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Listeria monocytogenes - inactivation by essential oils and High Pressure, and contribution of genetic variation to stress resistance

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To my parents

CONTENTS

CHAPTER 1

Introduction

1. *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive facultative anaerobic bacterium, which can cause gastroenteritis and more importantly listeriosis, a serious disease with high mortality in immunocompromised people, unborn children, and neonates (Cox 1989). It is regarded as one of the most important foodborne pathogens of the last years (Lovett, 1989; Sci. Com of E.C., 1999), and numerous foodborne outbreaks and sporadic cases of listeriosis have been reported, mostly in North America and Europe (Cox 1989; Lovett, 1989; Sci. Com of E.C., 1999). There has been an increase in incidents of listeriosis over the last years, but it is unclear if this is due to better diagnostics and reporting and/or awareness of the disease (Sci. com of E.C., 1999). Cases of listeriosis are rare (2-15 cases per 10^6 inhabitants), and approximately 5-10% of the general population is carrier of the organism, usually without developing listeriosis (Sci. Com of E.C., 1999; Lou and Yousef 2000; Notermans and Hoornstra 2000). However, when listeriosis occurs, the mortality can range from 20 to 40 %, and even up to 75% for immunocompromised individuals (Sci. Com of E.C., 1999).

L. monocytogenes is able to grow at temperatures ranging from – 0.4 to 44^{o}C (Lovett, 1989; Walker *et al.*, 1990), pH values from pH 5 to 9, NaCl concentrations up to 10%, and this organism generally survives mild preservation treatments (Linton 1992). These features make this bacterium a difficult but very important target organism to be eliminated from the food chain. As proposed by the scientific committee on veterinary measures related to public health of the E.C. in September of 1999, *L. monocytogenes* levels should be lower than 100 cfu g^{-1} throughout the shelf life of the food product (Sci. Comm. of E.C., 1999).

2. Mild preservation and novel techniques in food processing

Heat treatment has been one of the main methods of food preservation for centuries, because of its prominent inactivating effects on enzymes and microorganisms. Often, heat treatment results in the reduction of the nutritional quality of foods and it can affect the organoleptic value of the treated product (Smelt, 1998). Such negative effects of heat treatment and, in addition, the possible negative health effects or the alteration of the natural organoleptic characteristics that some additives could cause (e.g. nitrates, NaCl), prompted the exploration of novel processing methods and new combinations of (mild) food preservation techniques. The concept of ‘Hurdle technology’ (Leistner, 1999) gained momentum, with the overall aim to produce nutritious and mildly or minimally processed foods that are safe for consumption. Clearly, a prerequisite of mild food processing methods is their capacity to efficiently suppress

outgrowth and/or reduce the numbers of dangerous pathogenic bacteria, like *Listeria monocytogenes*.

2.1 Essential oils

2.1.1 General

Various herbs that have been used by humans for thousands of years, such as oregano and basil, contain volatile compounds with antimicrobial properties. These compounds, which can be extracted in the so-called essential oil fraction, have been explored for their use in the preservation of foods in recent years. Essential oil components include S-carvone from caraway seed, carvacrol from oregano, thymol from thyme and oregano, and cinnamaldehyde from cinnamon (Table 1) (Hartmans *et al.*, 1995; Smid and Gorris, 1999). An important characteristic of these essential oil compounds is their hydrophobic nature, which allows them to accumulate in the bacterial cytoplasmic membrane, eliciting toxic effects to the cells (Sikkema *et al.*, 1994). The main disadvantage of applying these substances in foods is their low solubility in water and their usually distinct odor. In addition, their antimicrobial properties are affected by the membrane fluidity of the targeted cells. These characteristics limit their broad use in a wide range of products. However, in certain cases, these antimicrobial compounds can be applied successfully, and in the context of Hurdle Technology, they can offer an additional obstacle for microbial growth.

2.1.2 Mode of action

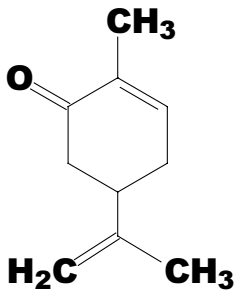
As mentioned above, the antimicrobial properties of several essential oil compounds results from their highly hydrophobic character. Their accumulation in the cellular membrane perturbs the membrane, resulting in increased permeability to protons and ions and eventually cell-death (Sikkema *et al.*, 1995). Different essential oil compounds with comparable hydrophobicities can demonstrate different antimicrobial effects (Smid and Gorris, 1999; Ultee *et al.*, 2001). The presence of specific reactive groups in the compounds plays an additional role on their antimicrobial properties. This was demonstrated clearly in the case of carvacrol and its natural precursor cymene (see Table 1 for chemical structures), that does not contain a hydroxyl group (Ultee et al., 2001). These authors demonstated that the antimicrobial activity of carvacrol is approximately 10-fold higher than that of cymene, indicating the importance of the hydroxyl group. An additional feature that increases the antimicrobial effect of these essential oil compounds is the presence of a system of conjugated double bonds (Ultee et al., 2001, Ultee *et al.*, in press), as indicated by a demonstrated ~ 10-fold lower antimicrobial activity of menthol compared with carvacrol. In addition, the antimicrobial activity of these compounds is not restricted to the lipid part of the membrane; proteins embedded in the membrane are also affected (Sikkema *et al.*, 1995).

2.2 High Hydrostatic Pressure (HHP)

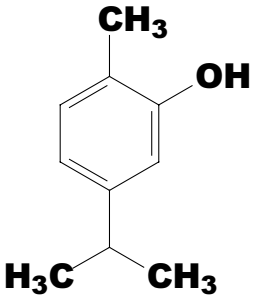
2.2.1 HHP treatment of foods

At the surface of the earth the ambient pressure is 0.1 MPa, while at the deepest point of the oceans the pressure is about 100 MPa, and at the center of the earth it is about 360 GPa (Palou *et al.*, 1999). The term “high hydrostatic pressure” (HHP) in food applications represents

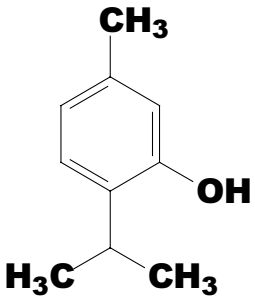
Table. 1 The chemical structures of essential oil components, namely, S-carvone from caraway, carvacrol and thymol from oregano, cinnamaldehyde from cinnamon, cymene from cumin, menthol which is the biological precursor of carvacrol, and n-decanal from apple.



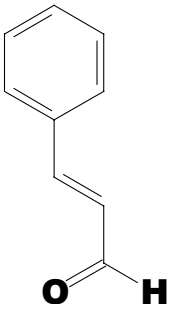
Carvone



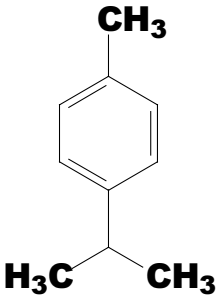
Carvacrol



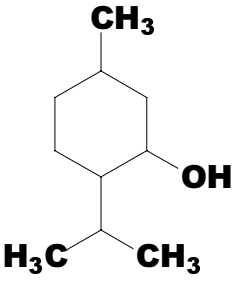
Thymol



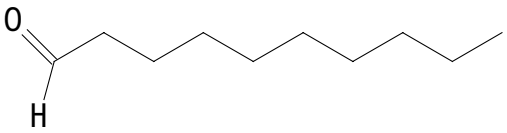
Cinnamaldehyde



Cymene



Menthol



n-Decanal

pressures in the range of 50 to 1000 MPa. The antimicrobial effect of HHP was demonstrated as early as 1899 in experiments done by Hite (Hite, 1899). HHP treated foods have been commercially available only since 1990 in Japan and since 1996 in Europe and the USA (Knorr *et al.* 1998). HHP treatment of foods effectively reduces the numbers of vegetative cells of microorganisms present, and it inactivates enzymes, without greatly affecting the nutritional value and the organoleptic characteristics of the food. Furthermore, as a result of the instant distribution of pressure, it is effective throughout the food product, independent of size and geometry (Smelt, 1998). The latter is valid only in cases of liquid products, as pressure is not homogeneously distributed in solid food products and it could degrade their structure. In addition, HHP can only be used for pasteurisation purposes, as the inactivation of bacterial spores requires pressures up to 1000 MPa (Smelt, 1998), which would lead to unacceptable increases in costs of a product.

2.2.2 Mode of action of HHP

In general, increase in pressure causes decrease in volume. According to Le Chatelier's principle, a system in equilibrium tends to minimise the effect of any external factor that is perturbing it. Every increase in pressure would favour changes that cause a decrease in volume and thus would increase the order of the system (Heremans, 1982; Mozhaev, *et al.*, 1996). In this view, it is apparent that an increase in temperature is antagonistic to pressure, because elevation of the temperature would cause a volume increase, and thus a decrease in the order of the system. Every phenomenon that affects the volume of a system is thereby influenced strongly by pressure changes. In general, electrostatic and hydrophobic interactions are highly affected by pressure, while covalent and hydrogen bonds are mainly unaffected (Heremans, 1982; Mozhaev, *et al.*, 1996).

A phenomenon associated with HHP treatment is a decrease in pH. A pressure increase causes separation of electrical charges, allowing water molecules to be positioned with a higher range of order, resulting in the decrease of the total volume of the system. This phenomenon is called electrostriction and causes ionic dissociation, resulting in a pH reduction, with numerous implications on various biological functions. HHP also favours phase transitions that would decrease the volume of the system. For example, a pressure increase would cause increase of the melting temperature of lipids (10^{o}C for every 100 MPa) and decrease of the melting temperature of water. These phenomena are triggered by the increased volume of lipids in liquid phase compared to the solid state, and the increased volume of the crystals of ice compared with water in liquid phase, respectively (Cheftel, 1995).

As apparent from the above, HHP affects a combination of processes that take place in the cell, and does not inhibit or disrupt only one specific function (Metrick *et al.*, 1989). It is obvious that different cellular functions are inactivated at different pressures depending on various factors, like the stability of the enzymes or the chemical substances involved. Furthermore, an important factor is the significance of the disrupted function for the survival of the cell. One of the most important biological structures is the cell membrane, which has been proven to play an important role to the survival of cells upon HHP. In general, less fluid membranes are more sensitive to HHP (MacDonald *et al.*, 1992). Also, Gram positive bacteria are more piezotolerant than Gram negative bacteria, probably as a result of a more robust cell envelope that contains a high percentage of peptidoglycan and teichoic acids (40-90%), in contrast to only 10-20% peptidoglycan and no teichoic acids for the Gram negative bacteria

(Salton, 1994; Palou *et al.*, 1999). In eukaryotes, the nuclear, mitochondrial, vacuolar and cellular membranes are affected by HHP (Shimada *et al.*, 1993; Kobori *et al.*, 1995; Sato *et al.*, 1995; Sato *et al.*, 1996)

HHP furthermore has an influence on protein functions, and can lead to irreversible denaturation above a certain pressure threshold. The effect of pressure on proteins has major implications for cellular functions, since proteins constitute 25-55% of the dry weight of the cell. Pressure does not have an effect on the primary structure of the proteins, which are assembled by covalent bonds that are virtually unaffected by pressure. HHP slightly affects the secondary, but mainly the tertiary and quaternary structure of proteins that are stabilised by electrostatic and hydrophobic interactions. In general, HHP highly affects membranes and proteins, and to a lesser extent nucleic acids. DNA and RNA contain covalent bonds, and in the case of DNA also hydrogen bonds, which are not disrupted by HHP.

In addition to the detrimental effects that HHP can have on cellular components, it can also disturb macromolecular synthesis. In *Escherichia coli*, the synthesis of DNA, proteins and RNA stops at 50, 58, and 77 MPa, respectively (Yayanos and Pollard, 1969). Seemingly, there are many cellular targets on which HHP can act, and it is very difficult to define a specific mode of action for HHP, as pressure is a parameter affecting a broad range of biological and physical phenomena.

2.2.3 Resistance to HHP

In the distant past (3.5 billion years ago), the ultraviolet light from the sun was probably too strong on the land or in shallow water to permit the development of living organisms, and life likely originated in the deep sea. The first organisms in this environment must have developed systems that allowed them to live in that relatively high pressure environment. Since the majority of the earth is covered by sea, high pressure might be a familiar environment for life. In evolutionary terms, it could be more appropriate to talk about adaptation of life to ambient environment rather than adaptation of life to a high pressure environment (Turley, 2000). Evidence for that is the fact that a high-pressure resistant system for gene expression is found not only in the deep-sea-adapted bacteria, but also in atmospheric-pressure-adapted bacteria such as *E. coli*, indicating that systems developed in a high pressure environment may be conserved in microorganisms adapted to atmospheric pressure (Kato, 1995).

Unfortunately, there is little information regarding the mechanisms of bacterial adaptation and resistance to high pressure. The ultimate pressure resistant living structures are the bacterial spores, which are capable of withstanding pressures of 1000 MPa (Palou *et al.*, 1999). Their high resistance compared with other forms of life results from their rigid structure and the absence of metabolic activity. With respect to vegetative cells, HHP tolerance –or piezotolerance- depends on the genetic make-up of the organism and the physiological state of the cell. Features regarding those two characteristics could explain the increased piezotolerance of certain cells within microbial populations.

