness of fish in Research and Industry*. Proceeding of the Conference "Evalution of fish freshness AIR3CT94 2283" (ed. International Institute of Refrigeration)

**Cantoni, O., Brandi, G., Schiavano, G.F., Albano, A. & Cattabeni, F.** (1989) Lethality of $H_2O_2$ in wild type and SOD-mutants of *Escherichia coli*. *Chemical Biological Interactions*, 70: 281-288

**Carlin, F., Nguyen-The, C., Cudennec, P. & Reich, M.** (1989) Microbiological spoilage of fresh, ready-to-use grated carrots. *Science des Aliments*, 9: 371-386

**Carlin, F., Nguyen-The, C. Hilbert, G. & Chambroy, Y.** (1990a) Modified atmosphere packaging of fresh, "ready-to-use" grated carrots in polymeric films. *Journal of Food Science* 55: 1033-1038

**Carlin, F., Nguyen-The, C. Chambroy, Y. & Reich, M.** (1990b) Effects of controlled atmospheres on microbial spoilage, electrolyte leakage and sugar content of fresh 'ready-to-use' carrots. *International Journal of Food Science and Technology*, 25: 110-119

**Carlin, F., Nguyen-The, C., Abreu-da-Silva, A. & Cochet, C.** (1996) Effects of carbon dioxide on the fate of *Listeria monocytogenes*, of aerobic bacteria and on the development of spoilage in minimally processed fresh endive. *International Journal of Food Microbiology*, 32: 159-172

**Cecchini G., Sices, H., Schroder, I. & Gunsalus, R.** (1995) Aerobic inactivation of fumarate reductase from *Escherichia coli* by mutation of the [3Fe-4S]-Quinone binding domain. *Journal of Bacteriology*, 177: 4587-4592

**Chan, H.S.S**. (1987) Oxygen free radicals in food. *Proceedings of the Nutrition Society*, 46: 35-41

**Chang, S., Ostric-Matijasevic, B., Hsieh, O.A.L. & Huang, C.** (1977) Natural antioxidants from rosemary and sage. *Journal of Food Science*, 42: 1102-1106

**Chang, S.K., Hassan, H.M.** (1997) Characterization of superoxide dismutase in *Streptococcus thermophilus. Applied & Environmental Microbiology* 63: 3732-3735

**Cheah, P.B. & Ledward, D.A.** (1997) Catalytic mechanism of lipid oxidation following high pressure treatment in pork fat and meat. *Journal of Food Science*, 62: 1135-1138

**Cheftel, J.C.** (1992) Effects of high hydrostatic pressure on food macromolecules: an overview. In: *High Pressure and Biotechnology* (ed. Hayashi, R., Heremans, K. & Masson, P.) Colloque INSERM John Libbey Eurotext Ltd., vol. 224, p.195-197

**Cheftel, J.C.** (1995) High pressure, microbial inactivation and food preservation. *Food Science and Technology International*, 1: 75-87

**Chirife, J.** (1993) Physicochemical aspects of food preservation by combined factors. *Food Control*, 4: 210-215

**Chubey, B.B. & Nylund, R.E.** (1969) Surface browning of carrots. *Canadian Journal of Plant Science*, 49: 421-426

**Church, P.N.** (1993) Meat and meat products In: *Principles and Applications of MA packaging of food* (ed. Parry, R.T.), Blackie Academic & Profesional, Glasgow, pp. 170-187

**Church, I.J. & Parsons, A.L.** (1994) Modified atmosphere packaging technology: A review. *Journal of the Science of Food and Agriculture*, 67: 143-148

**Cisneros-Zevallos, L., Saltveit, M.E. & Krochta, J.M.** (1995) Mechanism of surface white discoloration of peeled (minimally processed) carrots during storage. *Journal of Food Science*, 60: 320-323, 333

**Cisneros-Zevallos, L., Saltveit, M.E. & Krochta, J.M.** (1997) Hygroscopic coatings control surface white discoloration of peeled (minimally processed) carrots during storage. *Journal of Food Science*, 62: 363-366, 398

**Claiborne, A., Ross, R.P. & Parsonage, D.** (1993) Flavin linked peroxide reductases: protein sulfenic acids and the oxidative stress response. *Trends in Biochemical Sciences*, 17: 183-186

**Cogan, J.F., Walsh, D. & Condon, S.** (1989) Impact of aeration on the metabolic end products formed from glucose and galactose by *Streptococcus lactic. Journal of Applied Bacteriology*, 66: 77-84

**Collins, E.B. & Aramaki, K.** (1980) Production of hydrogen peroxide *by Lactobacillus acidophilus*. *Journal of Dairy Science*, 63: 353-357

**Condon, S.** (1987) Responses of lactic acid bacteria to oxygen. *FEMS Microbiological Reviews*, 46: 269-280

**Cross, A.R. & Jones, O.T.G.** (1991) Enzymatic mechanism of superoxide production *Biochimica and Biophysica Acta*, 1057: 281-298

**Daniels, J.A., Krishnamurthi, R. & Rizvi, S.S.H.** (1985) A review of effects of carbon dioxide on microbial growth and food quality. *Journal of Food Protection*, 48: 532-537

**Davies, K.J.A. & Lin, S.W.** (1988) Degradation of oxidatively denatured proteins in *Escherichia coli*. Free Radical in Biology and Medicine, 5: 215-224

**Davies, K.J.A** (1995) Oxidative stress: the paradox of aerobic life. *Biochemistry*, Society Symposium, 61: 1-31

**Day, B.P.F** (1992) Guidelines for the good manufacturing and handling of modified atmopshere packed food products. Technical Manual, Campden Food and Drink Research Association 34, 79pp.

**Day, B.P.F** (1996a) High oxygen modified atmosphere packaging for fresh prepared produce. *Postharvest News and Information* Conference Proceedings, 5: 31N-34N

**Day, B.P.F** (1996b) Novel MAP for fresh prepared produce. *The European Food and Drink Review*, Spring, 73-80

**Day, B.P.F** (2000) Novel MAP for freshly prepared fruit and vegetable products, *Postharvest News & Information.* 11: 27-31

**Day, B.P.F** (2001) Fresh prepared produce: GMP for high oxygen MAP and non-sulphite dipping. Guideline No. 31 Campden & Chorleywood Food Research Association Group, Chipping Campden, Gloshister, pp. 76

**Debs-Louka, E., Louka, N., Abraham, G., Chabot, V., & Allaf, K.** (1999) Effect of compressed $CO_2$ on microbial cell viability. *Applied & Environmental Microbiology*, 62: 626-631

**Decker, E.A.** (1998) Strategies for manipulating the prooxidative/antioxidative balance of foods to maximize oxidative stability. *Trends in Food Science & Technology* 9: 241-248

**Declerck, D.** (1976) Organoleptic, chemical and microbiological aspects of vacuum packed and unpacked smoked pink salmon. Mededeling van het Rijksstation voor Zeevisserij (CLO Gent), Publicatie no. 113-VB/VV (IWONL) 18: 1-9

**Demple, B. & Halbrook, J.** (1983) Inducible repair of oxidative DNA damage in *Escherichia coli*. *Nature*, 304: 446-448

**Demple, B.** (1991) Regulation of bacterial oxidative stress genes. *Annual Review in Genetics*, 25: 315-37

**Deng, J.C., Matthews, R.F. & Watson, C.M.** (1977) Effect of chemical and physical treatments on rancidity development of frozen mullet (*Mugil cephalus*) filles. *Journal of Food Science*, 42: 344

**Dixon, N.M. & Kell, D.B.** (1989) The inhibition by $CO_2$ of the growth and metabolism of micro-organisms. *Journal of Applied Bacteriology*, 67: 109-136

**Dodd, C.E.R., Sharman, R.L., Bloomfield, S.F., Booth, I.R. & Stewart, G.S.A.B.** (1997) Inimical processes: bacterial self-destruction and sub-lethal injury. *Trends in Food Science and Technology*, 8: 238-241

**Doyle, M.P.** (1983) Effect of carbon dioxide on toxin production by *Clostridium botulinum. European Journal of Applied Microbiology & Biotechnology*, 17: 53-56

**Duwat, P., Ehrlich, D.S. & Gruss A.** (1995a) The recA gene of *Lactococcus lactis:* characterization and involvement in oxidative and thermal stress. *Molecular Microbiology* (Reading), 17: 1121-1131

**Duwat, P., De-Oliveira, R., Ehrlich, S.D. & Boiteux, S**. (1995b) Repair of oxidative DNA damage in Gram-positive bacteria: The *Lactococcus lactis* Fpg protein. *Microbiology* (Reading), 141: 411-417

**Duwat, P., Sourice, S., Cesselin, B., Lamberet, G., Vido, K., Gaudu, P., le Loir, Y., Violoet, F., Loubiere, P. & Gruss, A.** (2001) Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. *Journal of Bacteriology,* 183: 4509-4516

**Eisenstark, A., Calcutt, M.J., Becker-Hapak, M. & Ivanova, A.** (1996) Role of *Escherichia coli* rpoS and associated genes in defence against oxidative damage. *Free Radicals in Biology and Medicine,* 21: 975-93

**Eklund, T. & Jarmund, T.** (1983) Microcultural studies on the effect of several gas atmospheres on microbial growth at different temperatures. *Journal of Applied Bacteriology*, 55: 119-125

**Eklund, T.** (1984) The effect of $CO_2$ on bacterial growth and on uptake processes in bacterial membrane vehicles. *International Journal of Food Microbiology,* 1: 179-185

**El-Goorani, A. & Sommer, N.F.** (1981) Effects of modified atmospheres on postharvest pathogens of fruits and vegetables. *Horticultural Reviews,* 11: 412-461

**Enfors, S.O. & Molin, G.** (1980) Effect of high concentrations of $CO_2$ on growth rate of *Pseudomonas fragi*, *Bacillus cereus* and *Streptococcus cremoris. Journal of Applied Bacteriology,* 48: 409-416

**Engesser D.M. & Hammes, W.P.** (1994) Non-Heme catalase activity of lactic acid bacteria. *Systematic & Applied Microbiology,* 17: 11-19

**Enomoto, A., Nakamura, K., Nagai, K., Hashimoto, T. & Hakoda, M.** (1997) Inactivation of food micro-organisms by high-pressure carbon dioxide treatment with or without explosive decompression. *Bioscience, Biotechnology & Biochemistry,* 61: 1133-1137

**Eyles, M.J., Moir, C.J. & Davay, J.A.** (1993) The effect of modified atmospheres on the growth of psychrotrophic pseudomonas on a surface in a model system. *International Journal of Food Microbiology,* 20: 97-10

**Eytan, O., Weinert, I.A.G. & McGill, A.E.J.** (1992) Effect of salad dressing and citric acid dip on storage quality of shredded cabbage and carrots packed under modified atmosphere. *Lebensmittel Wissenschaft & Technologie*, 25: 445-450

**Fain, A.R.** (1996) A review of the microbiological safety of fresh salads. *Dairy Food and Environmental Sanitation,* 16: 146-149

**Farber, J.M.** (1991a) Microbiological aspects of modified atmosphere packaging technology-a review. *Journal of Food Protection,* 54: 58-70

**Farber, J.M.** (1991b) *Listeria monocytogenes* in fish products. *Journal of Food Protection,* 54: 922-924, 934

**Farr, S. B. & Kogoma, T.** (1991) Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiological Reviews,* 55: 561-585

**Fey, M.S. & Regenstein, J.M.** (1982) Extending shelf-life of fresh wet red hake and salmon using $CO_2$-$O_2$ modified atmosphere and potassium sorbate ice at 1°C. *Journal of Food Science,* 47: 1048-1054

**Fisher, C.W., Lee, D., Dodge, B.A., Hamman, K.M., Robbins, J.B. & Martin., S.E**. (2000) Influence of catalase and superoxide dismutase on ozone inactivation of *Listeria monocytogenes*. *Applied & Environmental Microbiology*, 66: 1405-1409

**Flint, D.H., Tuminello, J.F. & Emptage, M.H.** (1993a) The inactivation of Fe-S cluster containing hydro-lyases by superoxide. *Journal of Biological Chemistry,* 268: 22369-22376

**Flint, D.H., Smyk-Randall, E., Tuminello, J.F., Draczynska-Lusiak, B. & Brown, O.R.** (1993b) The inactivation of dihydroxy-acid dehydratase in *Escherichia coli* treated with hyperbaric oxygen occurs because of the destruction of its Fe-S cluster, but the enzyme remains in the cell in a form that can be reactivated. *Journal of Biological Chemistry,* 268: 25547-25552

**Food and Drug Administration** (1997) Irradiation in the Production, Processing and Handling of Food, Final rule 21, CFR Part 179, Vol. 62, No. 232 pp. 64107-64121

**Foster, J.W. & Spector, M.P.** (1995) How *Salmonella* survive against the odds. *Annual Review in Microbiology,* 49: 145-74

**Francis G.A. & O'Beirne, D.** (1997) Effect of gas atmosphere, antimicrobial dip and temperature on the fate of *Listeria innocua* and *Listeria monocytogenes* on minimally processed lettuce. *International Journal of Food Science and Technology*, 32: 141-151

**Fridovich, I.** (1985) Handbook of Methods of Oxygen Radical Research (ed. Greenwald, R.A.) CRC Press, Boca Raton, pp. 51-53

**Fridovich, I.** (1986) Biological effects of the superoxide radical. *Archives in Biochemistry and Biophysics*, 247: 1-11

**Fridovich, I.** (1999) Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? *Annual New York Academy of Sciences*, 893: 13-8.