The physiological state of a microorganism can influence its piezotolerance. Increased resistance to HHP has been demonstrated for cultures that were grown at relatively high temperatures, for cultures in stationary growth phase, and for starved cells (Iwahashi *et al.*, 1991; Mackey *et al.*, 1995; Casadei and Mackey 1997). The physiological stress response induced by these conditions protects cells against HHP, probably by the increased synthesis of stress proteins. In *E. coli*, induced expression of 55 proteins was observed upon exposure to a pressure upshift to 55 MPa, and many of these proteins were previously identified as heat

shock or cold shock proteins (Welch *et al.*, 1993). In this thesis, we furthermore demonstrate that increased HHP tolerance of *L. monocytogenes* coincided with increased expression of the ClpP heat shock protein. It seems that the ability of the cell to cope with misfolded and denaturated proteins that may be toxic to the cell is essential for the observed resistance to both heat and HHP. This might explain observations by other authors who reported increased heat resistance of HHP resistant microorganisms (Iwahashi *et al.*, 1993; Smelt, 1998).

Different bacterial species demonstrate different resistances to HHP. In addition, great variability in the piezotolerance has been reported among strains of the same species (Metrick *et al.*, 1989; Hauben *et al.*, 1997; Alpas *et al.*, 2000) and even for cells within the same population (Hauben *et al.*, 1997; this thesis). The variability in HHP resistance of different species, strains, and even cells within a population make the task of designing HHP treatments that cause adequate reductions of bacteria quite difficult, and insight in the underlying resistance mechanisms is therefore highly desirable.

2.3 Combined processing

Variable resistance of bacterial populations to inactivation treatments can pose problems to ensure microbiological food safety and quality of foods. To overcome these problems, the intensity of the treatment applied could be increased. However, this in turn could be economically not feasible in the case of HHP treatments, or detrimental to the quality of the product in the case of *e.g.* heat treatment. A possible solution in such cases is the employment of an additional hurdle that could act synergistically or additively against microorganisms. In this thesis we demonstrate that essential oils can play a role as an additional preservation factor. A similar approach could be followed for other antimicrobial compounds such as bacteriocins, lysozyme, α-terpinene, (R)-(+)-limonene (Adegoke *et al.* 1997, Pol and Smid, 1999). An important aspect is that combined processing could also contribute to the further development of classical techniques like heat treatment (this thesis). It is apparent that the concept of combined processing could be applied with a plethora of treatments and generate new combinations of preservation methods.

3. Outline of this thesis

In **Chapter 1** a short introduction to novel preservation techniques and combined treatments in the food industry is given. Particular attention is paid to the foodborne pathogen *Listeria monocytogenes* and two relatively novel food preservation techniques, namely, the use of essential oils and High Hydrostatic Pressure.
In **Chapter 2** volatiles from plant-derived essential oils, like S-carvone, and mild heat are demonstrated to have a synergistic effect on inactivation of *L. monocytogenes*.
In **Chapter 3** the synergistic antimicrobial effect of High Hydrostatic Pressure and carvacrol or thymol on *L. monocytogenes* is reported.
Chapter 4 describes the isolation and the phenotypic characterisation (*e.g.* multiple stress resistance) of a High Pressure tolerant -or piezotolerant- strain of *L. monocytogenes*, designated AK01. Also it is shown that the increased piezotolerance of this strain compared to the wild type is not a result of altered cellular membrane properties.

In **Chapter 5**, we demonstrate that increased resistance of strain *L. monocytogenes* AK01 to HHP and other stresses is caused by a mutation in the *ctsR* (class three stress gene repressor) gene. It is furthermore demonstrated that this mutation leads to reduced virulence and immobility.
In **Chapter 6** the major findings presented in this work are discussed. Some additional information regarding the functions of genes mentioned in this thesis, as well as their role in the regulatory network of *L. monocytogenes* is presented. Also further implications of the work presented in this thesis are discussed.

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CHAPTER 2

Combined Action of S-Carvone and Mild Heat Treatment on *Listeria monocytogenes* Scott A

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ABSTRACT

The combined action of the plant-derived volatile, S-carvone and mild heat treatment on the foodborne pathogen, *Listeria monocytogenes*, was evaluated. The viability of exponential phase cultures grown at 8°C could be reduced by 1.3 log units after exposure to S-carvone (5 mmol l^{-1}) for 30 min at 45°C, while individual treatment with S-carvone or exposure to 45°C for 30 min did not result in a loss in viability. Other plant-derived volatiles, namely carvacrol, cinnamaldehyde, thymol, and decanal, were also found to reduce the viability of *L. monocytogenes* in combination with the same mild heat treatment at concentrations of 1.75 mmol l^{-1}, 2.5 mmol l^{-1}, 1.5 mmol l^{-1}, or 2 mmol l^{-1}, respectively. These findings show that essential oil compounds can play an important role in minimally processed foods, and can be used in the concept of Hurdle Technology to reduce the intensity of heat treatment or other individual hurdles.

INTRODUCTION

A recent trend towards the consumption of natural healthy foods has strongly favoured the use and development of mild processes in food preservation. These processes rely on low intensity levels and low doses of treatment, which thereby keep the fresh characteristics of products intact. To ensure the microbial safety and quality of mildly processed foods, it is critical that the proliferation of spoilage bacteria or foodborne pathogens is inhibited or eliminated. The application of only one specific mild treatment might not be effective in controlling the outgrowth of undesirable micro-organisms. However, this may be achieved by combining several mild preservation techniques simultaneously, as advocated by the Hurdle Technology concept. Using an intelligent combination of hurdles (i.e. preservation factors) can effectively improve the microbial safety and maintain the sensory and nutritional quality of foods (Leistner 1999).

A particular concern for the food industry and public health is the outgrowth of foodborne pathogens that may be able to survive mild preservation treatments. A prominent pathogen is *Listeria monocytogenes*, as this Gram-positive facultative anaerobic has caused numerous outbreaks and sporadic cases of listeriosis, mostly in North America and Europe (Cox 1989; Lovett 1989; Bunduki *et al.* 1994; Vines and Swaminathan 1998; Leistner 1999; Vasseur *et al.* 1999). This bacterium is widely distributed in food products (Papageorgiou and

Marth 1988; Brackett 1988) and is able to proliferate at refrigeration temperatures (Vasseur *et al.* 1999).

L. monocytogenes has been shown to survive mild heat treatments (Linton 1992). However, an effective control of this bacterium might be achieved by applying such treatments in the presence of antimicrobial substances. Certain plant-derived flavour compounds can have antimicrobial activity (Smid and Gorris. 1999) and may serve as a suitable additional hurdle. Plant-derived volatiles such as S-carvone, carvacrol, cinnamaldehyde, thymol, and decanal are known for their antimicrobial activity (Juven *et al.* 1994; Ultee *et al.* 1998; Smid and Gorris 1999) and might be suitable for use in Hurdle Technology in combination with other hurdles (e.g. heat treatment). A common characteristic of these compounds is their hydrophobic nature that allows for accumulation in the bacterial cytoplasmic membrane, where they can elicit several toxic effects that may eventually lead to cell death (Juven *et al.* 1994; Sikkema *et al.* 1994). Differences in the mode of action of these compounds may be related to the presence of specific reactive groups (Smid and Gorris 1999), as was demonstrated for cinnamaldehyde and S-carvone (Smid *et al.* 1995).

The main objective of this study was to assess whether the combined action of mild heat treatment and S-carvone could reduce the viability of *L. monocytogenes*. In addition, other plant-derived essential oil compounds such as carvacrol, cinnamaldehyde, thymol, and decanal were tested for their ability to reduce the viability of *L. monocytogenes* when combined with mild heat.

MATERIALS AND METHODS

Micro-organism and growth

L. monocytogenes Scott A (Department of Food Science, Wageningen University Research Centre, The Netherlands) was used throughout this study. The stock culture was kept at –80^{o}C in 15% (v/v) glycerol. This culture was transferred to 9 ml of sterile Brain Heart Infusion (BHI broth; Oxoid) and incubated at 35^{o}C overnight. The overnight culture was subcultured in 9 ml of sterile Brain Heart Infusion (BHI broth; Oxoid) and incubated at 35^{o}C overnight prior use.

Chemicals

S-carvone, carvacrol, cinnamaldehyde, thymol, and decanal, were obtained from Fluka Chemie AG (Buchs, Switzerland). All purified volatiles were kept in stock solutions (1mol l^{-1}) in ethanol. Chloroform and methanol were obtained from Lab-scan (Dublin, Ireland). Sodium sulphate and n-hexane were obtained from Merk (Darmstadt, Germany), and naphthalene from J.T. Baker (Deventer, The Netherlands).

Effect of S-carvone on the growth of L. monocytogenes

A 0.1 ml inoculum of the second overnight culture was added to 100 ml of BHI broth with or without S-carvone (5 mmol l^{-1}). Cultures were incubated in a shaking incubator (160 rev min^{-1}) at 8^{o}C and growth was monitored by measuring the optical density at 660nm ($O.D._{660}$). Viable counts were determined at regular time intervals by preparing decimal dilutions of samples in peptone-physiological salt (1.5 g l^{-1} peptone and 8.5 g l^{-1} NaCl) and plating these on BHI agar (1,2 % w/v). Colony forming units (cfu) were determined after incubation at 30^{o}C

for 3 days and from these counts, the exponential growth rate was calculated using the following equation: $\mu=(\ln N_t - \ln N_0) \cdot (t-t_0)^{-1}$
where N_t is the cell population at the time t (Schlegel 1992).

Treatment of L. monocytogenes with essential oil compounds and heat

A 0.1 ml inoculum of the second overnight culture of *L. monocytogenes* was added to 100 ml BHI medium with or without S-carvone (5 mmol l^{-1}). Cultures in BHI broth, or in BHI broth containing S-carvone, were incubated in a shaking incubator (160 rev min^{-1}) at 8, 35 or 45°C or at 8 or 45°C, respectively. Cells were harvested at an $O.D._{660}$ between 0.1 and 0.4 in the case of mid-exponential phase cells, and stationary phase cells were harvested at an $O.D._{660}$ of approximately 0.9; they were then centrifuged at 10 000 *g* for 10 min at 8°C. Cultures were washed twice in N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) buffer pH 7.0, kept at 8°C, and resuspended in HEPES buffer to an $O.D._{660}$ of 0.1. S-carvone was added to the cell suspensions to a final concentration of 5 mmol l^{-1}, using a freshly prepared stock solution of 1mol l^{-1} S-carvone in ethanol. Control suspensions received equivalent amounts of ethanol, and no adverse effects of ethanol on growth and survival of *L. monocytogenes* were observed. Suspensions were incubated at either 8°C, or in a boiling waterbath until 45°C had been reached (within 80 s), followed by incubation in a water-bath at 45°C for a maximum of 30 min. At regular time intervals, samples were taken, diluted in peptone-physiological salt (1.5 g l^{-1} peptone and 8.5 g l^{-1} NaCl), and the viability of the cells in the suspension determined by plating onto BHI agar (1,2 % w/v) followed by incubation at 30°C for 3 days.

In addition to studying the effect of S-carvone in combination with heat treatment on survival of *L. monocytogenes*, the survival of this pathogen was determined after treatment with different concentrations of carvacrol, cinnamaldehyde, decanal, and thymol in combination with a mild heat treatment (45°C, 30 min). The procedure followed was the same as for S-carvone (see the protocol described above), except that the volatiles were added to suspensions after a temperature of 45°C had been reached.

Determination of S-carvone in cultures

The presence of S-carvone in the medium was tested in a 5- and a 25-day old culture. Cultures were sterilized by filtration, using 0.2 μm filters (Gyro Disc CA, Orange Scientific, Waterloo Belgium). To determine whether S-carvone was retained on the filter during filtration, the concentration of S-carvone in filtered and unfiltered BHI medium containing 5 mmol l^{-1} S-carvone, was compared.

Extraction of S-carvone was carried out according to a modified method of Bligh and Dyer (1959). In short, a 40 ml sample was added to a solution of 100 ml methanol and 50 ml chloroform, and mixed for 2 min. Subsequently, 50 ml chloroform was added, and the solution was mixed for 30 s. Finally, 50 ml of distilled water was added followed by mixing for 30 s. The mixture was left overnight for phase separation of the chloroform and aqueous layers. The chloroform phase was treated with sodium sulphate to remove the remaining water. After filtration, chloroform was removed using a rotovapor (Büchi, Flawil, Switzerland) and samples were diluted in *n*-hexane. Naphthalene was added as an internal standard. S-carvone was determined using thermal desorption gas chromatography.

Thermal desorption was performed with a Tenax tube (Chrompack, Bergen op Zoom, the Netherlands) on a thermal desorption autosampler (Carlo Erba, Milano, Italy) (flow: 20 ml

min^{-1}), followed by gas chromatography (Carlo Erba HRGC 5300, gas chromatograph), using a fused silica WCOT CP-Sil-19 CB column (25 m * 0.32 mm, Chrompack), at gas flow rates of, 2.5:300:20 ml min^{-1} for He_2, air, and H_2, respectively. Detection was performed using flame ionisation at 260°C.

RESULTS

Antibacterial activity of S-carvone

The effect of S-carvone on the growth of *L. monocytogenes* was determined by culturing the cells at 8°C in the presence and absence of 5 mmol l^{-1} S-carvone and determining the viable counts (Fig. 1). The maximum specific growth rate (μ_{max}) of *L. monocytogenes* was reduced in the presence of S-carvone (0.024 h^{-1}) compared with unexposed cultures (0.035 h^{-1}). In addition, lower final population densities in medium containing S-carvone ($6.3x10^8$ cfu · ml^{-1}) than in the absence of S-carvone ($3.3x10^9$ cfu · ml^{-1}) were observed (Fig. 1).

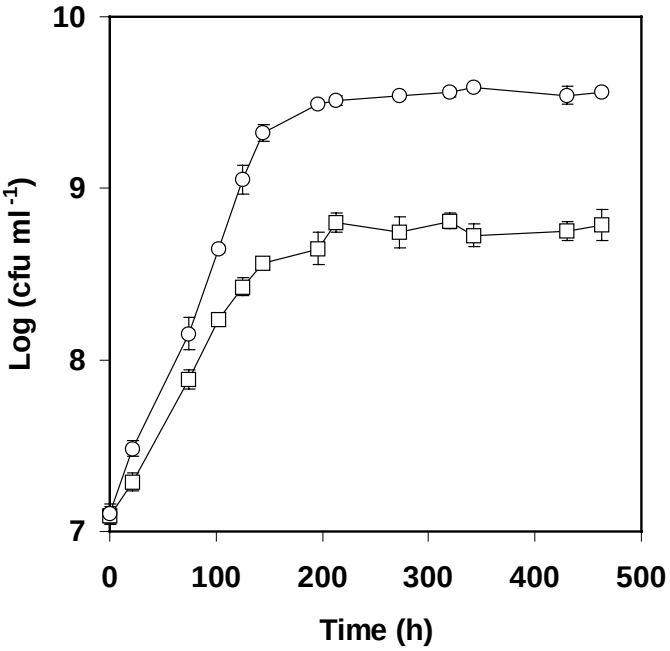

Fig. 1 Growth of *Listeria monocytogenes* in the presence of 5 mmol l^{-1} of S-carvone (□), and in absence of S-carvone (○). Cells were grown in BHI broth at 8°C with shaking (160 rev min^{-1}).

Essential oils and heat treatment

Addition of 5 mmol l^{-1} S-carvone to a washed cell suspension of *L. monocytogenes* grown and kept at 8°C, did not reduce its viability. Similarly, subjecting cells to a mild treatment of 45°C only produced no reduction of the viability. However, the combined treatment with S-carvone (5 mmol l^{-1}) at 45°C resulted in a 1-2 log reduction in viability (Fig. 2A). The D-value (time needed for 1 log reduction of a microbial population) was 22 min.

When cells from the mid-exponential phase grown at 35°C or 45°C were subjected to S-carvone (5 mmol l^{-1}) at 45°C, no reduction in viable counts during 30 min was observed (data not shown). This indicates that the growth temperature prior to treatment is a critical factor in successfully reducing the numbers of *L. monocytogenes* by combined treatment.

Furthermore, it was observed that the growth phase of cells influenced the effect of 5 mmol l^{-1} S-carvone at 45°C. Subjecting stationary phase cells of *L. monocytogenes* (grown at 8°C or 45°C) to treatments similar to those mentioned above did not result in reduced viable numbers (data not shown).

To determine whether *L. monocytogenes* cells are able to adapt to S-carvone, and thereby loose sensitivity to the combined treatment, cells were cultured in the presence of S-carvone (5 mmol l^{-1}) at 8°C, harvested in the mid-exponential phase of growth, and subsequently exposed to S-carvone (5 mmol l^{-1}) in combination with mild heat treatment of 45°C. The reduction in viability for cells pre-grown in the presence or absence of S-carvone was similar (1-2 log reduction of viability in 30 min, Fig. 2B), indicating that pre-exposure to S-carvone did not result in adaptation to the combined treatment.

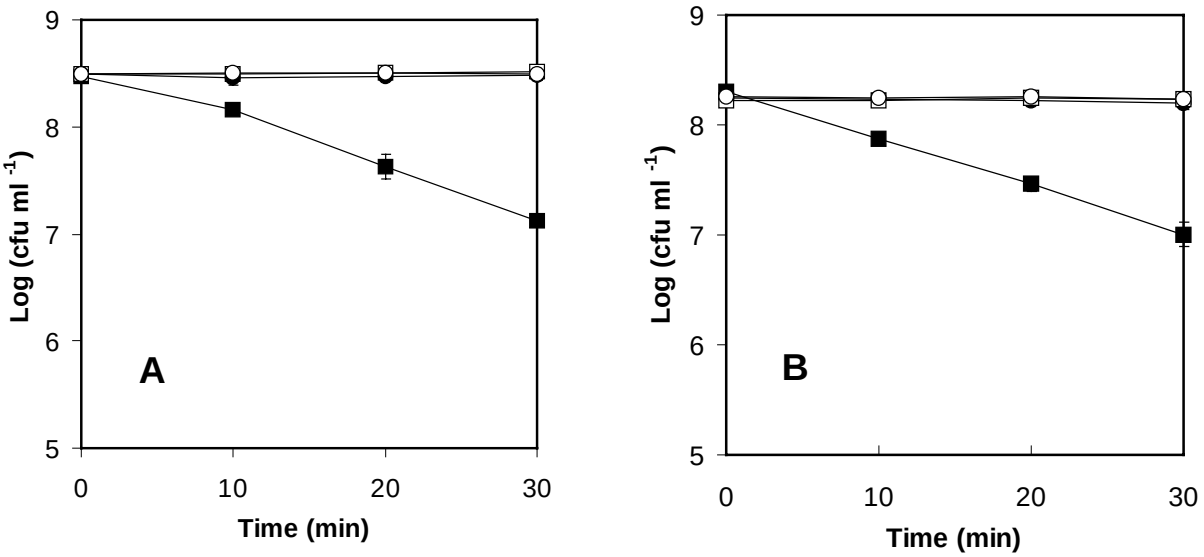

Fig. 2 Viable count of *Listeria monocytogenes* [log (cfu ml^{-1})] at different time intervals after addition of 5 mmol l^{-1} S-carvone at 8°C (□), exposure to 45°C (●), combined treatment of 5 mmol l^{-1} S-carvone and 45°C (■), or no treatment (○). Cells were grown aerobically at 8°C in BHI broth in (A) the absence and (B) the presence of 5 mmol l^{-1} S-carvone, washed and maintained in 50 mmol l^{-1} of HEPES buffer at pH 7.0. Values are means of triplicate measurements. Bars represent the standard deviation (*n*=3).

In further studies, it was investigated whether mild heat treatment in combination with other antimicrobial volatiles from essential oils could effectively reduce the viable numbers of *L. monocytogenes*. The antilisterial activity of carvacrol, thymol, cinnamaldehyde, and decanal was assessed at 45°C for 30 min, using exponential phase cultures grown at 8°C (Fig. 3). A decline in viable numbers of *L. monocytogenes* was observed at 45°C during combined treatment at concentrations of carvacrol, thymol, cinnamaldehyde, or decanal were 1.75 mmol l^{-1}, 1.5 mmol l^{-1}, 2.5 mmol l^{-1}, or 2 mmol l^{-1}, respectively. Carvacrol and thymol were more effective at higher concentrations, indicating a dose-effect correlation.

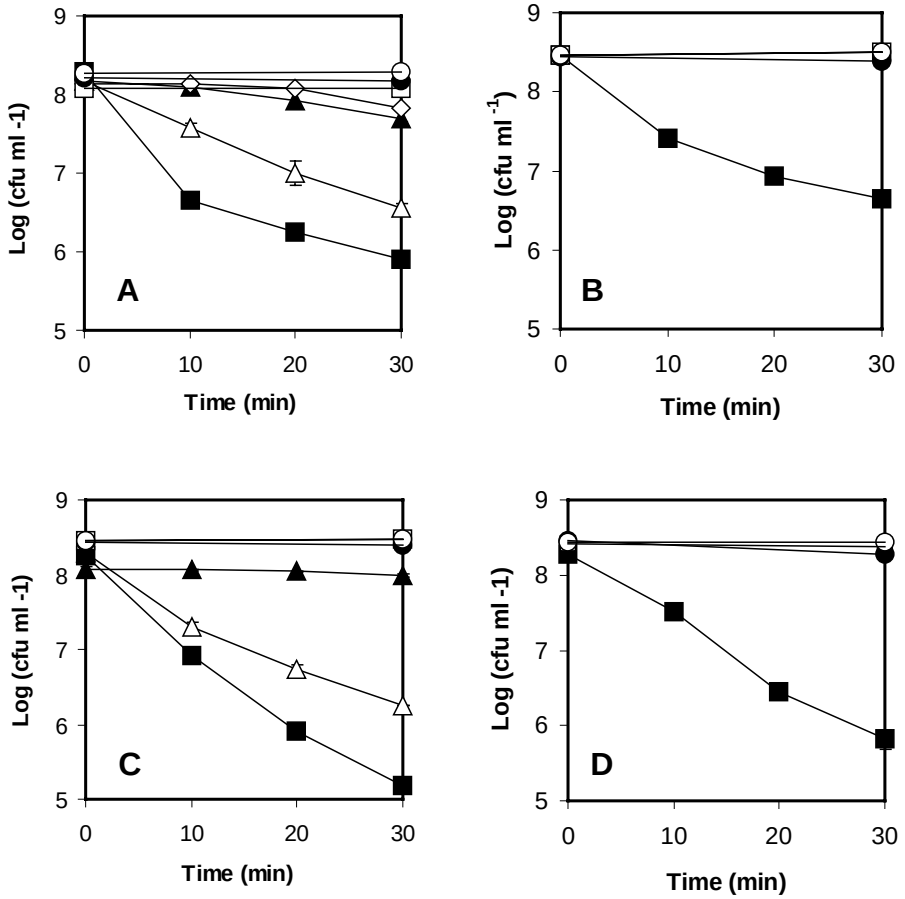

Fig. 3 Viability of *L. monocytogenes* [log (cfu ml^{-1})] at different time intervals. (○) No treatment, (●) exposure to 45^{o}C, or

(A) heat treatment of 45^{o}C in combination with (◊) 0.5 mmol l^{-1}, (▲) 1.5 mmol l^{-1}, (△) 1.75 mmol l^{-1}, (■) 2 mmol l^{-1} or (□) no heat treatment in the presence of 2 mmol l^{-1} carvacrol or

(B) heat treatment of 45^{o}C in combination with (■) 2.5 mmol l^{-1} or (□) no heat treatment in the presence of 2,5 mmol l^{-1}cinnamaldehyde or,

(C) heat treatment of 45^{o}C in combination with (▲) 0.5 mmol l^{-1}, (△) 1.5 mmol l^{-1}, (■) 2 mmol l^{-1} or (□) no heat treatment in the presence of 2 mmol l^{-1} thymol or

(D) heat treatment of 45^{o}C in combination with (■) 2 mmol l^{-1} or (□) no heat treatment in the presence of 2 mmol l^{-1} decanal.

Cells were cultivated aerobically at 8^{o}C in BHI broth, washed and maintained in 50 mmol l^{-1} HEPES buffer at pH 7.0. Values are means of triplicate measurements. Bars represent the standard deviation (n=3)

Stability of S-carvone

As S-carvone is sensitive to air and light, and can be subjected to bioconversion by some microorganisms (Oosterhaven *et al.* 1996; van der Werf *et al.* 1997), its stability was determined in a 5- and a 25-day-old culture of *L. monocytogenes* containing S-carvone.

The recovery of S-carvone from filtered or unfiltered BHI broth containing S-carvone was 77.1% or 94.4%, respectively; 18,3% of the S-carvone was retained by the filter. The recovery of S-carvone from the 5 day old culture was 82,7% and was similar to that of the

filtered uninoculated BHI broth containing S-carvone (77.1%); the recovery from the 25-day-old culture was lower (65,1%). This indicates that the levels of S-carvone in inoculated cultures probably decrease as a result of decomposition through exposure to air and light, but not because of bioconversion by *L. monocytogenes*. The latter would result in a stronger reduction of the amounts of S-carvone in the medium.

DISCUSSION

In this study, we demonstrated that the viability of exponential phase cells of *L. monocytogenes* grown at 8°C can be reduced by a combined treatment with the plant-derived volatile S-carvone, and mild heat. More specifically, exposure to S-carvone (5 mmol l^{-1}) for 30 min at 45^{o}C caused a decrease in viable numbers of *L. monocytogenes* of approximately 1.3 log units, while separate treatment with S-carvone or exposure to 45°C for 30 min did not result in a loss in viability. Sublethal concentrations of other plant-derived volatiles, namely carvacrol, cinnamaldehyde, thymol, and decanal, also caused a decline in viable numbers of the *L. monocytogenes* at 45^{o}C. These findings show that essential oil compounds can play an important role in minimally processed foods, and can be used in the concept of Hurdle Technology to reduce the intensity of heat treatment or other individual hurdles.