**Gardner, P.R. & Fridovich, I.** (1991) Superoxide sensitivity of the *Escherichia coli* aconitase. *Journal of Biological Chemistry*, 266: 19328-19333.

**Gardner, P.R., Nguyen, D.D. & White, C.W.** (1994) Aconitase is a sensitive and critical target of oxygen poisoning in cultured mammalian cells and in rat lungs. *Proceedings National Academy of Science* USA, 91: 12248-12252.

**Gardner, P.R., Raineri, I., Epstein, L.B. & White, CW** (1995) Superoxide radical and iron modulate aconitase activity in mammalian cells. *Journal of Biological Chemistry*, 270: 13399-405.

**Gaudu, P., Touati, D., Niviere, V. & Fontecave, M.** (1994) The NAD(P)H:flavin oxidoreductase from *Escherichia coli* as a source of superoxide radicals. *Journal of Biological Chemistry*, 269: 8182-8188

**George, S.M., Richardson, L.C.C, Pol, I.E. & Peck, M.W.** (1998) Effect of oxygen concentration and redox potential on recovery of sublethally heat-damaged cells of *Escherichia coli* O157:H7, *Salmonella enteritidis* and *Listeria monocytogenes. Journal of Applied Microbiology*, 27: 313-317

**Gerschman, R., Gilbert, D.L., Nye, S.W., Dwyer, P. & Fenn, W.O.** (1954) Oxygen poisoning and X-irradiation: A mechanism in common sense. *Science*, 119: 623-629

**Gibson, C.M., Mallett, T.C., Claiborne, A. & Caparon, M.G.** (2000) Contribution of NADH oxidase to aerobic metabolism of *Streptococcus pyogenes*. *Journal of Bacteriology*, 182: 448-455

**Giese, J. (1996)** Antioxidants: Tools for preventing lipid oxidation. *Food Technology*, 50: 73-78**:** 80

**Gill, C.O. & Tan, K.H.** (1979) Effect of carbon dioxide on growth of *Pseudomonas fluorescens*. *Applied & Environmental Microbiology*, 38: 237-240

**Gill, C.O. & Tan, K.H.** (1980) Effect of carbon dioxide on growth of meat spoilage bacteria. *Applied & Environmental Microbiology*, 39, 317-319

**Gill, C.O. & Molin, G.** (1991) Modified atmospheres and vacuum packaging. In: *Food Preservatives* (ed. Russell, N.J. & Gould, G.W.) Blackie Academic & Profesional, Glasgow, pp. 172-199

**Gille, G & Sigler, K.** (1995) Oxidative stress and living cells. *Folia Microbiologia*, 40: 131-152

**Gonzalez, S.N., Apella, M.C., Romero, N., Pesce-de-Ruiz-Holgado, A.A. & Oliver, G.** (1989) SOD activity in some strains of lactobacilli: induction by manganese. *Chemical Pharmaceutical Bulletin* 37: 3026-3028

**Gonzalez, S.N., Nadra-Chaud, C.A., Apella, M.C., Strasser de Saad, A.M. & Oliver G.** (1991) Evidence of superoxide dismutase in *Lactobacillus acidophilus. Chemical Pharmaceutical Bulletin*, 39: 1065-1067

**Gonzalez-Flecha, B. & Demple, B.** (1997) Homeostatic regulation of intracellular hydrogen peroxide in aerobically growing *Escherichia coli*. *Journal of Bacteriology*, 179: 382-388

**Gonzelez Roncero, G. & Day, B.P.F.** (1998) The effects of novel MAP on fresh prepared produce microbial growth. In: Proceedings of the COST 915 Conference, Ciudad Universitaria, Madrid, Spain, 15-16 October

**Gorris, L.G.M. & Peppelenbos, H.W.** (1992) Modified atmosphere and vacuum packaging to extend the shelf-life of respiring produce. *HortTechnology*, 2: 303-315

**Gorris, L.G.M. & Peck, M.W.** (1998) Microbiological safety considerations when using hurdle technology with refrigerated processed foods of extended durability. In: *Sous Vide and Cook Chill Processing for the food Industry* (ed. Ghazala, S.) Aspen Publishers, Gaithersburg MD, pp. 206-233

**Gorris, L.G.M. & Taucher, B.** (1999) Quality and safety aspects of minimally processed technologies. In: *Processing Foods: Quality optimisation and Process Assessment* (ed. Oliveira, F.A.R. & Oliveira, J.C.) C.R.C. Press, New York pp. 325-338

**Gorris, L.G.M.** (2000) Hurdle technology, a concept for safe minimal processing of foods. In: *Encyclopedia of Food Microbiology* (ed. Robinson, R., Batt, C. & Patel, P.) London Academic Press, pp. 1071-1076

**Gort, A.S. & Imlay, J.A.** (1998) Balance between endogenous superoxide stress and antioxidant defences. *Journal of Bacteriology*, 180: 1402-1410

**Goto, H., Kajiyama, N. & Noguchi, A.** (1993) In: *High Pressure Bioscience and Food Science* (ed. Hayashi, R.) San-ei Publications, Kyoto, pp. 300-306

**Gotz, F., Elstner, E.F., Sedewitz, B. & Lengfelder, E.** (1980) Oxygen utilization by *Lactobacillus plantarum* II. Superoxide and Superoxide Dismutase. *Archives in Microbiology*, 125: 215-220

**Gram, L.** (1992) Evaluation of the bacteriological quality of seafood. *International Journal of Food Microbiology*, 16: 25-39

**Gregory, E.M. & Fridovich, I.** (1974) Oxygen metabolism in *Lactobacillus plantarum*. *Journal of Bacteriology*, 117: 166-169

**Gutteridge, J.M.C.** (1987) Ferrous salt promoted damage to deoxyribose and benzoate. *Biochemistry Journal*, 243: 709-714

**Haard, N.F. & Lee,Y.Z.** (1982) Hypobaric storage of Atlantic salmon in a carbon dioxide atmosphere. *Canadian Institute of Food Science and Technology Journal*, 15: 68-71

**Halliwell, B. & Gutteridge, J.M.C.** (1984) Lipid peroxidation, oxygen radicals, transition metals and disease. *Biochemistry Journal*, 219: 1-4

**Halliwell, B. & Gutteridge J.M.C.** (1989) Protection against oxidants in biological systems the superoxide theory of oxygen toxicity. In*: Free Radicals in Biology and Medicine* (2nd ed., ed. Halliwell, B. & Gutteridge, J.M.C.) Clarendon Press Oxford, pp. 86-187

**Halliwell, B. & Gutteridge, J.M.C.** (1990) Iron and free radical reactions: two aspects of antioxidant protection. *Trends in Biochemistry Science*, 11: 372-375

**Halliwell, B. & Gutteridge, J.M.C.** (1992) Biologically relevant metal ion-dependent hydroxyl radical generation: An update. *FEBS Letters*, 307: 108-112

**Halliwell, B. & Gutteridge, J.M.C.** (1995) Protections against oxidants in biological systems: the superoxide theory of oxygen toxicity. In: *Free radicals in Biology and Medicine* (ed. Halliwell, B. & Gutteridge, J.M.C) Oxford University Press, pp. 86-188

**Halliwell, B., Murcia, M.A., Chirico, S. & Aruoma, O.I.** (1995) Free radicals and Antioxidants in Food and *in vivo*: What they do and how they work. CRC *Food Science and Nutrition*, 35: 7-20

**Hampton, M.B. & Winterbourn, C.C.** (1995). Modification of neutrophil oxidant production with diphenyleneiodonium and its effect on bacterial killing. *Free Radicals in Biology & Medicine*, 18: 633-639

**Hansson, L. & Haggstrom, M.H.** (1984) Effects of growth conditions on the activities of SOD and NADH oxidase/NADH peroxidase in *Streptococcus lactis*. *Current Microbiology*, 10: 345-352

**Harth, G. & Horwitz, M.A.** (1999) Export of recombinant *Mycobacterium tuberculosis* superoxide dismutase is dependent upon both information in the protein and mycobacterial export machinery. A model for studying export of leaderless proteins by pathogenic mycobacteria. *Journal of Biological Chemistry*, 274: 4281-4292

**Hartke, A., Bouche, S., Laplace, J.M., Benachour, A., Boutibonnes, P. & Auffray, Y**. (1995) UV-inducible proteins and UV-induced cross-protection against acid, ethanol, $H_2O_2$ or heat treatment *in Lactococcus lactis* subsp. *lactis*. *Archives in Microbiology*, 163: 329-336

**Hassett, D.J., Ma, J.F., Elkins, J.G., McDermott, T.R., Ochsner, U.A., West, S.E., Huang, C.T., Fredericks, J., Burnett, S., Stewart, P.S., McFeters, G., Passador, L. & Iglewski, B.H.** (1999) Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. *Molecular Microbiology*, 34: 1082-93

**Heimdal, H., Kuhn, B.F., Poll, L. & Larsen, L.M.** (1995) Biochemical changes and sensory quality of shredded and MA-packaged iceberg lettuce. *Journal of Food Science*, 60: 1265-1268, 1276

**Higuchi, M.** (1984) The effect of $O_2$ on the growth and mannitol fermentation of *Streptococcus mutants*. *Journal of General Microbiology*, 130: 1819-1826

**Hoffman, P.S., George, H.A., Krieg, N.R. & Smibert, R.M.** (1979) Studies on the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni*. II Role of exogenous superoxide anions and hydrogen peroxide. *Canadian Journal of Microbiology*, 25: 8-16

**Hoogerwerf, S., Moezelaar, R. & Kets, E.** (2000) Effect of novel high oxygen modified atmospheres on growth of food associated moulds. *Abstract in 2nd Int. Symposium on Food Packaging-Ensuring the safety and quality of foods, 8-10 November 2000, Vienna, Austria*, 107

**Hoover, D.G., Metrick, C., Papineu, A.M., Farkas, D.F. & Knorr, D.** (1989) Biological effects of high hydrostatic pressure on food micro-organisms. *Food Technology*, 43: 99-106

**Hotchkiss, J.H.** (1988) Experimental approaches to determining the safety of food packaged in modified atmospheres. *Food Technology*, 42: 55, 60-62, 64.

**Howard, L.R. & Griffin, L.E.** (1993) Lignin formation and surface discoloration of minimally processed carrot sticks. *Journal of Food Science*, 58: 1065-1067

**Howard, L.R., Griffin, L.E. & Lee, Y.** (1994) Steam treatment of minimally processed carrot sticks to control surface discoloration. *Journal of Food Science*, 59: 356-358

**Howard, L.R. & Dewi, T.** (1996) Minimal processing and edible coating effects on composition and sensory quality of mini-peeled carrots. *Journal of Food Science*, 61: 643-645, 651

**Huang, X.F., Huang, D.C., Novel, G. & Novel, M.** (1995) Two *Lactococcus lactis* genes, including lacX, cooperative to trigger an SOS response in a recA-negative background. *Journal of Bacteriology*, 177: 283-289

**Imlay, J.A. & Linn, S.** (1986) Bimodal pattern of killing of DNA-repair-defective or anoxically grown *Escherichia coli* by hydrogen peroxide. *Journal of Bacteriology*, 66: 519-27.