The observation that the maximum specific growth rate of *L. monocytogenes* at 8°C was 33% lower in the presence of S-carvone (5 mmol l^{-1}) than in its absence was consistent with previously reported antimicrobial effects of this compound on a great variety of micro-organisms, such as *Helminthosporium solani*, *Fusarium sulphureum* and other fungi (Hartmans *et al.* 1995), as well as *Streptococcus thermophilus*, *Escherichia coli* and *Lactococcus lactis* (Oosterhaven *et al.* 1995).

Combined treatment of S-carvone (5 mmol l^{-1}) and mild heat (45^{o}C, 30 min) led to a decline in viable numbers of exponential phase *L. monocytogenes* cultures incubated at 8^{o}C. However, exponential phase cells that were grown at 35^{o}C or 45^{o}C were not susceptible to the same combined treatment. This observation might be explained by differences in the phospholipid composition of the cytoplasmic membrane of cells that have been grown at different temperatures. When *L. monocytogenes* is grown at 7°C instead of 30°C, the fluidity of the membrane can be maintained by (*i*) increasing the amount of C15:0 fatty acids at the expense of C17:0 (Russell *et al.*1995) and (*ii*) increasing the amount of the unsaturated fatty acid C18:1 (Russell *et al.*1995). When these cells are subsequently transferred to higher temperatures, their membranes will have a higher fluidity than the membranes of cells that were already cultured at elevated temperatures. As a consequence, higher amounts of S-carvone can dissolve into the lipid bilayer. The partitioning of this lipophilic compound in the membrane may lead to a loss of membrane integrity and dissipation of ion gradients, eventually leading to cell death, and can furthermore affect the function of proteins and enzymes embedded in the membrane (Sikkema *et al.* 1995). The observation that cells grown at higher temperatures were not susceptible to S-carvone at 45^{o}C indicates that partitioning of S-carvone (5 mmol l^{-1}) in membranes of cells with a relative lower membrane fluidity was not sufficient to cause cell death.

Cells from stationary phase cultures grown at 8 or 45^{o}C were also not susceptible to S-carvone at 45^{o}C. When cells enter the stationary phase, they undergo physiological changes, such as decreased membrane fluidity and permeability and synthesis of stress proteins (Lou

and Yousef 1996). Such changes may correlate with the increased resistance of the stationary phase cells to the combined treatment.

Carvacrol, cinnamaldehyde, thymol, and decanal reduced the viable numbers of *L. monocytogenes* cells grown at 8^{o}C, by 1.5, 1.3, 1.7 and 2.1 log units at concentrations of 1.75 mmol l^{-1}, 2.5 mmol l^{-1}, 1.5 mmol l^{-1} and 2 mmol l^{-1}, respectively, when combined with a 45^{o}C heat treatment for 20 min. Numerous workers have reported the antimicrobial activity of carvacrol and thymol, and of several essential oils that contain these compounds (Lis-Balchin and Deans 1997; Ultee *et al.* 1998). The synergistic antimicrobial effect of all of the above volatiles with mild heat (45^{o}C, 30 min), is probably similar to that of S-carvone. The similarity lies in a basic common characteristic of these compounds, namely, their high hydrophobicity. Due to this feature, the compounds partition into biological lipid bilayers. The lipophilicity of the compound, and the fluidity of the cellular membrane (Oosterhaven *et al.* 1995) mainly affect this phenomenon. Increased membrane fluidity leads to a change in the partition coefficient and subsequently to accumulation of the volatiles in the membranes and impairment of different membrane functions, including metabolic energy conservation (Oosterhaven *et al.* 1995). However, the hydrophobicity of these compounds is not the only characteristic that affects their antimicrobial activity; specific reactive groups may also play a role (Hartmans *et al.* 1998).

The design of effective combined processing is a complicated task that depends on a great number of factors (e.g. the microbial target, the nature of the food and, in addition, consumer requirements and legislation). Therefore, a specific combination of hurdles needs to be tailored to individual products and micro-organisms. This study shows that plant-derived volatiles could effectively be employed in food preservation, especially when combined with other hurdles, such as mild heat.

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CHAPTER 3

The Combined Action of Carvacrol and High Hydrostatic Pressure on *Listeria monocytogenes* Scott A

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ABSTRACT

Aims
The aim of the study was to investigate the combined antimicrobial action of the plant-derived volatile carvacrol and High Hydrostatic Pressure (HHP).

Methods and Results
Combined treatments of carvacrol and HHP have been studied at different temperatures, using exponentially growing cells of *Listeria monocytogenes*, and showed a synergistic action. The antimicrobial effects were higher at 1^{o}C than at 8 or 20°C. Furthermore, addition of carvacrol to cells exposed to sub-lethal HHP treatment caused similar reductions in viable numbers as simultaneous treatment with carvacrol and HHP. Synergism was also observed between carvacrol and HHP in semi-skimmed milk that was artificially contaminated with *L. monocytogenes*.

Conclusions
Carvacrol and HHP act synergistically and the antimicrobial effects of the combined treatment are greater at lower temperatures.

Significance and impact of the study
The study demonstrates the synergistic antimicrobial effect of essential oils in combination with HHP and indicates the potential of these combined treatments in food processing.

INTRODUCTION

The Gram-positive facultative anaerobic bacterium *Listeria monocytogenes* is an environmental pathogen responsible for the occurrence of listeriosis, a disease of humans and animals (Cox 1989). *Listeria monocytogenes* is found in many food products, and has been associated with foodborne outbreaks and sporadic cases of listeriosis, mostly in North America and Europe (Cox 1989; Vasseur *et al.* 1999). It is regarded as a major problem because of the mortality of the disease it causes, particularly in the unborn child and neonates.

Listeria monocytogenes is able to proliferate at refrigeration temperatures (Vasseur *et al.* 1999) and can survive mild preservation treatments. These features make it difficult to eliminate this microorganism from foods.

Conventional thermal processing is a reliable method of reducing the viable numbers of *L. monocytogenes* in food. However, heat treatment often causes a reduction in the nutritional quality of food products. Therefore, novel mild preservation techniques and new combinations of preservative treatments have been introduced in food processing. One such food processing technique is High Hydrostatic Pressure (HHP) treatment. High hydrostatic pressure treated foods have been commercially available since 1990 in Japan and since 1996 in Europe and the USA (Knorr *et al.* 1998). This technique offers major benefits to the food industry compared with heat treatment, because its adverse effects on product characteristics such as taste, flavour, or vitamin content are reduced (Smelt 1998). Furthermore, as a result of the instant distribution of pressure, it is effective throughout the food product, independent of size and geometry (Smelt 1998).

The antimicrobial effect of HHP was demonstrated as early as the end of the 19th century in experiments performed by Hite (1899). The HHP treatment of microorganisms leads to a number of changes in the cell. Pressures in the range of 20 to 50 MPa inhibit cell division more than cell growth, causing single cells to form long filaments (Zobell *et al.* 1963). Pressure affects motility (Kitching 1957) and in *Escherichia coli*, it was demonstrated that DNA synthesis stops at around 50 MPa, protein synthesis around 58 MPa, and RNA synthesis around 77 MPa (Yayanos and Pollard 1969). Relatively moderate pressures affect a variety of cellular processes and result mostly in sublethal injury of bacteria, whereas at higher pressures the cellular membrane appears to be the primary site of damage and a rapid increase in the death rate occurs (Morita 1975; Hoover *et al.* 1989; Kalchayanand *et al.* 1998). However, the exact mechanisms of cellular damage by HHP have not been elucidated and may be complex (Iwahashi *et al.* 1993; Rönner 1998).

Although HHP treatments can effectively reduce the viable numbers of bacteria in food products, it may adversely alter the texture and colour of certain foods (Cheftel 1995). The intensity of pressures required to inactivate microorganisms might be reduced in the presence of antimicrobial compounds, since moderate pressurisation or short exposures can cause sublethal injury to bacterial cells, making them more susceptible to antibacterial compounds such as plant derived volatiles (Adegoke *et al.* 1997). A suitable compound for combined use with HHP is carvacrol, a plant-derived flavour compound known for its antimicrobial activity (Ultee *et al.* 1998; Smid and Gorris 1999). Its hydrophobic nature allows for accumulation in the bacterial cytoplasmic membrane, where it can elicit several toxic effects that may eventually lead to cell death (Sikkema *et al.* 1994; Ultee *et al.* 1999). In this study, the effects of a simultaneous combined treatment of carvacrol (2, 2.5 or 3 mmol l^{-1}) and HHP (150, 200, 250 or 300 MPa) at 1, 8 or 20^{o}C on the viability of exponentially grown cells of *L. monocytogenes* was investigated. In addition, the viability of this strain was evaluated on the application of pressure, followed by carvacrol treatment. Finally, the most effective combined treatment was applied using semi-skimmed milk, artificially contaminated with *L. monocytogenes*, to study the loss of viability of this organism in a food matrix.

MATERIALS AND METHODS

Microorganism and growth conditions

Listeria monocytogenes Scott A (Department of Food Science, Wageningen Agricultural University, The Netherlands) was used throughout this study. The stock culture was kept at –80^{o}C in 15% (v/v) glycerol. The stock cultures were transferred to 9 ml sterile Brain Heart Infusion (BHI) broth (Oxoid, Hampshire, UK) and incubated at 30^{o}C overnight. Subsequently, a 0.3% (v/v) inoculum of *L. monocytogenes* was added to 100 ml of BHI broth. Cultures were then incubated in a shaking incubator (160 rpm) at 8^{o}C and harvested at mid-exponential phase (OD_{660} 0.1-0.4).

Carvacrol and HHP treatment

Listeria monocytogenes was harvested at an $O.D._{660}$ of 0.1-0.4 by centrifugation (10 000x *g*, 10 min, 8^{o}C). The cells were washed twice in 50 mmol l^{-1} N-[2-acetamido]-2-aminoethanesulfonic acid (ACES buffer; Sigma-Aldrich, Steinheim, Germany), pH 7.0. The pellet was resuspended in ACES buffer to an $O.D._{660}$ of 0.1 and 10-ml aliquots were transferred into sterile plastic tubes (Greiner, Kremsmünster, Austria). Exposure of cells to HHP was performed in ACES buffer in order to maintain pH 7.0 during HHP treatments (Smelt and Hellemons 1998). Carvacrol (Fluka Chemie AG, Buchs, Switzerland) was added to the cell suspensions to final concentrations of 2, 2.5 or 3 mmol l^{-1}, using a carvacrol stock solution of 1 mol l^{-1} in ethanol. In control experiments, equivalent amounts of ethanol [maximum 0.1% (v/v)] were added to cells. No adverse effects of ethanol on the survival of *L. monocytogenes* were observed. Suspensions were placed in sterile plastic stomacher bags (Seward, London, UK) that were sealed while avoiding excess of air bubbles. These pouches were submerged in glycol, which acted as hydrostatic fluid medium in the press (Resato, Roden, The Netherlands). Subsequently, cell suspensions were exposed to pressures of 150, 200, 250 or 300 MPa in a High Pressure unit (Resato) at 1, 8, or 20^{o}C for 20 min. The viability of *L. monocytogenes* was determined before and after pressure treatment. Decimal dilutions of samples were prepared in peptone-physiological salt (1.5 g l^{-1} peptone and 8.5 g l^{-1} NaCl) and plated in triplicate onto BHI agar (1,2 % w/v agar). Plates were incubated at 30^{o}C for 5 days.

Application of high hydrostatic pressure followed by addition of carvacrol

In accordance to the protocol above, cell suspensions were placed in plastic pouches in the absence or presence of carvacrol (2 mmol l^{-1}) and subjected to a Pressure treatment of 200 MPa at 20^{o}C for 20 min. After the pressure treatment, 2 mmol l^{-1} of carvacrol was added to cell suspensions that were pressurised in the absence of carvacrol. After 20 min, decimal dilutions of all samples were made in peptone buffer, and the viability of *L. monocytogenes* was determined.

Effect of combined treatment on the viability of Listeria monocytogenes *in semi-skimmed milk*

Cells were grown and harvested as described in the above protocol, suspended in semi-skimmed milk (Coberco, Arnhem, The Netherlands), and left for 15 min to adjust to the new medium before the addition of 3 mmol l^{-1} carvacrol. Viable numbers of cells were determined,

and the suspensions transferred to pouches which were sealed and pressurised at 300 MPa for 20 min at 1°C, representing the most effective inactivation treatment in ACES buffer. Subsequently, viabilities were determined in pressurised or non-pressurised suspensions in the presence or absence of carvacrol. Samples were plated in triplicate and incubated at 30°C for 5 days.

RESULTS

Combined effect of high hydrostatic pressure and carvacrol on viability of L. monocytogenes

The reductions in viable numbers [log (initial cfu ml^{-1})-log (final cfu ml^{-1})] of mid-exponential phase cells of *L. monocytogenes* grown at 8°C were assessed using different combinations of pressure treatments (0, 150, 200, 250, or 300 MPa) and concentrations of carvacrol (0, 2, 2.5, 3 mmol l^{-1}). Pressurisation was performed at 1, 8 or 20°C.

The reductions in viable numbers of *L. monocytogenes* after a 150 MPa pressure treatment in absence of carvacrol were only 0.2 and 0.3 log units on pressurisation at 1 and 20°C respectively (Fig. 1 and 2). At increasing pressures, the reductions were more substantial, indicating a dose-effect correlation: at 300 MPa, 2 log unit reductions in viable counts were observed at 1 and 20°C. Overall, pressurisation at 1°C (Fig. 1) was slightly more effective in reducing the viable counts of *L. monocytogenes* than at 20°C (Fig. 2) or 8°C (similar to effects at 20°C; data not shown).

The antimicrobial effect of carvacrol on *L. monocytogenes* was higher at increasing carvacrol concentrations. Reductions in viable numbers increased from 0.2 to 0.6 log units, using 2 or 3 mmol l^{-1} carvacrol at 1°C, respectively (Fig. 1). At 20°C, carvacrol treatment was more effective: the reductions in viable numbers increased from 0.3 to 1.8 log units at 2 or 3 mmol l^{-1} carvacrol, respectively (Fig. 2). Results at 8°C (data not shown) were similar to those obtained at 20°C (Fig. 2).

Combined treatments with carvacrol and HHP resulted in significantly greater reductions in viable counts of *L. monocytogenes* compared with the reductions caused by carvacrol or HHP alone (Fig. 1 and 2). At 1°C, more than 5 log reductions in viable counts were realised by applying pressures of 250 MPa in combination with 2.5 or 3 mmol l^{-1} carvacrol, or 300 MPa in combination with 2, 2.5 or 3 mmol l^{-1} carvacrol. At 20°C, similar effects were observed, although less pronounced: the reductions in viable numbers varied from 3.5 to 5 log units for the above-mentioned treatments. Overall, the applied temperature during pressurisation in the presence of carvacrol was an important factor: the reductions in viable numbers of *L. monocytogenes* upon the combined treatment were higher at 1°C (Fig. 1) than at 20°C (Fig. 2) or 8°C (data not shown).

In additional experiments, the combined action of HHP and the plant-derived antimicrobial compound thymol against *L. monocytogenes* was investigated. Pressure treatments of 150, 200, 250 or 300 MPa at 1, 8, or 20°C, and thymol concentrations of 2, 2.5, or 3 mmol l^{-1} were applied (data not shown). Similarly to carvacrol, thymol was more effective in reducing the viable numbers of *L. monocytogenes* at increasing concentrations. Combined treatments of thymol and high pressures were more effective than applying thymol or pressure treatments alone, and reductions of the viable counts were highest at 1°C (data not shown).

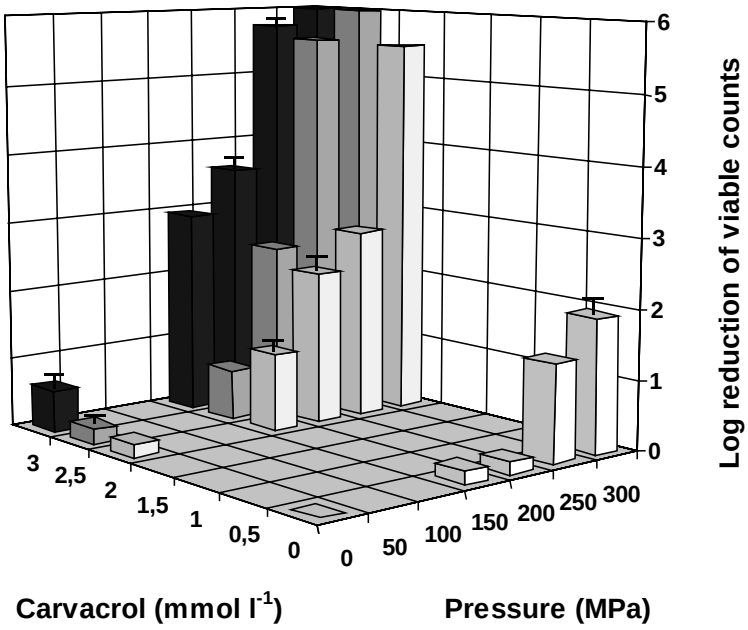

Fig. 1 Combined effect of different concentrations of carvacrol (0, 2, 2.5 and 3 mmol l^{-1}) and pressures (0,150, 200, 250 and 300 MPa) at 1°C on exponentially growing cells of *Listeria monocytogenes.* Cells were grown in brain heart infusion broth at 8°C with shaking (160 rev min^{-1}), harvested and maintained in N-[2-acetamido]-2-aminoethanesulfonic acid buffer, pH 7.0. The detection limit was a 6 log reduction in the viable counts. Experiments were performed in duplicate and error bars represent the S.D.

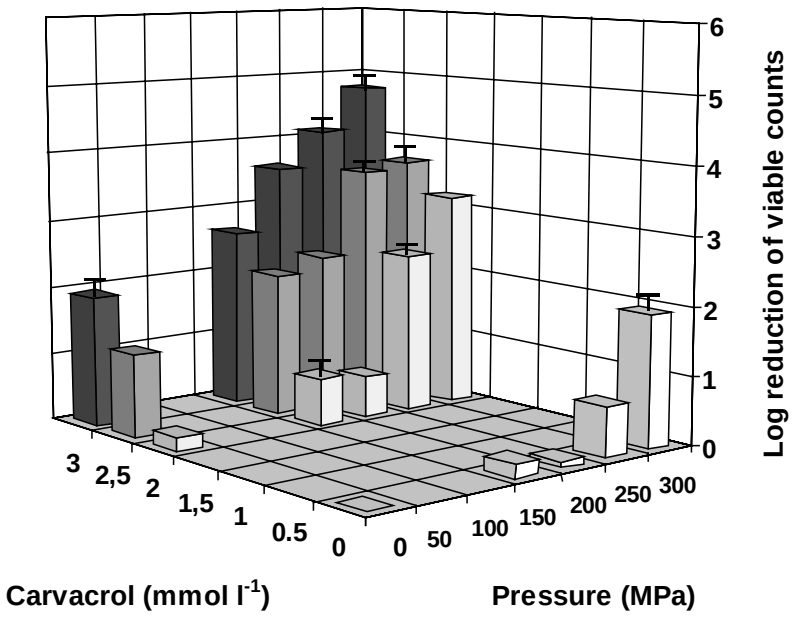

Fig. 2 Combined effect of different concentrations of carvacrol (0, 2, 2.5 and 3 mmol l^{-1}) and pressures (0, 150, 200, 250 and 300 MPa) at 20°C on exponentially growing cells of *Listeria monocytogenes* grown in brain heart infusion broth at 8°C with shaking (160 rev min^{-1}), harvested and maintained in N-[2-acetamido]-2-aminoethanesulfonic acid buffer, pH 7.0. Experiments were performed in duplicate and error bars represent the S.D.

Effect of addition of carvacrol after high hydrostatic pressure treatment

To investigate wether the synergistic effect of the simultaneous application of carvacrol and HHP could also be achieved by HHP treatment followed by the addition of carvacrol, mid-exponential phase cells of *L. monocytogenes* were suspended in ACES buffer (pH 7.0), first subjected to 200 MPa for 20 min at 20^{o}C and subsequently to 2 mmol l^{-1} of carvacrol for 20 min. This combined treatment resulted in an approximate 1 log reduction in viable numbers of *L. monocytogenes*, (Fig. 3), which was not significantly different from the reduction after simultaneous application of 200 MPa for 20 min at 20°C in the presence of 2 mmol l^{-1} carvacrol (0.9 log) (Fig. 3). Separate treatments had no effect on the viability of cells (Fig. 3).

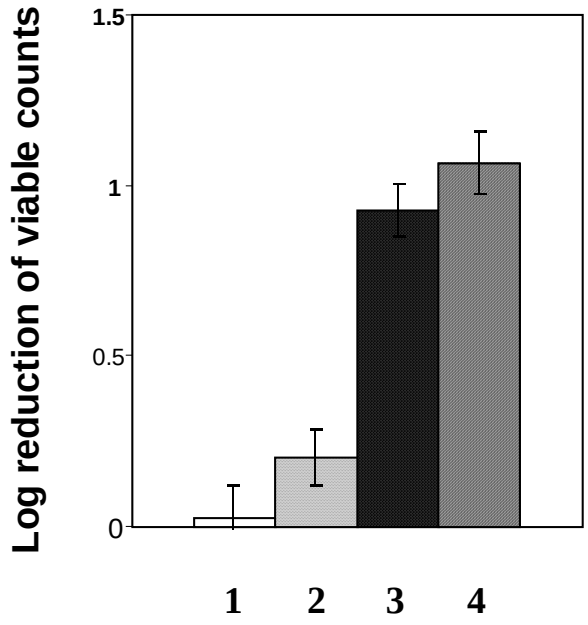

Fig. 3 Log reduction of the viable counts of *Listeria monocytogenes* after treatment with (1) high pressure at 200 MPa; (2) 2 mmol l^{-1} carvacrol; (3) a combination of high pressure (200 MPa) and carvacrol (2 mmol l^{-1}); and (4) 2 mmol l^{-1} carvacrol after high pressure treatment of 200 MPa. All treatments were performed for 20 min at 20^{o}C in N-[2-acetamido]-2-aminoethanesulfonic acid buffer, pH 7.0. Values are means of triplicate measurements. Bars represent the S.D. (*n*=3)

Effectiveness of combined pressure treatment with carvacrol in a food matrix

Mid-exponential phase cells of *L. monocytogenes* grown at 8^{o}C were suspended in milk as a model food substrate and subjected to separate or combined treatments with carvacrol (3 mmol l^{-1}) or pressure (300 MPa for 20 min at 1^{o}C). The application of 3 mmol l^{-1} of carvacrol did not reduce the viable numbers of *L. monocytogenes*, while a 2.3 log reduction in viable counts was observed after the pressure treatment alone (Fig. 4). Combined treatment of pressure and carvacrol was most effective, and resulted in a 3.2 log reduction in the viable counts. In the absence of any treatment, no increase in the viable numbers of *L. monocytogenes* in milk, at 20°C for 20 min, was observed.

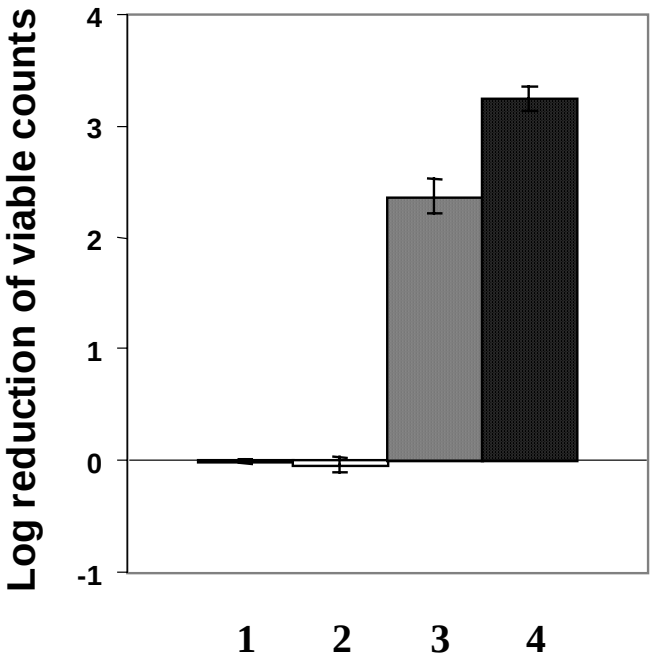

Fig. 4 Log reduction of the viable counts of *Listeria monocytogenes* after treatment with (1) 3 mmol l^{-1} carvacrol; (2) no treatment; (3) high pressure at 300 MPa and (4) a combination of high pressure (300 MPa) and carvacrol (3 mmol l^{-1}). Treatments were applied in semi-skimmed milk at 1°C for 20 min. Values are means of triplicate measurements. Bars represent the S.D. (*n*=3)

DISCUSSION

In the present study, we demonstrated that the viable numbers of *L. monocytogenes* are significantly more reduced by a combined treatment of HHP and carvacrol than by separate treatments with HHP or carvacrol. Mid-exponentially grown cells of *L. monocytogenes* were subjected to different combinations of high pressures and carvacrol concentrations, and pressurisation was performed at 1, 8 or 20°C. At all of these temperatures, the reductions in viable counts of *L. monocytogenes* after combined treatments were significantly higher than the sum of the reductions caused by the individual treatments, suggesting a synergistic effect. The cellular targets at which carvacrol and high pressures act may account for this synergism. Carvacrol is a lipophilic agent that is believed to preferentially insert into the cytoplasmic membrane (Sikkema *et al.* 1994). In the Gram-positive bacterium *Bacillus cereus*, carvacrol causes increased permeability of the membrane for cations like H^+ and K^+ (Ultee *et al.* 1999). The dissipation of the ion gradients leads to impairment of essential processes in the cell and finally to cell death (Sikkema *et al.* 1994; Ultee *et al.* 1999). High hydrostatic pressure treatment is also believed to cause damage to the cell membrane (Morita, 1975). This is probably related to the increase of the melting temperature of lipids (triglycerides) by more than 10°C per 100 MPa. Thus, membrane lipids present in a liquid state at room temperature will crystallise under high pressure, resulting in changes in the structure and permeability of the cell membrane (Cheftel 1995). The observed synergistic effect may, therefore, be related to this common cellular target.