**Imlay, J.A., Chin, S.M. & Linn, S.** (1988) Toxic DNA damage by $H_2O_2$ through the Fenton reaction *in vivo* and *in vitro*. *Science*, 240: 640-642

**Imlay, J.A. & Linn, S.** (1988) DNA damage and oxygen radical toxicity. *Science*, 240: 1302-1309.

**Imlay, J.A. & Fridovich, I.** (1991) Assay of metabolic superoxide production in *Escherichia coli*. *Journal of Biological Chemistry*, 266: 6957-6965

**Imlay, J.A.** (1995) A metabolic enzyme that rapidly produces superoxide, fumarate reductase of *Escherichia coli*. *Journal of Biological Chemistry*, 270: 19767-19777

**Ishikawa, H., Shimoda, M., Kawano, T. & Osajima, Y.** (1995) Inactivation of enzymes in an aqueous solution by micro-bubbles of supercritical carbon dioxide. *Bioscience, Biotechnology & Biochemistry*. 59: 628-631

**Ison, R.W. & Gutteridge, C.S.** (1987) Determination of the carbonation tolerance of yeasts. *Letters in Applied Microbiology*, 5: 11-33

**Iuchi, S. & Weiner, L.** (1996) Cellular and molecular physiology of *Escherichia coli* in the adaptation to aerobic environments. *Journal of Biochemistry*, 120: 1055-1063

**Izumi, H. & Watada, A.E.** (1994). Calcium treatments affect storage quality of shredded carrots. *Journal of Food Science*, 59: 106-109

**Izumi, H., Watada, A.E., Ko, N.P. & Douglas, W.** (1996) Controlled atmosphere storage of carrot slices, sticks and shreds. *Postharvest Biology and Technology*, 9: 165-172

**Iverson, T.M., Luna-Chavez, C., Cecchini, G. & Rees, D.C.** (2000) Structure of the *Escherichia coli* fumarate reductase respiratory complex. *Science*, 284 (5422): 1961-1964

**Jacxsens, L., Devliegere, F. & Debevere, J.** (2000) Designing equilibrium modified atmosphere packages for fresh-cut produce subjected to changing temperatures. *Lebensmittel Wissenschaft und Technologie*, 33: 178-187

**Jacxsens, L., Devliegere, F., van der Steen C. & Debevere, J.** (2001) Effect of high oxygen modified atmosphere packaging on microbial growth and sensorial qualities of fresh-cut produce. *International Journal of Food Microbiology*, (in press)

**Jay, J.M.** (2000) Food Preservation with Modified atmospheres. In: *Modern Food Microbiology* 6th ed., (ed. Jay, J.M.) Aspen Publishers, Inc. pp. 283-300

**Jensen, C., Flensted-Jensen, M., Skibsted, L.H. & Bertelsen, G.** (1998) Effects of dietary rape seed oil, copper(II) sulphate and vitamin E on drip loss, colour and lipid oxidation of chilled pork chops packed in atmospheric air or in high oxygen atmospheres. *Meat Science*, 50: 211-221.

**Jeroni, D. & Brashears, M.M.** (2000) Production of hydrogen peroxide by *Lactobacillus delbruckii* subsp. *lactis* influenced by media used for propagation of cells. *Journal of Food Science*, 65: 1033-1036

**Jones, R.P. & Greenfield, P.F.** (1982) Effect of $CO_2$ on yeast growth and fermentation. *Enzyme Microbiology and Technology*, 4: 210-223

**Juliot, K.N., Lindsay, R.C. & Ridley, S.C.** (1989) Directly-acidified carrot slices for ingredients in refrigerated vegetable salads. *Journal of Food Science*, 54: 90-95

**Kader, A.A.** (1986) Biochemical and physiological bases for effects of controlled and modified atmospheres on fruits and vegetables. *Food Technology*, 5: 99-105

**Kader, A.A., Zagory, E.L. & Kerbel, E.L.** (1989) Modified atmosphere packaging of fruit and vegetables. CRC *Critical Reviews in Food Science and Nutrition*, 28: 1-30

**Kader, A.A. & Shimshon B.Y.** (2000) Review: effects of superatmospheric oxygen levels on postharvest physiology and quality of fresh fruits and vegetables. *Postharvest Biology and Technology*, 20: 1-13

**Kakiomenou, K., Tassou, C. & Nychas, G.J.** (1996) Microbiological, physicochemical and organoleptic changes of shredded carrots stored under modified storage. *International Journal of Food Science & Technology*, 31: 359-366

**Kallander, K.D., Hitchins, A.D., Lancette, G.A., Schmieg, J.A., Garcia, G.R., Solomon, H.M. & Sofos, J.N.** (1991) Fate of *Listeria monocytogenes* in shredded cabbage stored at 5 and 25$^{o}$C under a modified atmosphere. *Journal of Food Protection*, 54: 302-304

**Kargalioglou, Y. & Imlay, J.A.** (1995) Importance of anaerobic superoxide dismutase synthesis in facilitating outgrowth of *Escherichia coli* upon entry into an aerobic habitat. *Journal of Bacteriology* 176: 7653-7658

**Kato-Noguchi, H. & Watada, A.E.** (1996) Regulation of glycolytic metabolism in fresh-cut carrots under low oxygen atmosphere. *Journal of the American Society of Horticultural Science*, 121: 123-126

**Kato-Noguchi, H. & Watada, A.E.** (1997a) Citric acid reduces the respiration of fresh-cut carrots. *HortScience*, 32:136-144

**Kato-Noguchi, H. & Watada, A.E.** (1997b) Effects of low-oxygen atmosphere on ethanolic fermentation in fresh-cut carrots. *Journal of the American Society of Horticultural Science*, 122:107-111

**Kehrer, J.P.** (2000) The Haber-Weiss reaction and mechanisms of toxicity. *Toxicology*, 149: 43-50.

**Kester, J.J. & Fennema, O.R.** (1986) Edible films and coatings: A review. *Food Technology*, 40: 47-59

**Keyer, A. & Imlay, J.A**. (1996) Superoxide accelerates DNA damage by elevating free-iron levels. *Proceedings of the National Academy of Science USA*, 93: 13635-13640

**Kim, J.G., Yousef, A.E. and Dave, S.** (1999) Application of ozone for enhancing the microbiological safety and quality of foods: a review. *Journal of Food Protection*, 62: 1071-1087

**King, A.D. & Nagel, C.W.** (1967) Growth inhibition of a Pseudomonas by $CO_2$ . *Journal of Food Science*, 32: 575-579

**Knauf, H.J., Vogel, R.F. & Hammes, W.P.** (1992) Cloning, sequence, and phenotypic expression of katA, which encodes the catalase of *Lactobacillus sake* LTH677. *Applied & Environmental Microbiology*, 58: 832-839

**Knorr, D** (1993) Effects of high hydrostatic pressure processed on food safety and quality. *Food Technology*, 47: 156-161

**Knorr, D.** (1995) Hydrostatic pressure treatment in food: Microbiology. In: *New Emerging Technologies for Food Preservation*. (ed. Gould, G.W.) Blackie Academic & Professional, Glasgow pp. 318-346

**Knorr, D.** (1999) Novel approaches in food-processing technology: new technologies for preserving foods and modifying function. *Current Opinion in Biotechnology*, 5: 485-91

**Kolter, R., Siegele, D.A. & Tormo, A.** (1993) The stationary phase of the bacterial life cycle. *Annual Review in Microbiology*, 47: 855-874

**Koshland, D.E. Jr** (1981) Biochemistry of sensing and adaptation in a simple bacterial system. *Annual Review in Biochemistry*, 50: 765-781

**Kot, E., Furmanov, S. & Bezkorovainy, A.** (1996) Hydrogen peroxide formation and oxidation of ferrous iron by *Lactobacillus delbrueckii* subsp. *bulgaricus. Journal of Dairy Science*, 79: 758-766

**Koutsoumanis, K. & Nychas, G.J.E.** (1999) Chemical and sensory changes associated with microbial flora of mediterranean bogue (*Boops boops*) stored aerobically at 0,3,7 and 10°C. *Applied & Environmental Microbiology*, 65: 698-706

**Kühne, K. & Knorr, D.** (1990). Effects of high pressure carbon dioxide on the reduction of micro-organisms in fresh celery. *ZFL*, 41: 55-57

**Kumagai, H., Hata, C. & Nakamura, K.** (1997) $CO_2$ sorption by microbial cells and sterilization by high pressure $CO_2$. *Bioscience, Biotechnology & Biochemistry*, 61: 931-935

**Lafuente, M.T., Lopez-Galvez, G., Cantwell, M. & Yang, S.F.** (1996) Factors influencing ethylene-induced isocoumarin formation and increased respiration in carrots. *Journal of the American Society of Horticultural Science*, 121: 537-542

**Lannelongue, M., Hanna, M.O., Finne, G., Nickelson, R. & Vanderzant, G.** (1982) Microbiological and chemical changes during storage of sword fish (*Zifias gladium*) steaks in retail packages containing $CO_2$-enriched atmospheres. *Journal of Food Protection*, 45: 440-444

**Layer, G.J. & Johnson E.A.** (1993) Acid adaptation induces cross protection against environmental stresses in *Salm. typhimurium. Applied & Environmental Microbiology*, 59: 1842-1847

**Ledward, D.A.** (1998). High pressure processing of meat and fish. In: *Fresh Novel food by High Pressure*, Symposium Proceedings, 21-22 Sept. 1998, VTT Helsinki, Finland (ed. VTT), pp. 165-155

**Leistner, L. & Rödel, W.** (1976) Inhibition of micro-organisms in food by water activity. *Society for Applied Bacteriology Symposium Series* 5: 219-237

**Leistner, L.** (1992) Food preservation by combined methods. *Food Research International*, 25: 151-158

**Leistner, L. & Gorris, L.G.M.** (1995) Food preservation by hurdle technology. *Trends in Food Science & Technology*, 6: 41-46

**Leistner, L.** (1995a) Emerging concept of food safety. Proceedings 41st ICoMST, San Antonio, Mexico TX pp. 321-322

**Leistner, L.** (1995b) Principles and application of hurdle technology In: *New Methods of Food Preservation*. (ed. Gould, G.W.) Blackie Academic & Professional, Glasgow, pp. 1-21

**Leistner, L**. (1999) Combined methods for food preservation. In: *Handbook of food preservation* (ed. Shafiur Rahman, M.) Marcel Dekker, New York, pp. 457-485

**Leistner, L.** (2000) Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology*, 55: 181-186

**Leshuk, J.A. & Saltveit, M.E. Jr.** (1991) Effects of rapid changes in oxygen concentration on the respiration of carrot roots. *Physiologia Plantarum*, 82: 559-568

**Li., P. & Barth, M.M.** (1998) Impact of edible coatings on nutritional and physiological changes in lightly processed carrots. *Postharvest Biology & Technology*, 14: 51-60

**Lin, H., Yang, Z., Chen, L.** (1992a) Inactivation of *Saccharomyces cerevisiae* by supercritical and subcritical carbon dioxide. *Biotechnology Progress*, 8: 458-461

**Lin, M.H., Yang, Z. & Chen, L.F.** (1992b) An improved method for disruption of microbial cells with pressurised carbon dioxide. *Biotechnology Progress*, 8: 165-166

**Lin, M.Y. & Yen, C.L.** (1999) Antioxidative ability of lactic acid bacteria. *Journal of Agricultural & Food Chemistry*, 47: 1460-1466

**Liochev, S.I. & Fridovich, I.** (1993) Modulations of fumarases of *Escherichia coli* in response to oxidative stress. *Archives of Biochemistry and Biophysics*, 301: 379-84

**Liochev, S.I. & Fridovich, I.** (1994) The role of $O_2^-$ in the production of $HO^\cdot$: in vitro and in vivo. *Free Radical in Biology & Medicine*, 16: 29-33

**Loewen, P.C.** (1997) Bacterial catalases. In: *Oxidative stress and the molecular biology of antioxidant defences* (ed. Scandalions, J.G.) Cold Spring Harbor Lab. Press, pp. 527-568

**Lou, Y. & Yousef, A.E.** (1996) Resistance of *Listeria monocytogenes* to heat after adaptation to environmental stresses. *Journal of Food Protection*, 59: 465-471

**Lougheed, E.C.** (1987) Interactions of oxygen, carbon dioxide, temperature and ethylene that may induce injuries in vegetables. *HortScience*, 22: 791-794

**Lowry, O.H., Rosebrough, N.J., Lewis-Farr, A. & Randall, R.J.** (1951) Protein measurements with the folin phenol reagent. *Journal of Biology and Chemistry*, 193: 265-275

**Lopez de Fellipe, F., Starrenburg, M.J.C. & Hugenholtz, J.** (1997) The role of NADH-oxidation in acetoin and diacetyl production from glucose in *Lactococcus lactis* subsp. *lactis* MG1363. *FEMS Microbiology Letters*, 156: 15-19

**Lopez de Fellipe, F. & Hugenholtz, J.** (1999) Pyruvate flux in NADH-oxidase-overproducing *Lactococcus lactis* strain as a function of culture conditions. *FEMS Microbiology Letters*, 179: 461-466

**Lund, B.M.** (1992) Ecosystems in vegetable foods. *Journal of Applied Bacteriology, Symposium-Supplement* 21: 115S-126S

**Lushckak, V.I.** (2001) Oxidative stress and mechanisms of protection against it in bacteria. *Biochemistry-Moscow*, 66: 476

**Ma, M. & Eaton J.W.** (1992). Multicellular oxidant defence in unicellular organisms. *Proceedings of the National Academy of Sciences USA*, 89: 7924-7928

**Marchetti, R., Casadei, M.A. & Guerzoni, M.E.** (1992) Microbial population dynamics in ready to use vegetable salads. *Italian Journal of Food Science*, 2: 97-108