High hydrostatic pressure treatment in the absence of carvacrol, followed by exposure to carvacrol caused reductions in viable numbers of *L. monocytogenes* similar to a simultaneous combined treatment. These findings suggest that HHP treatment alone causes a

sub-lethal injury to part of the *L. monocytogenes* population. These cells can recover on non-selective media (Kalchayanand *et al.* 1998); however, the addition of carvacrol to these injured cells (at concentrations that are not lethal to untreated cells) do not allow for recovery and cause cell death. Similarly, it was demonstrated that combined treatments of *L. monocytogenes* with thymol and HHP effectively reduce the viable numbers of this organism in a synergistic way (data not shown). In line with our observations, Adegoke *et al.* (1997) have previously shown that the plant volatiles α-terpinene and (R)-(+)-limonene enhanced the antimicrobial effects of HHP by preventing the recovery of pressure-injured *Saccharomyces cerevisiae* cells.

The loss in viability of *L. monocytogenes* due to combined treatment with carvacrol (or thymol) and HHP was higher at 1^{o}C than at 8 or 20^{o}C. It has been reported that the pressure resistance of microorganisms is highest at pressurisation temperatures of 15 to 30^{o}C and decreases significantly at higher or lower temperatures (Arroyo *et al.* 1999). The decreased resistance at high or low pressurisation temperatures may be due to changes in the membrane structure and fluidity, through weakening of hydrophobic interactions and crystallisation of phospholipids (Cheftel 1995). In our study, higher reductions in viable numbers of *L. monocytogenes* were observed after combined treatment at 1^{o}C than at 20°C. The observation of MacDonald (1992) that less fluid membranes are more sensitive to HHP might explain the observed effects on *L. monocytogenes* cultured at 8°C, since the membrane fluidity of these cells is lower at 1^{o}C than at 20^{o}C.

The synergistic effect of carvacrol and HHP also occurred in milk, showing that the combined treatment is effective in a model food system. However, the effect of carvacrol combined with HHP was at least two orders of magnitude lower in milk (3.2 log reduction) compared with buffer (> 6 log reduction). These results are in agreement with reports that the effectiveness of the essential oils can be decreased in foods due to the presence of components, such as proteins and fats, which immobilise and inactivate the essential oil components (Smid and Gorris 1999). Separate pressure treatments (300 MPa) in milk or buffer were equally effective in reducing the viability of *L. monocytogenes*. We did not observe a protective effect of milk against HHP compared with buffer, as has previously been reported by Styles *et al.* (1991). This discrepancy might be related to the lower fat content in the semi-skimmed milk that was used in our study. In short, this study shows that plant-derived volatiles, such as carvacrol and thymol can be effectively employed in food preservation, especially in combination with other mild treatments such as HHP. These essential oil compounds can play an important role in minimally processed foods, by reducing the intensity of HHP treatment (or of other treatments) enabling its economically attractive exploitation.

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CHAPTER 4

Characterisation of a *Listeria monocytogenes* Scott A Isolate With High Tolerance to High Hydrostatic Pressure

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Applied and Environmental Microbiology, in press

ABSTRACT

A high hydrostatic pressure (HHP) tolerant isolate of *L. monocytogenes* ScottA, named AK01, was isolated upon a single pressurization treatment of 400 MPa for 20 min, and further characterised. The survival of exponential and stationary phase cells of AK01 in ACES buffer, was at least 2 logs higher than that of the wild type over a broad range of pressures (150-500 MPa), while both strains showed higher HHP tolerance – or piezotolerance - in the stationary than in the exponential phase of growth. In semi-skimmed milk, exponential phase cells of both strains showed lower reductions upon pressurisation than in buffer, but again, AK01 was more piezotolerant than the wild type. The piezotolerance of AK01 was retained for at least 40 generations in rich medium, suggesting a stable phenotype. Interestingly, cells of AK01 lacked flagella, they were elongated, and showed slightly lower μ_{max} at 8°C, 22°C and 30°C than the wild type. Moreover, the piezotolerant strain AK01 showed increased resistance to heat, acid, and H_2O_2 compared with the wild type. The difference in HHP tolerance between the piezotolerant strain and the wild type strain could not be attributed to differences in membrane fluidity, since strain AK01 and the wild type had identical *in situ* lipid melting curves, as determined by FTIR. The demonstrated occurrence of a piezotolerant isolate of *L. monocytogenes* underscores the need to further investigate the mechanisms underlying HHP resistance of foodborne microorganisms, which in turn will contribute to the appropriate design of safe, accurate and feasible HHP treatments.

INTRODUCTION

Listeria monocytogenes is a Gram-positive facultative anaerobic bacterium, which can cause listeriosis, a serious disease with high mortality in immunocompromised individuals, unborn children, and neonates (4). Numerous foodborne outbreaks and sporadic cases of listeriosis have been reported, mostly in North America and Europe (4, 14). *L. monocytogenes* is able to grow at temperatures as low as – 0.4^{o}C (25), withstand osmotic stress, and survive mild preservation treatments (12, 14). These features make this bacterium a difficult but very important target organism to be eliminated from the food chain.

For thousands of years conventional thermal processing (cooking, boiling, roasting, pasteurisation, sterilisation, etc.) has been the most common method to ensure the microbiological safety of foods. However, this type of processing can have detrimental effects on the nutritional value of certain foods. Recent trends in processing are aimed at producing more healthy, nutritious and convenient food. As a result, new food preservation techniques and new concepts such as Hurdle Technology have been developed and are currently being used in the food industry. One of these newly used techniques is High Hydrostatic Pressure (HHP) treatment. The antimicrobial effects of HHP treatment were first demonstrated in 1899 by Hite (6), who proposed the use of this method for the pasteurisation of milk. It took about one century until High Pressure treated foods became commercially available, firstly in Japan in 1990 and 6 years later in Europe and the USA (11). The fundamental basis of all pressure effects stems from the changes in volume which accompany biochemical and physiological processes (3, 26). In contrast to thermal processing, HHP treatment can inactivate microorganisms and unfavourable enzymes at ambient or low temperatures without greatly affecting flavour, colour, or nutritional constituents within a food system (21).

Pressures between 600 and 700 MPa for 15 min (18) or 350 MPa for 40 min (16) are able to inactivate vegetative cells of fungi and bacteria, including most infectious foodborne pathogens (17, 21). Pressure treatments applied in the food industry can vary in that range, depending on several factors, such as the processing time and temperature, the kind of food and its constituents, and the microorganisms or enzymes to be inactivated (17, 21). By combining HHP with other treatments it may be possible to reduce costs and extend the range of products to which this technique can be applied (3, 10, 17).

A wide variety of HHP induced phenomena in living cells have been reported and reviewed, including changes in cellular morphology, biochemical reactions, and membrane integrity (3, 5, 17). High pressures manifest their effects on cellular processes in many ways, including disruption of protein and DNA synthesis, membrane-associated processes, and macromolecular quaternary structures (e.g. protein denaturation) (3, 17, 20, 27). Survival of bacteria upon pressure depends on the species and medium composition (17). In general, Gram-positive are more HHP tolerant – or piezotolerant (28) - than Gram-negative bacteria (17, 21), and different strains of a species can differ widely in their resistance to HHP (1). Bacterial growth is inhibited at pressures in the range of 20 to 130 MPa, while pressures of higher magnitudes (above 130 MPa) cause cell death (5). In *Escherichia coli*, the synthesis of DNA, proteins and RNA stops at 50, 58, and 77 MPa, respectively, while cell death occurs at pressures above 200 MPa (27).

A problem observed in HHP treatments is that a small portion of a bacterial population can be relatively resistant to a certain pressure applied (16). This phenomenon is called “tailing”, and it is of great importance for the food industry because of its possible implications to the food safety and the design of a HHP treatment. In 1989, Metrick *et al.* (16) observed tailing effects when *Salmonella typhimurium* was pressurised at 340 MPa, revealing a subfraction in the population with higher pressure resistance. However, when isolates derived from this subfraction were cultured, they displayed the normal pressure resistance. In addition, Hauben *et al.* (5) succeeded in isolating high pressure resistant *E. coli* mutants after numerous repeated cycles of selective HHP treatments. The mutants showed increased resistance to other stresses such as heat, but only below 62^{o}C, and superoxide stress generated by plumbagin. The existence of mutants like these could be an explanation for the tailing

phenomenon. So far, there is limited information regarding the mechanisms of bacterial survival and adaptation to high pressure. Research focused on these phenomena could increase our understanding and contribute to a more sophisticated use of HHP in food processing.

In this study we isolated a piezotolerant strain of *L. monocytogenes* following a single selection step of 400 MPa. We determined its growth characteristics, its HHP resistance at different pressures, and the effect of growth phase and temperature on its piezotolerance. Furthermore, we determined the resistance of this strain to heat, acid, and hydrogen peroxide. Finally, its survival in artificially contaminated semi-skimmed milk was determined to evaluate its behaviour in a food matrix.

MATERIALS AND METHODS

Bacterial strains, culturing conditions and selection of a piezotolerant strain

L. monocytogenes Scott A (Department of Food Science, Wageningen Agricultural University, The Netherlands) was used throughout this study. The stock culture was kept at –80^{o}C in 15% (v/v) glycerol. Stock cultures were transferred to 9 ml of sterile Brain Heart Infusion (BHI) broth (Oxoid, Hampshire, England), using a 0.3 % (v/v) inoculum, and incubated at 30^{o}C overnight before experiments. The piezotolerant isolate, designated *L. monocytogenes* AK01, was isolated from a population of *L. monocytogenes* Scott A that survived a HHP treatment of 400 MPa for 20 min. Cells from a single colony were cultured in BHI broth overnight using a 0.3 % (v/v) inoculum and then kept as a stock culture at –80^{o}C in 15% (v/v) glycerol. Several tests were carried out to confirm the identity of AK01 as *L. monocytogenes*, namely, growth on Palcam Listeria selective medium (Merck, Darmstadt, Germany), carbohydrate utilisation determined using the Apizym identification system (Biomérieux, Marcy-l'Etoile, France), and Ribotyping (TNO Food, Zeist, The Netherlands).

To conduct experiments, stock cultures of wild type (wt) *L. monocytogenes* Scott A and strain AK01 were inoculated in BHI broth, subcultured at 30^{o}C using 0.3 % (v/v) inocula, and subsequently, a 0.3 % (v/v) inoculum of each culture was added to 100 ml of BHI broth. Cultures were then incubated in a shaking incubator (160 rpm) at 8^{o}C, 22^{o}C, or 30^{o}C, and growth was monitored by measuring the optical density at 660 nm (OD_{660}). Viable counts were also determined at regular time intervals by preparing decimal dilutions of samples in peptone-physiological salt (1.5 g l^{-1} peptone and 8.5 g l^{-1} NaCl) and plating these on BHI agar (1.2 % w/v). Plates were incubated at 30^{o}C for 3 days. The mid-exponential, or the stationary phase of growth of wt and AK01 was determined, at the different temperatures, based on the OD_{660} and the viable count measurements. The maximum specific growth rates for the exponential phase were calculated using the following equation:

$\mu_{max} = (\ln N_t - \ln N_0) * (t - t_0)^{-1}$ where N_t is the cell population at the time t (19).

Electron microscopy

10 µl Of a mid-exponential culture of AK01 and wt was placed in a sterile petri dish and a Formvar-coated grid was put on top for 1 min. The grid was removed, the excess of liquid drained off, and the grid was placed on a drop of 2% phosphotungstic acid for 1 minute.

Subsequently, excess of phosphotungstic acid was removed and the grid was inspected with the use of an electron microscope (Jem-1200 EX II, Jeol Ltd., Tokyo, Japan).