**Marnett, L.J., Hurd, H.K., Hollstein, M.C., Levin, D.E., Ersterbauer, H. & Ames, B.N**. (1985) Naturally occurring carbonyl compounds are mutagens in *Salmonella* tester strain TA 104. *Mutation Research*, 148: 25-34

**Marquis, R.E. & Thom, S.R.** (1992) Hyperbaric sensitization of microbes to oxidative stress and disinfection. In: *High Pressure and Biotechnology* (ed. Balny C., Hayashi R., Heremans, K., Masson P.), John Libbey and Company Ltd, London

**Marty-Teysset, C., de la Torre, F., & Garel, J.R.** (2000) Increased production of hydrogen peroxide by *Lactobacillus delbrueckii* subsp. *bulgaricus* upon aeration: Involvement of an NADH oxidase in oxidative stress. *Applied & Environmental Microbiology*, 66: 262-267

**Massey, V., Strickland, S., Mayhew, S.G., Howell, L.G., Engel, P.C., Matthews, R.G., Schuman, M. & Sullival, P.A.** (1969) The production of superoxide anion radicals in the reaction of reduced flavins and flavoproteins with molecular oxygen. *Biochemistry & Biophysics Research Communication*, 36: 891-897

**Matsumoto, J., Higuchi, M., Shimada, M., Yamamoto, Y. & Kamio, Y.** (1996) Molecular cloning and sequence analysis of the gene encoding the $H_2O$-forming NADH oxidase from *Streptococcus mutans*. *Bioscience, Biotechnology & Biochemistry*, 60: 39-43

**McCord, J.M. & Fridovich, I.** (1969) Superoxide dismutase: an enzymatic function for erythrocuprein. *Journal of Biological Chemistry*, 244: 6049-6054

**McCormick, M.L., Buettner, G.R. & Britigan, B.E.** (1998) Endogenous superoxide dismutase levels regulate iron-dependent hydroxyl radical formation in *Escherichia coli* exposed to hydrogen peroxide. *Journal of Bacteriology*, 180: 622-625

**McMeekin, T.A., Presser, K., Ratkowsky, D., Ross, T., Salter, M. & Tienungoon, S.** (2000) Quantifying the hurdle concept by modelling the bacterial growth/no growth interface. *International Journal of Food Microbiology*, 55: 93-98

**Mehdi, M.C.** (1994) Active oxygen species in plant defence against pathogens. *Plant Physiology*, 105: 467-472

**Messner, K.R. & Imlay, J.A.** (1999) The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of *Escherichia coli*. *Journal of Biological Chemistry*, 274: 10119-10128

**Metrick, C., Hoover, D.G. & Farkas, D.F.** (1989) Effects of high hydrostatic pressure on heat-resistant and heat-sensitive strains of *Salmonella*. *Journal of Food Science*, 54: 1547-1549, 1564

**Meyer, A.S. & Isaksen, A.** (1995) Application of food enzymes as food antioxidants. *Trends in Food Science & Technology*, 6: 300-304

**Mikell, A.T., Parker, B.C. & Simmons, G.M.** (1983) Sensitivity of an oligotrophic lake planktonic bacterial community to oxygen stress. *Applied & Environmental Microbiology*, 46: 545-548

**Mikell, A.T., Parker, B.C. & Gregory, E.M.** (1986) Factors affecting high oxygen survival of heterotrophic micro-organisms from an antarctic lake. *Applied & Environmental Microbiology*, 52: 1236-1241

**Mohanan, P.V., Rathinam, K. & Devi, K.S.** (1997) Effect of sobatum on ultraviolet-induced damage and superoxide production. *Indian Journal of Pharmacology*, 29: 129-131

**Mohazzab, H.K.M., Kaminski, P.M., Fayngersh, R.P. & Wolin, M.S.** (1996) Oxygen-elicited responses in calf coronary arteries: Role of $H_2O_2$ production via NADH-derived superoxide. *American Journal of Physiology*, 270: H1044-H1053

**Mohazzab, H.K.M., Kaminski, P.M. & Wolin, M.S.** (1997) Lactate and $PO_2$ modulate superoxide anion production in bovine cardiac myocytes: Potential role of NADH oxidase. *Circulation*, 96: 614-620

**Molin, G.** (1983) The resistance to $CO_2$ of some food related baceria. *European Journal of Applied Microbiology and Biotechnology*, 18: 214-217

**Moleyar, V. & Narasimham, P.** (1994) Modified atmosphere packaging of vegetables: an appraisal. *Journal of Food Science & Technology*, 31: 267-278

**Moradas-Ferreira, P., Costa, V., Piper, P. & Mager, W.** (1996) The molecular defences against reactive oxygen species in yeast. *Molecular Microbiology*, 19: 651-658

**Morishita, T. & Yajima, M.** (1995) Incomplete operation of biosynthetic and bioenergetic functions of the citric acid cycle in multiple auxotrophic lactobacilli. *Bioscience, Biotechnology & Biochemistry*, 59: 251-255

**Murphey, M.G. & Condon, S.** (1984) Correlation of oxygen utilisation and hydrogen peroxide accumulation with oxygen induced enzymes in *Lactobacillus plantarum* cultures. *Archives in Microbiology*, 138: 44-48

**Murphey, M.G., O'Connor, L., Walsh, D. & Condon, S.** (1985) Oxygen dependent lactate utilization by *Lact. plantarum*. *Archives in Microbiology*, 141: 75-79

**Nakamura, K., Enomoto, A., Fukushima, H., Nagai, K. & Hakoda, M.** (1994) Disruption of microbial cells by the flash discharge of high-pressure carbon dioxide. *Bioscience, Biotechnology & Biochemistry* 58:1297-1301

**Nguyen-The, C. & Carlin, F.** (1994) The microbiology of minimally processed fresh fruits and vegetables. *CRC Critical Reviews in Food Science & Nutrition*, 34: 371-401

**Nunoshiba, T.** (1996) Two-stage gene regulation of the superoxide stress response soxRS system in *Escherichia coli*. *Critical Reviews in Eukaryotic Gene Expression*, 6: 377-389

**Nussinovitch, A. & Hershko, V.** (1996). Gellan and alginate vegetable coatings. *Carbohydrate Polymers*, 30: 185-192

**Nychas, G.J.E. & Tassou, C.C.** (1996) Growth/survival of *Salmonella enteritidis* on fresh poultry and fish stored under vacuum or modified atmphosphere. *Letters in Applied Microbiology*, 23: 215-219

**O'Sullivan, E. & Condon, S.** (1997) Intracellular pH is a major factor in the induction of tolerance to acid and other stresses in *Lactococcus lactis*. *Applied and Environmental Microbiology*, 63: 4210-4215

**O'Brien, J.K. & Marshall, R.T.** (1996) Microbiological quality of raw ground chicken processed at high isostatic pressure. *Journal of Food Protection*, 59: 146-150

**Ogihara, H., Kanie, M., Jano, N. & Haruta, M.** (1993) Effect of carbon dioxide, oxygen and their gas mixtures on the growth of some food borne pathogens and spoilage bacteria in modified atmosphere package of food. *Journal of Food Hygiene Society Japan*, 34: 283-288

**Ogrydziak, D.M. & Brown, W.D.** (1982) Temperature effects in modified-atmosphere storage of seafoods. *Food Technology* 36: 86, 88, 90-91, 94-96

**Ohlsson, T.** (1994) Minimal processing-preservation methods of the future: an overview. *Trends in Food Science and Technology*, 5: 341-344

**Ohshima, T., Ushio, H. & Koizumi, C.** (1993) High pressure processing of fish and fish products. *Trends in Food Science and Technology*, 4: 370-375

**Onken, U.** (1990) Batch and continuous cultivation of *Pseudomonas fluorescens* at increased pressure. *Biotechnology & Bioengineering*, 35: 983-989

**Pal, R.K., & Buescher, R.W.** (1993). Respiration and ethylene evolution of certain fruits and vegetables in response to carbon dioxide in controlled atmosphere storage. *Journal of Food Science & Technology Mysore,* 30: 29-32

**Palou, E., Lopez-Malo, A. & Barbosa-Kanovas, G.V.** (1999) High pressure treatment. In: *Handbook of Food Preservation* (ed. Rahman, M.S.) Marcel Dekker, Inc. New York, Basel pp. 547-576

**Parry, R.T.** (1993) Introduction. In: *Principles and Applications of Modified Atmosphere Packaging of Foods.* (ed. Parry, R.T.), Blackie Academic & Professional Glasgow, pp. 1-18

**Pastoriza, L., Sampedro, G., Herrera, J.J. & Cabo, M.L**. (1996) Effect of carbon dioxide atmosphere on microbial growth and quality of salmon slices. *Journal of the Science of Food & Agriculture,* 72: 348-352

**Patel, M.P., Marcinkeviciene, J. & Blanchard, J.S.** (1998) *Enterococcus faecalis* glutathione reductase: purification, characterization and expression under normal and hyperbaric oxygen conditions. *FEMS Microbiology Letters,* 166: 155-63

**Patterson, M.F., Quinn, M., Simpson, R. & Gilmour, A.** (1995) Effect of high pressure on vegetative pathogens. In: *High Pressure Processing of Foods* (eds. Ledward, D.A., Jonston, D.E., Earnshaw R.G. & Hasting R.P.M.), Nottingam, University Press

**Pebay, M., Holl, A.C., Simonet, J.M. & Decaris, B.** (1995) Characterization of the *gor* gene of the lactic acid bacterium *Streptococcus thermophilus* CNRZ368. *Research Microbiology,* 146: 371-383

**Phillips, C.A.** (1996) Modified atmosphere packaging and its effects on the microbiological quality and safety of produce. *International Journal of Food Science & Technology,* 31: 463-481

**Piard, L.C. & Desmazeaud, M.** (1991) Inhibition factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. *Lait,* 71: 525-541

**Picchioni, G.A., Watada, A.E., Whitaker, B.D. & Reyes, A.** (1996). Calcium delays senescence-related membrane lipid changes and increases net synthesis of membrane lipid components in shredded carrots. *Postharvest Biology & Technology*, 2: 235-245

**Pinheiro, R., Belo, I. & Mota, M.** (1997) Physiological behaviour of *Saccharomyces cerevisiae* under increased air and oxygen pressures. *Biotechnology Letters,* 19: 703-708

**Plihon, F., Taillandier, P. & Strehaiano, P**. (1995) Oxygen effect on batch cultures of *Leuconostoc mesenteroides*: Relationship between oxygen uptake, growth and end-products. *Applied Microbiology and Biotechnology,* 43: 117-122

**Purdy, D., Cawthraw, S., Dickinson, J.H., Newell, D.G. & Park, S.F.** (1999) Generation of SOD deficient mutant of *Campylobacter coli*: evidence for the significance of SOD in Campylobacter survival and colonization. *Applied & Environmental Microbiology,* 65: 2540-2546

**Reddy, N.R., Solomon, H.M., Yep, H., Roman, M.G. & Rhodehamel, E.J.** (1997) Shelf-life and toxin development by *Clostridium botulinum* during storage of modified-atmosphere-packaged fresh aquacultured salmon fillets. *Journal of Food Protection,* 60: 1055-1063

**Reyes, V.G., Simons, L. & Tran, C**. (1996). Preservation of minimally processed carrots by edible coating and acid treatment. Proceedings, Australasian Postharvest Horticulture Conference, Melbourne, Australia, 18-22 September 1995, pp. 451-456

**Reznick A.Z. & Packer, L.** (1994) Oxidative damage to proteins: spectrophotometric method for carbonyl assay. In: *Methods in Enzymology* (ed. Packer, L.) Academic Press, California, vol. 233, pp. 357-359

**Robey M., Park, S.M. & Mackey, B.M**. (1998) Role of oxidative stress in the inactivation of *Escherichia coli* by High Hydrostatic Pressure. International Conference High Pressure, Bioscience & Biotechnology, Heidelberg, Germany, Aug. 30 - Sept 3, 1998

**Roginski H., Hickey, M.W. & Legg, G.A.** (1991). Effect of the lactoperoxidase system in bacteriophage resistant mutants of lactococci. *Australial Journal of Dairy Technology,* 46: 31-35

**Ross, R.P. & Claiborne, A.** (1997) Evidence for regulation of the NADH peroxidase gene (npr) from *Enterococcus faecalis* by OxyR. *FEMS Microbiology Letters,* 151: 177-183

**Roth, L.A. & Clark, D.S.** (1975) Effect of lactobacilli and carbon dioxide on growth of *Microbacacterium thermosphactum* on fresh beef. *Canadian Journal of Microbiology*, 21: 629-632

**Rowe, M.T. & Kirk, R.** (1999) An investigation into the phenomenon of cross-protection in *Escherichia coli* O157:H7. *Food Microbiology*, 16: 157-164