High Hydrostatic Pressure treatment

L. monocytogenes wt and AK01 were cultured at 8 or 30 ^{o}C, and harvested by centrifugation (10,000x *g*, 10 min) at mid exponential phase (OD_{660} 0.3 and 0.2 for the wt and AK01, respectively). The cells were washed twice in 50 mmol l^{-1} N-[2-acetamido]-2-aminoethanesulfonic acid (ACES buffer; Sigma-Aldrich, Steinheim, Germany), pH 7.0. The pellet was resuspended in semi-skimmed milk (Friesche Vlag, Ede, The Netherlands), or in ACES buffer to an OD_{660} of 0.1. 10 ml Aliquots were transferred into sterile plastic tubes (Greiner, Kremsmünster, Austria). ACES buffer was selected since this buffer maintains pH 7.0 during High Pressure treatments (22). Suspensions were placed in sterile plastic stomacher bags (Seward, London, UK) that were sealed while avoiding excess of air bubbles. These pouches were submerged in glycol, which was the fluid medium through which the pressure was transferred (Resato, Roden, Holland). Subsequently, cell suspensions were exposed to pressures of 150, 200, 250, 300, 350, 400, 450 or 500 MPa in a High Pressure unit (Resato, Roden, Holland) at 20^{o}C for 20 min. The viability of *L. monocytogenes* was determined before and after pressure treatment. Decimal dilutions of samples were prepared in peptone-physiological salt solution and plated in triplicate onto BHI agar (1,2 % w/v agar). Plates were incubated at 30^{o}C for 5 days.

Stability of HHP resistance

To test the stability of the HHP resistant phenotype of *L. monocytogenes* AK01, cells were subcultured during 5 consecutive days using 0.3 % v/v inocula in 9 ml of fresh BHI medium (~ 70 generations). The wt *L. monocytogenes* was cultured the same way and used as a control. On day 2 and 5 (~30 and ~70 generations, respectively) overnight cultures were inoculated (0.3 % v/v) in 100 ml of BHI broth, incubated at 30^{o}C under shaking (160 rpm) till mid-exponential phase (OD_{660} 0.3 and 0.2 for the wt and AK01, respectively) and harvested. These cells were tested for resistance to 250 MPa or 300 MPa for 20 min.

Phase transition temperature

Cells from wt and AK01 *L. monocytogenes* were incubated at 30^{o}C, harvested at mid-exponential phase and washed in ACES buffer as described above. The pellet was placed between two circular CaF_2 windows (13 * 2 mm) and fitted in a liquid-nitrogen-cooled, temperature-controlled brass cell. The temperature of the sample was recorded and controlled using two PT-100 elements that were near the sample windows. Fourier-transform infrared spectra were recorded on a Fourier-transformed infrared (FTIR) spectrometer (Model 1725, Perkin-Elmer, Beaconsfield, Buckinghamshire, UK) equipped with an external beam facility to which a Perkin-Elmer IR microscope was attached. The microscope was equipped with a narrow-band mercury/cadmium/telluride LN_2-cooled IR detector. The sample was cooled to -40^{o}C and subsequently heated to 70^{o}C at a rate of 1.5^{o}C min^{-1}. During the heating, spectra were recorded every minute. Spectral analysis and display were carried out using the Infrared Data Manager Analytical Software, version 3.5 (Perkin Elmer). Band positions were calculated as the averages of central positions in 20 slices between 75% and 90% of the total peak height. The peak position of the CH_2-symmetric stretching vibration bands were analysed to estimate the extent of interaction between the acyl chains of the membrane lipids as a direct indication

of the membrane fluidity. Thus, transitions in membranes from the gel to the liquid-crystalline phase can be observed *in vivo* from plots of the vibrational frequency of the absorption peaks versus the temperature at which spectra were recorded (7). Estimation of the temperature at which half of the hydrocarbon containing compounds had been melted (T_m) was made by probit analysis. Using this technique we were able to monitor possible differences in membrane fluidity of these two strains.

Heat treatment

BHI broth (100 ml) was inoculated (0.1 v/v %) with an overnight culture of wt and AK01 *L. monocytogenes* and incubated under shaking (160 rpm) at 22°C or 30°C. Cells were harvested at mid-exponential phase (OD_{660} 0.3 and 0.2 for the wt and AK01, respectively), washed twice and resuspended in ACES buffer. Suspensions were placed in 10 ml plastic tubes (Greiner) and incubated in a waterbath at 55°C for maximally 20 minutes. At regular time intervals, samples were taken and viability was determined.

Acid treatment

L. monocytogenes wt and AK01 were cultured at 30°C and harvested as described above. Cells were resuspended in BHI broth, adjusted to pH 2.5 with HCl. Viable counts were determined immediately after resuspension of cells in the low pH BHI broth, and then at regular time intervals.

H_2O_2 treatment

The resistance of wt and AK01 *L. monocytogenes* to hydrogen peroxide (H_2O_2) was determined for cells cultured at 30°C to mid-exponential phase, by adding H_2O_2 (Merck, Hohenbrunn, Germany) to a concentration of 0.2 % w/v. Viable counts were determined just before and at regular time intervals after the addition of H_2O_2.

Growth in the presence of NaCl

To determine the maximum NaCl concentration at which *L. monocytogenes* wt and AK01 were able to grow, cells were inoculated (0.3% v/v) in BHI broth with final NaCl concentrations ranging from 8 to 14% w/v, increasing by 0.5% w/v. Growth and final population densities were monitored by measuring the OD_{660} at regular time intervals for maximally 50 h.

RESULTS

***Characterisation of strain AK01 as* L. monocytogenes**

The identity of AK01 was confirmed as *L. monocytogenes* by growth on Palcam Listeria selective medium, demonstration of characteristic metabolic activities of *L. monocytogenes* in the Apizym system, and by Ribotyping (94% similarity with *L. monocytogenes* DUP-1042).

Microorganism and growth

The OD_{660} and the viable counts of wt and AK01 *L. monocytogenes* were determined at 8°C, 22°C, or 30°C at regular time intervals. At all temperatures, the maximum specific growth rate (μ_{max}) of the wt was higher than the μ_{max} of strain AK01. The respective μ_{max} of the wt

and AK01 were 0.97 h^{-1} and 0.87 h^{-1} at 30°C (Fig 1A); 0.68 h^{-1} and 0.60 h^{-1} at 22°C; 0.10 h^{-1} and 0.09 h^{-1} at 8°C (data not shown). Furthermore, AK01 showed lower final OD_{660} than the wt (~0.6 versus ~0.9) (Fig 1B) and lower final population densities (~$2.3*10^9$ compared to ~$6*10^9$ for the wt) at all temperatures.

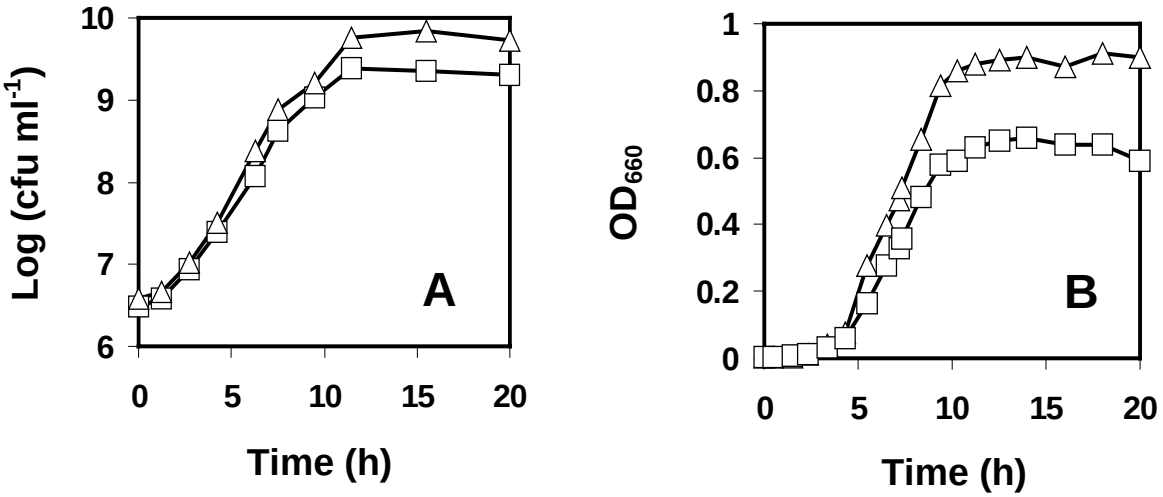

Fig 1. Growth of *Listeria monocytogenes* wild type (Δ), and the piezotolerant isolate AK01 (□), monitored by viable counts (A), and OD_{660} (B). Cells were grown in BHI broth at 30°C under shaking (160 rev min^{-1}).

Microscopic examination showed immobility of strain AK01, while wt *L. monocytogenes* was motile. In addition, we observed that cells of strain AK01 were roughly 2-fold longer than the wt cells. Further examination using electron microscopic analysis confirmed that the cells of AK01 were elongated, and in addition, flagella were not detected in strain AK01 (Fig 2A, 2B and 2C).

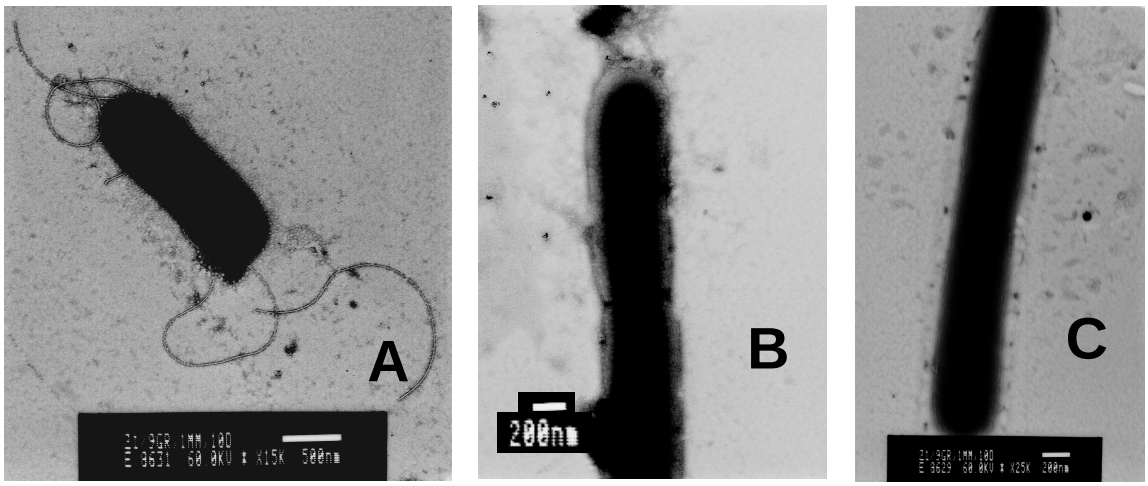

Fig 2. Visualisation of exponentially grown cells of wild type (A), and AK01 (B and C) with electron microscopy. The white bar in Fig 2A depicts 500 nm, while in Fig 2B and 2C it depicts 200 nm.

High Hydrostatic Pressure resistance

The HHP resistance of mid-exponential phase cultures of the piezotolerant *L. monocytogenes* AK01 (grown at 30°C) was higher than that of the wt strain over the range of pressures tested