**Sakamoto, M., Uchimura, T. & Komagata, K.** (1996) Comparison of $H_2O$-forming NADH oxidase from *Leuc. mesenteroides* subsp *mesenteroides* NRIC 1541(T) and $H_2O_2$-forming NADH oxidase from *Sporolactobacillus inulinus* NRIC 1133(T). *Journal of Fermentation and Bioengineering*, 82: 531-537

**Sakamoto, M., Tano, Y., Uchimura, T. & Komagata, K.** (1998) Aerobic growth of some lactic acid bacteria enabled by the external addition of peroxidase (horseradish) to the culture medium. *Journal of Fermentation & Bioengineering.* 85: 627-629

**Sanders, A., Eisenthal, R. & Harrison, R.** (1997) NADH oxidase activity of human xanthine oxidoreductase: generation of superoxide anion. *European Journal of Biochemistry*, 245: 541-548

**Sanders, J.W., Leenhouts, K.J., Haandrikman, A.J., Venema, G. & Kok, J.** (1995) Stress response in *Lactococcus lactis*: Cloning, expression analysis, and mutation of the lactococcal SOD gene. *Journal of Bacteriology* 177: 5254-5260

**Sanders, J.W.** (1997) Environmental stress responses in *Lactococcus lactis*: identification of genes and use of expression signals. Ph.D. thesis, Rijksuniversiteit Groningen, The Netherlands

**Sanders, J.W., Venema, G. & Kok, de J.** (1999) Environmental stress responses in *Lactococcus lactis. FEMS Microbiology Reviews*, 23: 483-501

**Sapers, G.M., Miller, R.L. & Simmons, G.** (1995). Effects of hydrogen peroxide treatment on fresh-cut fruits and vegetables. IFT Annual Meeting 1995, p. 272

**Sapers, G.M. & Simmons, G.F.** (1998). Hydrogen peroxide disinfection of minimally processed fruits and vegetables. *Food Technology*, 52: 48-52

**Sarkar, S.K. & Phan, C.T.** (1979). Naturally occurring and ethylene induced phenolic compounds in the carrot root. *Journal of Food Protection*, 42: 526-534

**Sawaya, W.N., Elnawawy, A.S., Adu-Ruwaida, A.S., Kwalafawa, S. & Sashti, B**. (1995) Influence of modified atmosphere packaging on shelf-life of chicken carcasses under refrigerated storage conditions. *Journal of Food Safety*, 15: 35-51

**Schmidt, H.L., Stocklein, W., Danzer, J., Kirch, P. & Limbach, B.** (1986) Isolation and properties of an $H_2O$ forming NADH oxidase from *Streptococcus faecalis. European Journal of Biochemistry*, 156: 149-155

**Scott, M.D., Meshnick, S.R. & Eaton, J.W.** (1987) Superoxide dismutase-rich bacteria. Paradoxical increase in oxidant toxicity. *Journal of Biological Chemistry*, 262: 3640-5

**Shimoda, M., Yamamoto, Y., Cocunubo-Castellanos, J., Tonoike, H., Kawano, T., Ishikawa, H. & Osajima, Y.** (1998) Antimicrobial effects of pressurised carbon dioxide in a continuous flow system. *Journal of Food Science*, 63: 709-712

**Sigler, K., Chaloupka, J., Brozmanova, J., Stadler, N. & Hofer, M.** (1999) Oxidative stress in micro-organisms – I - Microbial vs. higher cells - Damage and defences in relation to cell ageing and death. *Folia Microbiologia*, 44: 587-624

**Sihvonen, M., Järvenpää, E., Hietaniemi, V. & Huopalahti, R.** (1999) Advances in supercritical carbon dioxide technologies. *Trends in Food Science & Technology*, 10: 217-222

**Silliker, J.H. & Wolfe, S.K.** (1980) Microbiological safety considerations in controlled atmosphere storage of meats. *Food Technology*, 34, 59-63

**Silva, J.L. & White, T.D**. (1994) Bacteriological and color changes in modified atmosphere-packaged refrigerated channel catfish. *Journal of Food Protection*, 57: 715-719

**Simpson, B.K.** (1997) Innovative strategies for controlling fresh fish texture degradation during postharvest handling and storage. In: Seafood safety processing and biotechnology (eds. Shahidi, F. Jones, Y. & Kitts, D.D.) Technomic Publishing CO., Inc, Lancaster, Pensylvania

**Smelt, J.P.P.M.** (1998) Recent advances in the microbiology of high pressure processing. *Trends in Food Science & Technology*, 9: 152-158

**van Spreekens, K.J.A.** (1974). The suitability of a modification of Long and Hammer's medium for the enumeration of fastidious bacteria from fresh fish products. *Archives in Lebensmittel und Hygiëne,* 25: 213-219

**Stadtman, E.R.** (1986) Oxidation of proteins by mixed function oxidation systems implication in protein turnover, ageing and nutrophile function. *Trends in Biochemical Sciences,* 11: 11-12

**Stadtman, E.R.** (1990) Metal-ion catalyzed oxidation of proteins: Biochemical mechanism and biological consequences. *Free Radical Biology and Medicine,* 9: 315-325

**Stadtman, E.R. & Levine, R.L.** (2000) Protein oxidation. *Annual New York Academy Science,* 899: 191-208

**Stammen, K., Gerdes, D. & Caporaso T.** (1990) Modified atmosphere packaging of seafood. *Food Science & Nutrition,* 29: 301-331

**Stanley, D.W., Bourne, M.C., Stone, A.P. & Wismer W.V.** (1995) Low temperature blanching effects on chemistry, firmness and structure of canned green beans and carrots. *Journal of Food Science,* 60: 327-333

**Stead, D. & Park, S.F.** (2000) Roles of Fe SOD and Catalase in resistance of *Campylobacter coli* to Freeze-Thaw Stress. *Applied and Environmental Microbiology,* 59: 3110-3112

**Stier, R.F., Bell, L., Ito, K.A., Shafer, B.D., Brown, L.A., Seeger, M.L., Allen, B.H., Porcuna, M.N. & Lerke, P.A.** (1981) Effect of modified atmosphere storage on *Clostridium botulinum* toxigenesis and the spoilage microflora of salmon fillets. *Journal of Food Science,* 46: 1639-1642

**Stortz,G., Tartaglia, L.A. & Ames, B.N.** (1990) The OxyR regulon. *Antonie van Leeuwenhoek,* 58: 157-161

**Stortz, G. & Imlay, J.A.** (1999) Oxidative stress. *Current Opinion in Microbiology,* 2: 188-94

**Styles, M.F., Hoover, D.G. & Farkas, D.F.** (1991) Response of *Listeria monocytogenes* and *Vibrio parahaemolyticus* to high hydrostatic pressure. *Journal of Food Science,* 56: 1404-1407

**Swain, T. & Hillis, W.E.** (1959). The phenolic constituents of Prunus domestica I. The quantitative analysis of phenolic constituents. *Journal of the Science of Food & Agriculture,* 10: 63

**Tapia de Daza, M.S., Alzamora, S.M. & Welti-Chanes, J.** (1996) Combinations of preservation factors applied to minimal processing of foods. *CRC Reviews in Food Science & Nutrition,* 36: 629-659

**Tatsumi, Y., Watada, A.E. & Wergin, W.P.** (1991). Scanning electron microscopy of carrot stick surface determine cause of white translucent appearance. *Journal of Food Science,* 56: 1357-1359

**Taylor, B.L. (1983)** How do bacteria find the optimal concentration of oxygen? *Trends in Biochemical Sciences,* 8: 438-441

**Thom, S.R. & Marquis, R.E.** (1984) Microbial growth modification by compressed gases and hydrostatic pressure. *Applied and Environmental Microbiology,* 47: 780-787

**Thomas, E.L. & Pera, K.A.** (1983) Oxygen metabolism of streptococcus mutants: Uptake of oxygen and release of superoxide and hydrogen peroxide. *Journal of Bacteriology,* 154: 1236-1244

**Thomas, M.J.** (1995) The role of free Radicals and antioxidants: How do we know that they are working. *CRC Food Science & Nutrition,* 35: 21-39

**Touati, D., Jacques, M., Tardat, B. Bouchard, L. & Despied, S.** (1995) Lethal oxidative damage and mutagenesis are generated by iron in DELTA-fur mutagenesis of *Escherichia coli:* Protective role of SOD. *Journal of Bacteriology,* 177: 2305-2314

**Tseng C.P. & Montville, T.J**. (1990) Enzyme activities affecting end product distribution by *Lactobacillus plantarum* in response to changes in pH and $O_2$. *Applied and Environmental Microbiology,* 56: 2761-2763

**Vercet, A., Lopez, P. & Burgos, J.** (1998) Free radical production by manothermosonication. *Ultrasonics,* 36: 615-618

**Villegas, E. & Gilliland, S.E**. (1998) Hydrogen peroxide production by *Lactobacillus delbrueckii* subsp. *lactis* at 5$^{o}$C. *Journal of Food Science,* 63: 1070-1074

**Walkup, L.K.B. & Kogoma, T.** (1989) *Escherichia coli* proteins inducible by oxidative stress mediated by the superoxide radical. *Journal of Bacteriology,* 171: 1476-1484

**Wang, M.Y. & Brown, W.D.** (1983). Effects of elevated $CO_2$ atmosphere on storage of freshwater crayfish (*Pasifastacus leniusculus*). *Journal of Food Science,* 48: 158-161

**Warriner, K.S.R. & Morris, J.G.** (1995) The effects of aeration on the bioreductive abilities of some heterofermentative lactic acid bacteria. *Letters in Applied Microbiology*, 20: 323-327

**Wei, C.I., Balaban, M.O. Fernando, S.Y. & Peplow, A.J.** (1991) Bacterial effect of high pressure $CO_2$ treatment on foods spiked with *Listeria* or *Salmonella*. *Journal of Food Proteins*, 54: 189-193

**Wolf, G., Strahl, A. Meisel, J. & Hammes, W.P.** (1991) Heme-dependent catalase activity of lactobacilli. *International Journal of Food Microbiology*, 12: 133-140

**Wolfe W.C.** (1962) Spectophotometric determination of hydroperoxide in diethyl ether. *Analytical Biochemistry*, 34: 1328-1330

**Wolfe, S.K.** (1980) Use of CO and $CO_2$-enriched atmospheres for meats fish and produce. *Food Technology*, 34: 3-8

**Woodall, A.A., Wai-Ming-Lee, S., Weesie, R.J., Jackson, M.J. & Britton, G.** (1997) Oxidation of carotenoids by free radicals: relationship between carotenoid structure and reactivity. *Biochimica Biophysica Acta*, 1336: 33-42

**Woolfe, M., Pala, M., Hohn, E. & Somogyi, Z.** (1995) The safety of modified atmosphere packaged fruits and vegetables. In: "*COST 94. The post-harvest treatment of fruit and vegetables: modified atmosphere packaging*", Proceedings workshop, Istanbul, Turkey, 1-2 Oct. 1992 (ed. DG XIII-A. Luxembourg: Commission of the European Communities), pp. 65-77

**Wszelaki, A. & Mitcham, E**. (1999) Elevated oxygen atmospheres as a decay control alternative on strawberry. *Hortscience*, 34:514-515

**Wulff, K.** (1988) NADH/NADPH, Luminometric Method. Methods in Enzymatic Analysis Vol VII: Metabolites 2: Tri- and Dicarbonic acids, Purine and Pyrimidine Derivatives, Coenzymes, Inorganic Compounds. Verlag Chemie, Weinheim 3rd ed. (eds. Bergmeyer, J., Grassl, M.), pp. 280-284

**Yamauchi, R., Goto, Y., Kato., K. & Ueno, Y.** (1984) Prooxidant effect on hydroxyacetone and reducing sugars on the autoxidation of methyl emulsions. *Agricultural & Biological Chemistry*, 48: 843-848

**Yi X., Kot E. & Bezkorovainy, A.** (1998) Properties of NADH oxidase from *Lactobacillus delbrueckii* subsp *bulgaricus*. *Journal of the Science of Food & Agriculture*, 78: 527-534

**Zagory, D. & Kader, A.A.** (1988) Modified atmosphere packaging of fresh produce. *Food Technology*, 42: 70-74, 76-77

**Zagory, D.** (1995) Principles and practise of MA packaging of horticultural commodities In: Principle of Modified atmospheres and Sous Vide Product Packaging (eds. Farber, J.M. & Dodds, K.L.), Technomics Publ. Co., Lancaster, pp. 175-203

**ZoBell C.E. & Hittle, L.L.** (1967) Some effects of hyperbaric oxygenation on bacteria at increased hydrostatic pressure. *Canadian Journal of Microbiology*, 13: 1311-1319

## Summary

The exposure of micro-organisms to excessive amounts of $O_2$ confronts them with a certain level of oxidative stress which, in effect, can reduce their growth potential or can even cause loss of viability. In many bacteria, oxidative stress is mediated by a number of toxic compounds, referred to as Reactive Oxygen Species (ROS), such as $H_2O_2$ and $O_2^-$. Bacteria that tolerate $O_2$, apparently posses active anti-oxidative defence systems that destroy or inactivate ROS. The damage caused by ROS depends on the actual intracellular level at which they are present, which is the net result of the rate of their production and inactivation. Production may be directly proportional to the prevailing environmental concentration of $O_2$; inactivation depends on the active ROS-scavenging capability of a cell or a population. The environmental conditions and the activity of bacterial defence systems are essentially dynamic, as they depend on many factors endogenous and exogenous to the affected micro-organisms. Therefore, the environmental concentration of $O_2$ that poses a notable, detrimental oxidative stress upon a given population of bacteria can vary considerably. The aim of this thesis was to investigate whether the oxidative stress exerted by $O_2$ could effectively be used for the preservation of foods. A systematic investigation on this topic until now has not been conducted. Since it was recognised at the onset of this study that oxidative stress cannot be used as a single, lethal treatment, a choice was made to evaluate its potential use within the context of a hurdle technology strategy for preservation.

As explained in **Chapter 1**, a hurdle technology (or "combination preservation") strategy assures adequate control of undesirable bacteria (i.e. pathogens) possibly present in a certain food product, by using a combination of preservative factors ("hurdles") that individually challenge these bacteria only at a sub-lethal level but overall limit microbial growth or cause die-off as appropriate for consumer safety. Hurdle technology strategies are particularly employed for the preservation of minimally processed (MP) foods, in which the original quality features of the food ingredients are maintained to a large extend. In the preservation system of a number of MP foods, i.e. those foods in which aerobic pathogens can be present or that loose quality characteristics due to $O_2$, the removal of oxygen is used as a preservative factor for both safety and quality considerations. Examples are perishable produce, fatty fish products and red meat products. For such MP foods, Modified Atmosphere (MA) packaging systems have been developed and successfully introduced on many consumer markets throughout the world. While MA packaging takes advantage of low $O_2$ conditions combined with other hurdles (low temperature storage, $CO_2$), based on the potential anti-microbial effect of excessive $O_2$ as indicated above, one could alternatively hypothesise that a high $O_2$ level (>40%) could as well be used in a combined preservation system; this would possibly provide certain beneficial features not achievable now with low-$O_2$ MA packaging. This hypothesis is further developed in the thesis research (Chapters 2-4), focusing on the mechanisms that underlie oxidative stress towards specific undesirable bacteria, and verified for two food products, namely carrot and salmon (Chapters 5 and 6).

Substantiation of the potential anti-microbial effect of high $O_2$ (when combined with low temperature storage and high $CO_2$) on selected pathogenic bacteria, spoilage bacteria and spoilage yeasts, was obtained in a series of *in vitro* experiments, using a surface model system that mimic's growth on the a food surface (**Chapter 2**). The high $O_2$ gas atmospheres investigated consisted of 80 or 90% $O_2$ (balanced with 20 and 10% $N_2$, respectively, or with 20 and 10% $CO_2$, respectively); atmospheres containing an ambient oxygen level (20% $O_2$, 80% $N_2$) or containing a high $CO_2$ level (20 or 10% $CO_2$, balanced with $N_2$) only, were used as controls. Although the study was necessarily restricted in the number of pathogenic and spoilage micro-organisms investigated, the general conclusion was that, in most cases, microbial growth was not significantly affected when high $O_2$ or high $CO_2$ were used alone, but that growth characteristics ($\mu_{max}$, lag phase duration and final population density) were affected significantly when they were used in combination (i.e. 80% $O_2$ + 20% $CO_2$ and 90% $O_2$ + 10% $CO_2$). Notably, the combination of high $O_2$ and high $CO_2$ was consistently inhibitory towards the

various spoilage micro-organisms tested, whereas the impact on the pathogenic bacteria, that grew only slowly at the incubation temperature used, was more variable.
A group of spoilage bacteria of specific importance with respect to food preservation, are the lactic acid bacteria (LAB). To date, the effect of oxidative stress (particularly high $O_2$ stress) on LAB has not been studied as extensively as compared to that of other Gram(+) and that of Gram(-) bacteria. The mechanisms underlying high $O_2$ stress in a representative of the genus *Lactobacillus*, namely *L. sake*, were studied in detail on a cellular level (Chapter 3 and 4). A range of *L. sake* strains, that originated from different food products or were used in food fermentation processes, were included in these studies in order to minimise inter-species differences in high $O_2$ stress mechanisms whilst allowing to investigate intra-species variability in tolerance to high $O_2$.
The study reported in **Chapter 3** assessed the differences in the impact of high $O_2$ on several growth parameters of 10 different strains of *L. sake*, grown in laboratory media at 8$^oC$ under atmospheres containing either 50 or 90% $O_2$. It was found that individual *L. sake* strains differed substantially in their overall anti-oxidative capability, although a number of specific anti-oxidative properties were identified in every strain. *L. sake* strains that could tolerate high levels of oxygen typically were able to inactivate $H_2O_2$ and to control the intracellular $H_2O_2$ level. For example, strain *L. sake* NCFB 2813, which was found to be the most $O_2$-insensitive of all *L. sake* strains evaluated, was able to effectively control the intercellular concentration of $H_2O_2$ at very low levels when exposed to high $O_2$ conditions, whereas the $O_2$-sensitive strain *L. sake* DSM 6333 was much less effective. The apparent bacteriostatic effect of $H_2O_2$ could partly be explained by its involvement in the generation of the highly reactive HO· radical.
Using the $O_2$-insensitive strain *L. sake* NCFB 2813 and the $O_2$-sensitive strain *L. sake* DSM 6333, further mechanisms underlying high $O_2$ stress were investigated (**Chapter 4**). The loss of viability of *L. sake* DSM 6333 upon exposure to high $O_2$ conditions could not be attributed to extracellular generation of ROS. The relatively high growth rates observed under such conditions were probably responsible for the generation of intracellular toxic compounds at levels higher than those that the cells of this strain could cope with. In order to evaluate the role of the generation of intracellular $O_2^-$, one of the ROS possibly produced as a result of oxidative stress, intracellular levels of $O_2^-$ were assayed under oxygen stress. It was calculated that, theoretically, a steady-state level of $O_2^-$ radicals may develop in *L. sake* DSM 6333 as a result of the reduction of 22% of the total $O_2$ consumed. Most of the $O_2^-$ radicals are probably generated by cytosolic enzymes as only 5% of the total electron reduction by cells of was measured in the membrane fraction.
Furthermore it was investigated whether superoxide dismutase (SOD), an enzyme which is able to remove toxic $O_2^-$ radicals, plays an part in the defence of *L. sake* against oxygen stress. The presence and role of SOD in LAB has been under debate, but this study provides support to the hypothesis that SOD does play a role in $O_2$ detoxification in the case of *Lactobacillus* spp. For one, this is based on the finding that a specific trait of *L. sake* NCFB 2813 is the abundant presence of at least one type of SOD. Additional evidence was obtained from calculations of the theoretical level of intracellular $O_2^-$ radicals in cells of the $O_2$-sensitive *L. sake* DSM 6333 compared to that in cells of the $O_2$ insensitive mutant LSUV4 derived from the $O_2$-sensitive strain.
As reported in **Chapter 5,** microbiological spoilage of MP sliced carrots was significantly delayed when high $O_2$ and high $CO_2$ atmospheres were used in combination; the spoilage flora of minimally processed sliced carrots consisted mainly of *Pseudomonas* spp and lactic acid bacteria. Atmospheres composed of 70-90%$O_2$ combined with 30-10% $CO_2$, respectively, delayed growth of LAB to a significant degree. Conceivably, the increased level of $CO_2$ delayed spoilage by inhibiting Gram(-) bacteria, whilst the elevated level of $O_2$ delayed the growth of lactic acid bacteria.
In the current concept for modified atmosphere packaging of respiring products such as sliced carrots, the use of 1-3% $O_2$ in combination with $CO_2$ up to 10% is often advocated. In the current study, it was demonstrated that minimally processed carrots could tolerate $CO_2$ levels up to 30% and $O_2$ levels up to 50%. Oxygen levels of 70% were detrimental for product quality. Technological advantages of

employing atmospheres enriched in $O_2$ and $CO_2$, as compared to low-$O_2$ atmosphere, were clear reductions in water exudation and production of aberrant metabolites (i.e. ethanol, acetaldehyde and total phenols).

In **Chapter 6** the effect of hyperbaric gases on the natural microflora of salmon is evaluated. A compressed gas mixture of 50% $O_2$ + 50% $CO_2$ subjected to a pressure of 200 MPa for 30 min at a temperature of 5 ± 1°C during treatment, resulted in a practically sterile product. No further growth was observed during storage of the MA packed salmon. Treatment with compressed high $O_2$ and high $CO_2$ (at 150 MPa for 15 min), resulted in a substantial reduction of two types of pathogenic micro-organisms artificially inoculated on salmon. The effectiveness of the compressed gases treatment depended on the pressure level as well as the duration of the pressure treatment. Compressed gases have a stronger antibacterial effect on spoilage and pathogenic micro-organisms than pressure treatments of the same intensity in the absence of the gas mixture tested. The explanation for these observations is that under hyperbaric conditions the dissolved concentration of $O_2$ and $CO_2$ is increased. It was further demonstrated that, next to the positive effect on microbial shelf life, the combined application of high $O_2$, high $CO_2$ and high pressure had a positive impact on the texture and other organoleptic properties of salmon. However, because of a detrimental effect on the colour of the salmon product and an increase in oxidative rancidity, the technology as applied here did not qualify for practical application. Notably, this was the first study on the combined application of $O_2$ in supercritical and $CO_2$ in liquid state at the moment of application.

In **Chapter 7**, which briefly recapitulated some of the findings of this study, a model was proposed that describes the mechanisms underlying the process of cell damage by endogenous $O_2^-$ in *L. sake* DSM 6333 upon exposure to high $O_2$. Future studies might now be aimed at verifying this model by further unravelling of the responses of different LAB, specifically *L. sake*, to high $O_2$ by identifying the proteins and genes involved in generation and inactivation of the intermediate ROS ($H_2O_2$, $HO\cdot$ and $O_2^-$) and in the regulation of the SOD defence system.

## Samenvatting

Blootstelling van micro-organismen aan een overmaat van zuurstof ($O_2$) leidt tot oxidatieve stress die hun groei kan remmen en zelfs kan leiden tot een verlies van hun levensvatbaarheid. Bij veel micro-organismen wordt oxidatieve stress veroorzaakt door de vorming van reactieve zuurstof componenten (ROS) zoals $H_2O_2$ of $O_2^-$. Organismen die $O_2$ verdragen gebruiken zogenaamde antioxidant afweersystemen om de schade die ROS veroorzaken tegen te gaan. De mate van schade wordt bepaald door de snelheid waarmee ROS worden aangemaakt en de mate waarin ze geïnactiveerd worden. Aanmaak van ROS is evenredig met de omgevingsconcentratie van $O_2$; de inactivatie snelheid is afhankelijk van het niveau van de aanwezige hoeveelheid actieve ROS eliminerende enzymen. De omgevingconcentraties $O_2$ die vereist zijn om een oxidatieve stress binnen een bacteriepopulatie te veroorzaken kunnen sterk verschillen als gevolg van, al dan niet tijdelijke, variaties in het metabolisme of door dynamische omgevingsomstandigheden. De centrale vraag in het hier beschreven onderzoek was of de oxidatieve stress die wordt veroorzaakt door een hoge $O_2$ concentratie mogelijkerwijze kan worden gebruikt voor de conservering van levensmiddelen.

Zoals toegelicht in **Hoofdstuk 1** is de strategie van de zogenaamde "Horden Technologie" gericht op een adequate controle van onwenselijke bacteriën (e.g. pathogenen) die mogelijk aanwezig zijn op een bepaald voedselproduct. Door gebruik te maken van combinaties van conserverende factoren ("horden") die individueel voor een bepaald micro-organisme een niet lethaal effekt hebben, kunnen deze micro-organismen door het gezamenlijke effect alsnog in hun groei worden geremd of zelfs worden afgedood.. Horden technologie wordt speciaal toegepast voor de conservering van minimaal verwerkte voedingsmiddelen met als doel de intrinsieke kwaliteitskenmerken van de gebruikte ingrediënten en het eindproduct zoveel mogelijk te behouden. $O_2$ kan een nadelige invloed hebben omdat het de groei van micro-organismen bevorderd en een ook bepaalde kwaliteitskenmerken van tijdelijk houdbare producten nadelig kan beïnvloeden.. Het wegnemen van $O_2$ wordt aanbevolen als een horde die kan helpen de houdbaarheid van bederfelijke producten te verlengen. Voorbeelden van producten waarbij deze strategie in de praktijk wordt toegepast zijn bepaalde vette vissoorten en rood vlees. Voor deze levensmiddelen zijn verpakkingen ontwikkeld met een gewijzigde samenstelling van de gas-atmosfeer (MA). Dergelijke verpakkingen, waarin weinig $O_2$ en relatief veel $CO_2$ gas aanwezig is, worden over de hele wereld met succes worden toegepastvoor gekoelde bewaring  Echter, als wij de potentieel giftige werking van $O_2$ op micro-organismen in aanmerking nemen, zou men van de hypothese kunnen uitgaan dat ook een hoge $O_2$-concentratie (>40%) gebruikt zou kunnen worden om de werking van andere conserveringstechnieken te versterken. Dit zou mogelijk zelfs tot bepaalde voordelige effecten kunnen leiden die momenteel niet kunnen worden gerealiseerd door MA verpakkingen met lage $O_2$ gehaltes. Deze hypothese is verder ontwikkeld in dit onderzoek (Hoofdstukken 2-4), toegespitst op de onderliggende mechanismen van oxidatieve stress op ongewenste bacteriën, en is geverifieerd op twee voedselproducten, namelijk wortel en zalm (Hoofdstukken 5 en 6).

In **Hoofdstuk 2** worden studies gepresenteerd naar de werking van hoge $O_2$ en $CO_2$ concentraties op geselecteerde bederf- en ziekteveroorzakende bacteriën en op bepaalde gisten. De groei van micro-organismen op MA verpakte voedingsmiddelen werd hierbij nagebootst door een model systeem te gebruiken waarbij de voedingsbodem het opervlak van het voedingmiddel nabootst; in het systeem werden verschillende samenstellingen van de gasatmosfeer gebruikt, te weten 80 of 90% $O_2$ in combinatie met 10 of 20% $CO_2$ (allen aangevuld met $N_2$). Er werd een temperatuur aangehouden van 8$^oC$, hetgeen een gebruikelijke bewaartemperatuur is voor minimaal verwerkte producten. De groei onder deze condities is vergeleken met de groei onder normale atmosferische omstandigheden (20% $O_2$ en 1% $CO_2$) en met enkel een hoog $CO_2$ gehalte (10 of 20% $CO_2$). Hoewel deze studie zich beperkt tot een gelimiteerd aantal bederfveroorzakende en pathogene micro-organismen, is de algemene

conclusie dat de groei in de meeste gevallen niet significant wordt beïnvloed wanneer alleen $O_2$ of alleen $CO_2$ wordt toegepast. De groeikarakteristieken (tijd tot groei, specifieke maximale groeisnelheid en de uiteindelijke populatiedichtheid) worden wel beïnvloed wanneer verhoogde $O_2$ wordt gecombineerd met verhoogde $CO_2$. De gecombineerde toepassing van een hoog $O_2$ en $CO_2$ was met name consistent effectief tegen de verschillende bederfveroorzakende micro-organismen die waren getest, terwijl het effect op de, bij deze temperatuur langzaam groeiende, pathogene bacteriën meer variabel was.

Melkzuurbacteriën (LAB) zijn een groep van bederfveroorzakende bacteriën die erg belangrijk zijn met betrekking tot voedselconservering. Het effect van oxidatieve stress en in het bijzonder van hoge $O_2$ belasting op LAB is nog niet eerder goed bestudeerd, terwijl er voor Gram(+) en Gram(-) bacteriën wel al veel onderzoek naar gedaan is. De mechanismen die zijn betrokken bij het effect van verhoogde $O_2$ op een aantal *Lactobacillus sake* stammen zijn in dit proefschrift op cellulair niveau bestudeerd (Hoofdstukken 3 en 4). De stammen werden gekozen op grond van hun belang voor voedselbederf of voor gistingsprocessen, maar ook omdat ze opvallende verschillen vertoonden in hun reactie op verhoogde zuurstofconcentraties.

In **hoofdstuk 3** worden de effecten gerapporteerd van verhoogde $O_2$ concentraties (50 en 90% $O_2$) op de groei van 10 verschillende *L. sake* stammen, gekweekt in laboratoriummedia bij 8$^{o}$C. Individuele *L. sake* stammen bleken sterk te verschillen in hun antioxidatieve capaciteit, hoewel voor iedere stam een aantal antioxidatieve eigenschappen werden gevonden. De mate waarin *L. sake* stammen de hoge zuurstofconcentraties kunnen verdragen bleeksamen te hangen met de mate waarin zij $H_2O_2$ kunnen elimineren. Bijvoorbeeld, *L. sake* NCFB 2813, welke bijzonder $O_2$-ongevoelig bleek te zijn, kon de intracellulaire $H_2O_2$ concentratie tot op een laag niveau reduceren tijdens blootstelling aan hoge $O_2$ concentraties. De $O_2$-gevoelige *L. sake* stam DSM 6333 veel minder effectief hierin. Het bacteriestatische effect dat optreedt bij $H_2O_2$ -niveaus boven 400 µM kon voor een deel worden toegeschreven aan de vorming van de zeer giftige HO· radicaal.

Welke andere mechanismen ook een rol spellen bij de giftigheid door verhoogde $O_2$ concentratie is in **Hoofdstuk 4** onderzocht aan de hand van twee *L. sake* stammen met een zeer sterk verschillende gevoeligheid voor $O_2$. De schade die in de zuurstofgevoelige stam *L. sake* DSM 6333 (*L. sake*$^{sens}$) werd veroorzaakt bij blootstelling aan 90% $O_2$, kon niet worden toegeschreven aan schade als gevolg van extracellulaire vorming van ROS. De relatief hoge groeisnelheden van de stam in aanwezigheid van hoge $O_2$ concentraties waren wellicht verantwoordelijk voor de vorming van meer giftige bijproducten dan de cellen blijkbaar onder de heersende omstandigheden aankonden. Om te bepalen in hoeverre intercellulair gevormde ROS een rol spelen is het niveau van $O_2^-$ radicalen bepaald. Berekend is dat 22% van de totale hoeveelheid $O_2$ is gereduceerd tot een hoog en stabiel niveau van $O_2^-$ radicalen. Aangetoond is dat $O_2^-$ radicalen waarschijnlijk rechtstreeks betrokken zijn bij de schade ten gevolge van intercellulaire metabolieten. Het merendeel van de $O_2^-$ radicalen wordt gevormd door niet-membraan gebonden enzymen. Slechts 5% van de totale elektronreductie door *L. sake*$^{sens}$ cellen is in de membraan fractie gemeten.

Daarnaast is onderzocht of de aanwezigheid van superoxide dismutase (SOD) deel zou kunnen uitmaken van het afweersysteem van LAB tegen oxidatieve stress. SOD is een enzym dat de giftige $O_2^-$ radicalen kan elimineren. De aanwezigheid en rol van SOD in LAB is momenteel onderwerp van discussie en dit rapport ondersteunt de hypothese dat SOD een rol speelt bij de $O_2$ detoxificatie in *Lactobacillus* spp. Dit is ondermeer gebaseerd op de waarneming dat de ongevoelige stam *L. sake* NCFB 2813 (*L. sake*$^{ins}$) grote hoeveelheden van tenminste één type SOD enzym bezit. Additioneel bewijs wordt gevormd door de theoretische niveaus van intracellulair gevormde $O_2^-$ radicalen voor (*L. sake*$^{sens}$) in vergelijking met het niveau in cellen van de $O_2$ ongevoelige mutant LSUV4 die uit deze gevoelige stam is voortgekomen en die SOD in overvloed bevat. SOD is dus waarschijnlijk van groot belang voor de verwijdering van het giftige bijproduct $O_2^-$, in ongevoelige *L. sake* stammen.

In **Hoofdstuk 5** wordt aangetoond dat het microbiologisch bederf van minimaal verwerkte, gesneden wortelen duidelijk wordt vertraagd als verhoogde concentraties $O_2$ en $CO_2$ in combinatie worden

toegepast. De voor het bederf verantwoordelijke microflora bestaat bij dit product voornamelijk uit Gram(-) bacteriën, met name *Pseudomonas* spp. Waarschijnlijk worden onder een gasatmosfeer van 50% $O_2$ + 30% $CO_2$ de interactie en competitie tussen bacteriesoorten (bijvoorbeeld tussen LAB en *Pseudomonas* spp.) versterkt, hetgeen zijn eerslag heeft op de microflora als geheel. De verhoogde $CO_2$ concentratie vertraagt bederf van het product door de Gram(-) bacteriën in de microflora te remmen, terwijl het verhoogde $O_2$ niveau met name de groei van LAB remt. Bij $O_2$ concentraties van 70 tot 90%, gecombineerd met $CO_2$ concentraties van 30% respectievelijk 10%, wordt de groei van LAB duidelijk vertraagd, hetgeen wijst op een algeheel bacteriestatisch effect.
Binnen het huidige concept van gemodificeerde atmosfeer verpakking (MAP) van ademende producten zoals gesneden wortelen, wordt het gebruik van 1 tot 3% $O_2$ en 10% $CO_2$ geadviseerd; er komt enig verschil in deze percentages voor in de literatuur afhankelijk van het specifieke product type. In de onderhavige studie wordt aangetoond dat minimaal behandelde wortelen $CO_2$ percentages tot 30% kunnen verdragen, terwijl ze wel $O_2$ percentages van 50% maar niet van 70% of hoger kunnen verdragen. Zulke hoge $O_2$ percentages hebben namelijk een schadelijk effect op de fysisch-chemische karakteristiek van de wortelen. Een fundamenteel technologisch voordeel van een met $O_2$ en $CO_2$ verrijkte atmosfeer, vergeleken met een atmosfeer met weinig $O_2$, is dat de uitloop van water uit het product onder de eerstgenoemde voorwaarden duidelijk wordt gereduceerd. Daarbij komt nog dat verhoogde $O_2$ niveaus resulteren in een verminderde productie van metabolieten die de geur of smaak beïnvloeden (zoals ethanol en acetaldehyde) en het totaal aan fenolen.
In **Hoofdstuk 6** is het effect van verhoogde gasdruk op de natuurlijke microflora van zalm gepresenteerd. Een mengsel van 50% $O_2$ + 50% $CO_2$ bij een druk van 2000 bar (200 MPa) en een temperatuur van 5±1°C gedurende de 30 min durende behandeling, resulteerde in een praktisch steriel product. Gedurende de opslag van zo behandelde en MA verpakte zalm werd geen uitgroei waargenomen. Behandeling van met opzet met pathogene bacteriën besmet product met gecomprimeerde gassen bij 150 MPa gedurende 15 min resulteerde eveneens in een reductie van pathogene micro-organismen. De effectiviteit hangt zowel af van de hoogte van de druk als van de tijdsduur van de behandeling. Bij gelijke intensiteit van de drukbehandeling hebben gecomprimeerde gassen een sterker anti-bacterieel effect op bederfveroorzakende en pathogene micro-organismen dan vacuum condities. Een verklaring voor deze waarneming is dat onder condities van bovennormale druk de concentratie van de opgeloste gassen wordt verhoogd.
Verder is aangetoond dat de toepassing van verhoogd $O_2$ in combinatie met verhoogd $CO_2$ en hoge druk een duidelijk positief effect heeft op de structuur en sommige andere organoleptische eigenschappen van zalm. Alhoewel de houdbaarheid van minimaal behandelde zalm werd verbeterd, was er een nadelig effect op het kleur en was oxidatieve ranzigheid van het product merkbaar. Deze studie is de eerste waarbij superkritische $O_2$ en vloeibare $CO_2$ In combinatie zijn toegepast
In **Hoofdstuk 7**, waar de belangrijkste resultaten van de onderhavige studie integraal worden besproken, wordt een model voorgesteld waarmee het mechanisme wordt beschrven waardoor in *L. sake* DSM 6333 intracellulaire oxiderende beschadiging optreedt in aanwezigheid van verhoogde $O_2$ concentratie. Toekomstige studies zouden kunnen worden gericht op de verificatie van dit model en verdere opheldering van de reakties van verschillende LAB, in het bijzonder *L. sake*, op verhoogde $O_2$ niveaus. Identificatie van de enzymen en genen die een rol spelen bij de synthese en inactivatie van ROS ($H_2O_2$, $HO\cdot$ en $O_2^-$) en bij de regulatie van het SOD afweersysteem, zou van groot belang zijn voor een beter begrip van het afweersysteem van LAB tegen oxidatieve stress onder hoog zuurstof condities.

# Περίληψη

Ο σκοπός της παρούσας διατριβής είναι να μελετηθεί η προοπτική της χρησιμοποίησης του οξειδωτικού στρες που παράγεται ως αποτέλεσμα της έκθεσης σε υψηλές συγκεντρώσεις οξυγόνου ($O_2$) για την συντήρηση τροφίμων που έχουν υποβληθεί σε σχετικά σύντομη και ήπια επεξεργασία. Το σκεπτικό της υπό μελέτης υπόθεσης στηρίζεται στη θεωρία της «τεχνολογίας τών εμποδίων». Η θεωρία αυτή πρεσβεύει ότι η παρεμπόδιση της ανάπτυξης των ανεπιθύμητων αλλοιογόνων ή/και παθογόνων μικροοργανισμών που αποτελούν δυνητικούς κινδύνους για την συντήρηση πολλών τροφίμων μπορεί να επιτευχθεί πιο αποτελεσματικά εάν περισσότερες απο μία μέθοδοι συντήρησης και επεξεργασίας των τροφίμων χρησιμοποιηθούν σε συνδυασμό. Τα επίπεδα στα οποία οι μέθοδοι αυτοί απαιτούνται στο συνδυασμό είναι χαμηλότερα από αυτά που θα απαιτούνταν, εάν, καθένας από αυτούς χρησιμοποιούνταν κατά μόνας για να παρεμποδιστεί η ανάπτυξη των μικροοργανισμών στον ίδιο βαθμό.

Μια συστηματική μελέτη της χρησιμοποίησης τού υψηλού $O_2$ σύμφωνα με το σκεπτικό της θεωρίας των εμποδίων δεν έχει αναφερθεί στη βιβλιογραφία, όπως φαίνεται στο **Κεφάλαιο 1** της παρούσας διατριβής. Παρόλα αυτά και με βάση την προυπάρχουσα βιβλιογραφία, η βασική αρχική θέση που λαμβάνεται και η οποία επιβεβαιώνεται στο **Κεφάλαιο 2** είναι ότι, υψηλές συγκεντρώσεις $O_2$ δεν μπορούν να χρησιμοποιηθούν σαν μοναδικός παράγοντας για να παρεμποδιστεί η ανάπτυξη των μικροοργανισμών. Οι συνθήκες που επικρατούν στον περιβάλλοντα του μικροοργανισμού χώρο δεν είναι στατικές και εξαρτώνται απο πολλούς ενδογενείς και εξογενείς παράγοντες. Κατά συνέπεια, η συγκέντρωση του $O_2$ που απαιτείται για να περιοριστεί η μεταβολική δράση και τελικά να θανατωθεί ο υπό μελέτη μικροβιακός πληθυσμός ποικίλει σημαντικά. Η ανάπτυξη οξειδωτικού στρες στούς προκαρυωτικούς οργανισμούς καταλύεται απο συγκεκριμένα στοιχεία που ειναι τοξικά προς αυτούς και που είναι γνωστά ως «ενεργά παράγωγα τού οξυγόνου» (ΕΠΟ). Τέτοια στοιχεία είναι το υπεροξείδιο τού υδρογόνου ($H_2O_2$) και το σουπεροξείδιο του οξυγόνου ($O_2^-$). Οι μικρογανισμοί εκείνοι για τους οποίους το οξυγόνο δεν είναι τοξικό κατέχουν αντιοξειδωτικά αμυντικά συστήματα προστασίας έναντι των παραγώγων αυτών. Το τελικό αποτέλεσμα της έκθεσης σε ΕΠΟ είναι το καθαρό αποτέλεσμα του ισοζυγίου παραγωγής /εξουδετέρωσης των ΕΠΟ.

΄Οπως αποδεικνύεται στη συγκεκριμένη διδακτορική διατριβή η χρησιμοποίηση υψηλού $O_2$ σε χαμηλές θερμοκρασίες προκαλεί μείωση τής ανάπτυξης και επηρεάζει τη ζωτικότητα των μικροβιακών κυττάρων λόγων διαταραχών της κυταρικής ομοιόστασης και ισορροπίας των κυττάρων. Διάφορα στελέχη γαλακτικών βακτηρίων τού γένους *Lactobacillus sake* που εξετάστηκαν στην παρούσα μελέτη κατέχουν διαφορετικά επίπεδα ενδογενών αντιοξειδωτικών (όπως χειλικές και αναγωγικές ιδιότητες) και αντιοξειδωτικών ενζύμων (όπως η δισμουτάση του σουπεροξειδίου του οξυγόνου) η παρουσία των οποίων αποτελεί ικανή και αναγκαία συνθήκη για τη διασφάλιση της ζωτικότητας των κυττάρων. Τα επίπεδα στα οποία τα στοιχεία αυτά απαντώνται στα κύτταρα διαφέρουν μεταξύ στελεχών του ίδιου είδους ανάλογα με την προέλευση και την προιστορία κάθε στελέχους, όπως αποδεικνύεται στα **Κεφάλαια 3** & **4**. **Στο Κεφάλαιο 7** παρουσιάζεται ένα προτεινόμενο μοντέλο που περιγράφει τις συνθήκες που οδηγούν στιν ανάπτυξη οξειδωτικού στρες στον ενδοκυταρικό χώρο στελεχών τού γένους *Lactobacillus sake*. Το μοντέλο αυτό, μπορεί να περιγράψει το μηχανισμό ανάπτυξης οξειδωτικού στρες και για άλλα γαλακτικά βακτήρια στα οποία η επίδραση του υψηλού $O_2$ υπό χαμηλές θερμοκρασίες δεν έχει μελετηθεί συστηματικά έως σήμερα.

Η πρακτική εφαρμογή του σκεπτικού της θεωρίας των εμποδίων για τη συντήρηση των τροφίμων με υψηλό $O_2$ ως πρόσθετο παράγοντα, εξετάζεται με δύο παραδείγματα ευαλοίωτων προιόντων με εμπορικό ενδιαφέρον (ψάρια και φρέσκα λαχανικά) στα **Κεφάλαια 5** & **6** της διατριβής. Στα παραδείγματα αυτά, υψηλό $O_2$ χρησιμοποιείται σε συνδυασμό με άλλες μεθόδους συντήρησης, όπως χαμηλή θερμοκρασία, διοξείδιο τού άνθρακα ή/και υψηλή πίεση για την συντήρηση προιόντων όπως τεμαχισμένα καρότα (που χρησιμοποιούνται ως συστατικά έτοιμων για κατανάλωση σαλατών) και φρέσκος σολομός. Επίπεδα $O_2$ που κυμαίνονται μεταξύ 50 και 90% απαιτούνται για να

παρεμποδίσουν σημαντικά την ανάπτυξη των αλλοιογόνων και παθογόνων μικροοργανισμών στα προιόντα αυτά, ανάλογα με το είδος και τα επίπεδα των άλλων παραγόντων που χρησιμοποιούνται σε συνδυασμό με το υψηλό $O_2$. Η ανεκτικότητα των προιόντων στις υψηλές συγκεντρώσεις του $O_2$ είναι ένας παράγοντας που θα πρέπει να ληφθεί υπόψην εάν το σκεπτικό του υψηλού $O_2$ πρόκειται να χρησιμοποιηθεί σε εμπορική κλίμακα για την συντήρηση των συγκεκριμένων προιόντων, καθώς οξειδωτική αλλοίωση των φυσικοχημικών συστατικών παρατηρήθηκε κάτω από τις υπό μελέτη συνθήκες συντήρησης.

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## Curriculum vitae

Athina Amanatidou was born in 10 September 1970 in Egaleo, Athens. She completed the basic studies at the Public Lyceum in Egaleo in 1989. After she succeeded the panhellenic exams, she started a five-year study at the department of Food Technology of the Agricultural University of Athens. From June until September 1992 and from June until September 1993 she did her training periods at the Heineken BV, in Renti, Athens and at the National Agricultural Research Foundation, (NAGREF), in Lycovrisi, Athens. The last year of the study she was specialised in Food Microbiology. Research training periods were done at the Department of Dairy Products of the AUA (Prof. Dr. P. Kalantzopoulos) and at the Department of Applied Microbiology of NAGREF (Prof. Dr. G.J.E. Nychas) on the topics of Dairy and Food Microbiology, respectively. She obtained her M.Sc. degree in Food Technology & Engineering in November 1995. Between November 1994 and November 1995, she was employed at the AUA, where she assisted Prof. Dr. G.J.E. Nychas in setting up and management of the newly founded Laboratory of Food Biotechnology & Applied Microbiology. At the same time, she worked as a junior research scientist in the EU project "Predictive Modelling of Shelf Life of Fish and Meat Products".
Between August and October 1995 she worked at the department of Microbiology & Preservation at ATO-DLO in Wageningen, The Netherlands (Dr. M.J.H. Bennik and Dr. L.G.M. Gorris) in terms of a co-operation between ATO and the Lab of Food Biotechnology & Applied Microbiology of AUA for the EU project "Improving Safety and quality of Refrigerated Ready-to-Eat Foods using Mild Preservation Methods". Between January 1995 and June 1999 she did her Ph.D. research at the Lab of Microbiology & Safety, under the supervision of Dr. L.G.M. Gorris, Dr. E.J. Smid and Prof. Dr. Ir. F.M. Rombouts in a research project that was funded by the Dutch Government, the European Union and ATO. She got married in September 1998 and in November of that year she worked as a guest researcher at the Department of Food Biotechnology and Food Process Engineering of the Technical University of Berlin, Germany (Prof. Dr. D. Knorr). Between August 1999 and August 2001 she worked at Unilever Research Vlaardingen, the Netherlands. She was involved in multidisciplinary research and development projects at the department of Preservation & Safety of this company.

## List of publications

### Peer reviewed articles

**Amanatidou, A., Smid, E.J., & Gorris, L.G.M.** (1999) Effect of elevated oxygen and carbon dioxide on the surface growth of vegetable associated micro-organisms. *Journal of Applied Microbiology*, 86: 429-438

**Amanatidou, A., Slump, R., Gorris, L.G.M & Smid, E.J.** (2000) Microbiological and physicochemical changes of sliced carrots stored under high oxygen-controlled atmospheres. *Journal of Food Science*, 65: 61-66

**Amanatidou, A., Schlüter, O., Lemcau, K., Gorris, L.G.M, Smid, E.J. & Knorr, D.** (2000) Effect of combined application of high pressure treatment and modified atmospheres on shelf life of fresh atlantic salmon. *Innovative Food Science and Emerging Technologies*, 1: 87-98.

**Amanatidou, A., Bennik, M.J.H., Gorris, L.G.M. & Smid, E.J.** (2001) Superoxide dismutase plays an important role in the survival of *Lactobacillus sake* upon exposure to elevated oxygen. Archives in Microbiology 176: 79-88

**Amanatidou, A., Smid, E.J., Gorris, L.G.M.** (2001) Antioxidative properties of *Lactobacillus sake* upon exposure to elevated oxygen (*FEMS Microbiology letters, in press*)

**Amanatidou, A., Slump, R.A, Smid, E.J.** (2002) Microbial interactions under elevated oxygen and carbon dioxide on minimally processed carrots" *Acta Horticulturae Proceedings Int. symposim CA 2001*, Rotterdam 9-14 July 2001 (*in press*)

**Amanatidou, A., Pin, K., Baranyi, J., Gorris, L.G.M. & Smid, E.J.** Modelling interactions of the spoilage flora of minimally processed carrots in relation to temperature and modified atmospheres (in preparation)

### Abstracts and other publications

**Amanatidou A., Kets, E., Smid E.J. & Gorris, LG.M. (**1997) High oxygen modified atmosphere packaging, a novel approach with minimally processed vegetables COPERNICUS workshop "Process Optimisation & Minimal Processing of Food", Leuven, Belgium, 13-14 October 1997

**Amanatidou, A., Tijskens, P.L.M. Bennik M.J.H, Smid, E.J and Gorris, L.G.M.** (1998) Growth of spoilage flora of vegetables in a high oxygen modified atmosphere system in relation with keeping quality. Proceeding of the COPERNICUS workshop "Shelf-life Prediction for Safety and Quality of Food", Wageningen, The Netherlands, 26-27 November, 1997

**Amanatidou A., Slump, R.A. & Smid E.J.** (1998) Minimal processing, a tool for extending shelf-life of sliced carrots A-case study. Poster presented in T.U.B. Berlin, Germany, July 1998

**Amanatidou A., Gorris, LG.M. & Smid E.J.** (1999) "Responses of food related lactic acid bacteria to high oxygen: an update". Proceedings Int. Symposium: Microbial Stress and Recovery in Food, Quimper, France, 14-16 June 1999

**Amanatidou, A., Schlüter, O., Smid, E.J. & Knorr, D.** (1999) Antimicrobial activity of $O_2$ and $CO_2$ under pressure on food-related pathogens". Proceedings Int. Symposium: Microbial Stress and Recovery in Food, Quimper, France, 14-16 June 1999

**Amanatidou, A., Knorr, D., Smid, E.J. & Gorris, L.G.M.** (1999) Effect of compressed gases on survival and growth of spoilage flora of fresh salmon. Proceedings Int. Symposium Food Micro 99, Velthoven, the Netherlands, 13-17 September 1999

**Amanatidou, A. & Nychas L.G.M.** (2001) Comparative study of the application of different methods to identify lactic acid bacteria isolated from Greek meat products. Proceedings Int. Synposium Microbiologia Balcanica, Thessaloniki, Greece, 22-24 November 2001

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