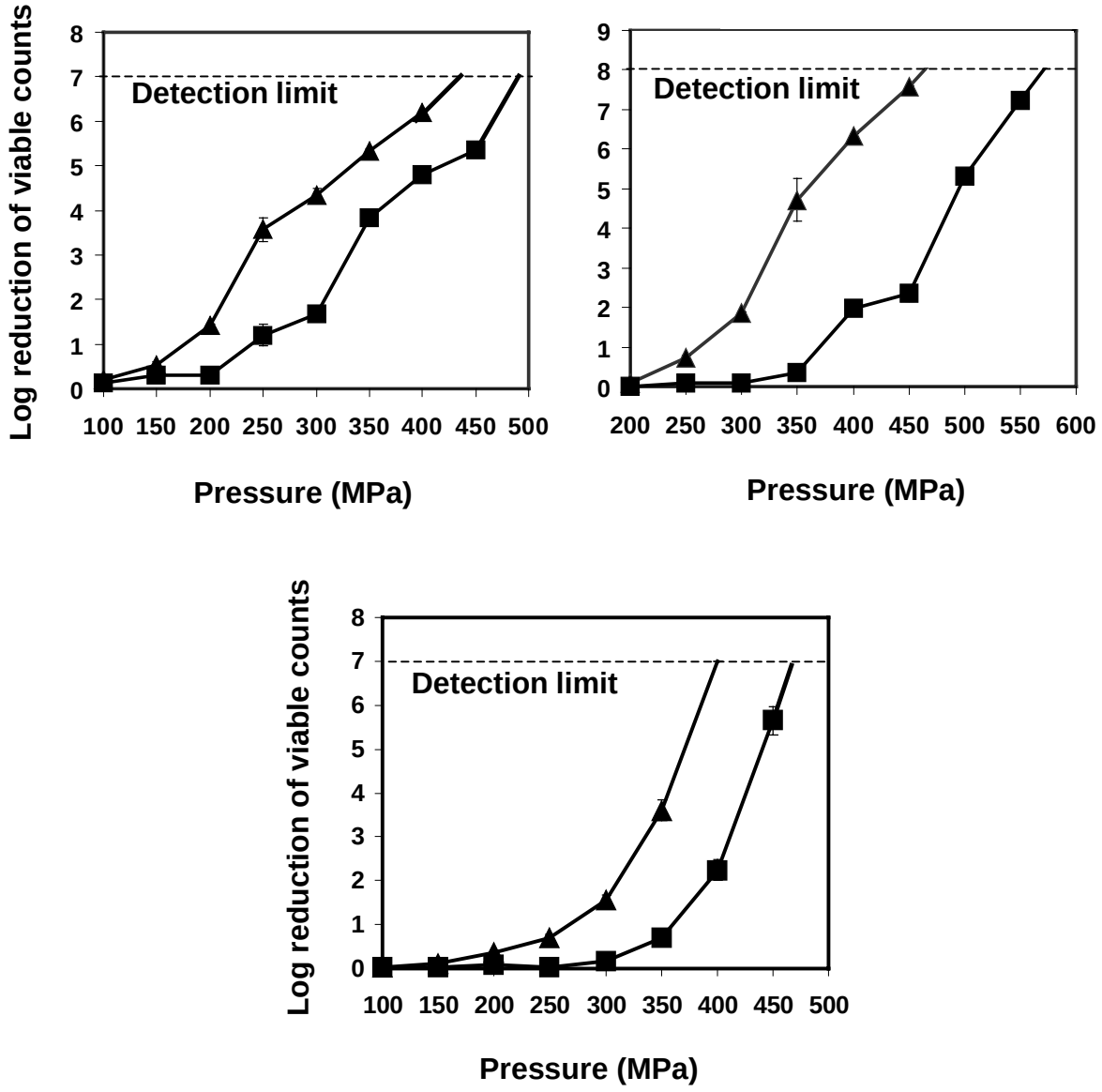

Fig 3. Reductions in viable numbers of *L. monocytogenes* wild type (▲) and AK01 (■) after exposure to different pressures for 20 min at 20°C. Cells were grown in BHI broth at 30°C under shaking (160 rpm). (A) Cells were harvested in mid-exponential phase and resuspended in ACES buffer before treatment. The detection limit was 7 log cycles reduction of the viable counts, which was exceeded by the wt at 450 MPa, and by AK01 at 500 MPa. (B) Cells were harvested in stationary phase and resuspended in ACES buffer before treatment. The detection limit was 8 log cycles reduction of the viable counts which was exceeded by the wt at 500 MPa, and by AK01 at 600 MPa. (C) Cells were harvested in mid-exponential phase and resuspended in semi-skimmed milk before treatment. The detection limit was 7 log cycles reduction of the viable counts, which was exceeded by the wt at 400 MPa, and by AK01 at 500 MPa. Experiments were performed in duplicate and error bars represent the standard deviation

(150, 200, 250, 300, 350, 400, 450 or 500 MPa), showing maximally a 2.5 log cycle difference (Fig 3A). Notably, more than 6 logs reduction in viable counts were achieved, for both the wt and AK01, using pressures greater than 400 MPa. A similar difference in resistance to HHP between the wt and strain AK01 was observed for cells grown at 8^{o}C and harvested at mid exponential phase. Overall, cells grown at 8^{o}C were maximally 1 log more resistant to the whole range of pressures tested than those grown at 30^{o}C (data not shown). The growth phase of cells affected the HHP resistance of both the wt and strain AK01. When cultures reached stationary phase, their HHP resistance increased strongly, but also in this case, strain AK01 had a piezotolerance that was higher (maximally 5.2 log cycles), than that of the wt (Fig 3B). While pressure inactivation of more than 6 log units was achieved at 450 MPa for stationary phase cells of the wt, pressures close to 600 MPa were required for cells of AK01.

The log reductions of the viable numbers of mid exponential phase cells from strain AK01 and the wt (grown at 30^{o}C) were overall 2-3 log cycles lower in milk than in ACES buffer over the whole range of pressures (150 – 500 MPa). Again, AK01 was more resistant to HHP than the wt (Fig 3C).

Stability of HHP resistance

Exponential cultures of AK01 had the same HHP resistance after ~30 and ~70 generations. Their HHP resistance was tested at 250 MPa and 300 MPa, resulting in a 1.2 and 1.6 log reduction of the viable counts, respectively, independent the number of generations, versus 3.4 and 4.4 log reduction of the wt control cultures (data not shown). This suggests that AK01 retained its HHP resistant character, indicating a stable phenotype.

Phase transition temperature

The *in situ* membrane fluidity as well as the melting curve of *L. monocytogenes* AK01 was identical to that of the wt strain as determined by FTIR (data not shown). The T_m was centered between 10 and 11°C, with the beginning of melting around -3°C and the end of the melting around 23°C.

Heat treatment

Exponential phase cultures of AK01 grown at 22^{o}C were more resistant to heat treatment than the wt control cells. After 20 min at 55^{o}C only a 0.4 log reduction in viable numbers was observed for the piezotolerant strain, compared to a 3.7 log reduction of viable numbers for the wt (Fig 4). Similarly, subjecting mid-exponential phase cells of AK01 grown at 30^{o}C to heat treatment (55^{o}C for 20 minutes) resulted in no reduction of the viable counts, while a 2.3 log reduction in viable numbers was observed for the wt (data not shown).

Acid treatment

Exponential phase cells of the wt *L. monocytogenes* grown at 30^{o}C showed an approximate 1 log higher reduction of their viable counts during exposure to pH 2.5 for 45 min than those of strain AK01 grown at the same temperature (Fig 5).

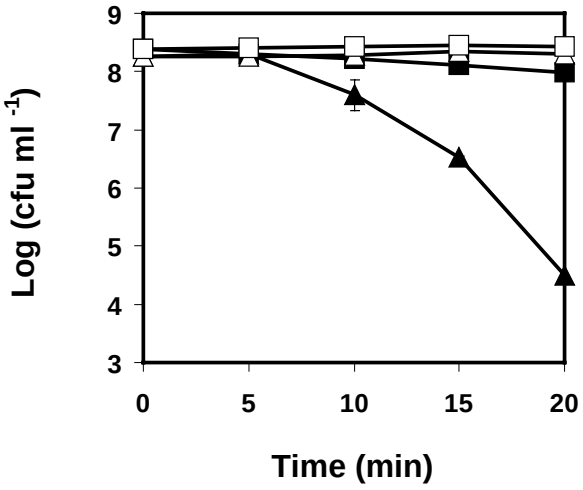

Fig 4. The effect of heat treatment at 55°C on viable numbers of *L.monocytogenes* wild type (▲) and AK01 (■). The viability of both cultures in absence of heat treatment is also represented for the wild type (Δ) and AK01 (□). Cells were cultivated aerobically at 22°C in BHI broth (pH 7), harvested and resuspended in ACES buffer, 50mM, pH 7.0, before treatment. Values are means of triplicate measurements of a representative experiment. Bars represent the standard deviation (n=3)

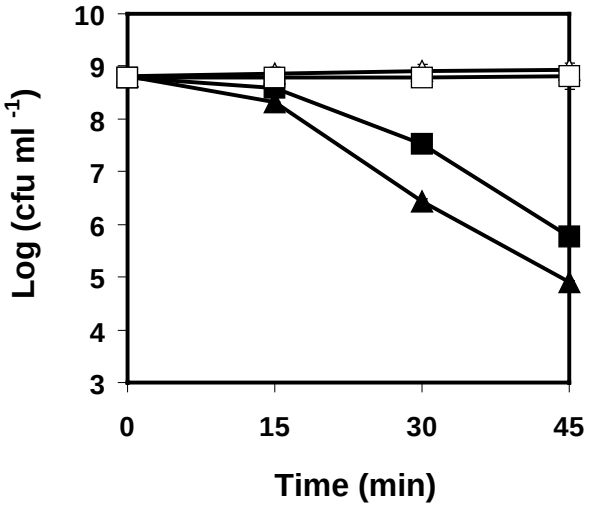

Fig 5. Effect of exposure to pH 2.5 on viable numbers of *L. monocytogenes* wild type (▲) and the piezotolerant strain AK01 (■). The viability of both cultures in absence of HCl at pH 7 is also represented for the wild type (Δ) and the piezotolerant strain (□). Cells were cultivated aerobically at 30°C in BHI broth (pH 7). Experiments were performed in BHI broth adjusted to pH 2.5 with HCl. Values are means of triplicate measurements of a representative experiment. Bars represent the standard deviation (n=3)

H_2O_2 treatment

Exposure of both *L. monocytogenes* strains to 0.2 % w/v hydrogen peroxide showed that AK01 had a higher resistance to H_2O_2 than the wt. During the first 40 minutes of exposure the population of AK01 showed a reduction of only ~0.5 log in the viable numbers, compared to a ~4.5 log reduction in viable numbers for the wt population. After 60 min the viable counts of AK01 started to decrease but remained at least 1 log higher than those of the wt after 150 min (Fig 6).

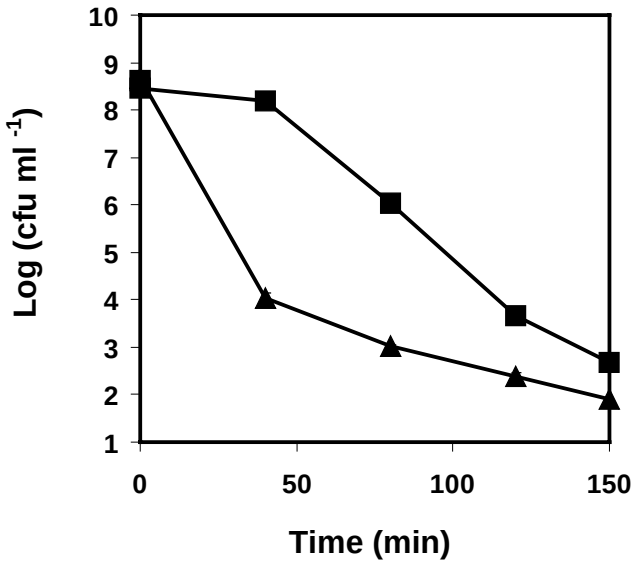

Fig 6. Effect of exposure to 0.2 % H_2O_2 on viable numbers of wild type *L. monocytogenes* (▲) and the piezotolerant strain AK01 (■). Experiments were performed in BHI broth, at 30°C, under shaking, in the absence of light. Values are means of triplicate measurements of a representative experiment. Bars represent the standard deviation (n=3)

Maximum growth NaCl concentration

NaCl negatively affected the growth of wt and AK01 *L. monocytogenes* at increasing NaCl concentrations. We did not observe marked differences between the wt and strain AK01, and the maximum concentration of NaCl at which growth occurred was 12.5 % w/v for both strains throughout 50 h.

DISCUSSION

In this study, we characterised the piezotolerant strain AK01 of *L. monocytogenes* ScottA, which was isolated from a wt population after exposure to HHP treatment of 400 MPa for 20 min. The survival of exponential and stationary phase cells of strain AK01 upon pressurisation was more than 2 logs higher than that of wt *L. monocytogenes* over a broad range of pressures tested. The piezotolerance of AK01 was retained for at least 40 generations in rich medium. These results suggest a stable phenotype, likely resulting from altered cellular properties rather than a short-lived adaptation. The HHP phenotype was accompanied by a slightly lower μ_{max} than that of the wt, and altered morphological characteristics, namely, the absence of flagella and elongation of cells.

To date, only a limited number of studies have demonstrated the occurrence of piezotolerant strains derived from microorganisms that can be present in foods. To our knowledge, this is the first report describing a *L. monocytogenes* piezotolerant strain. In a study performed by Iwahashi *et al.* (8), piezotolerant mutants of *Saccharomyces cerevisiae* were obtained upon treatment of cells with mutagenic substances such as *N*-methyl-*N´*-nitro-*N*-nitrosoguanidine. Furthermore, Hauben *et al.* (5) reported the isolation of HHP mutants of *E. coli* after numerous subsequent selective HHP cycles. By contrast, *L. monocytogenes* AK01 was selected upon a single HHP cycle that reduced the initial wt population of ~ 10^9 cfu ml^{-1} to such an extent that the only colony formed on the agar was AK01.

We investigated whether the difference in HHP tolerance between the piezotolerant strain and the wt strain could be attributed to altered membrane properties, since there is evidence that an increased membrane fluidity of natural and artificial membranes gives rise to an increased high pressure resistance (15). Importantly, strain AK01 and the wt had identical *in situ* membrane fluidities, indicating that the piezotolerant phenotype was not originating from a higher membrane fluidity of strain AK01 than that of the wt. Similar findings have been reported for piezotolerant *E. coli* mutants that showed no significant differences in the fatty acid composition and the outer membrane properties compared with the wt strain (5). While the difference in piezotolerance of strain AK01 and the wt could not be linked to their membrane fluidity, the wt and AK01 cells grown at 8°C had similarly increased HHP resistances compared with cells grown at 30°C upon pressurisation at 20°C. This can be explained by a higher membrane fluidity of *L. monocytogenes* cells cultured at low temperatures compared with 30°C (24).

The piezotolerant strain *L. monocytogenes* AK01 had increased resistance to heat, acid, and H_2O_2 compared with the wt. An increased heat resistance of piezotolerant strains of *E. coli* compared to the wt has also been described by Hauben *et al.* (5), who demonstrated that *E coli* piezotolerant mutants were thermotolerant at 58 and 60°C but not at higher temperatures. In addition, Iwahashi *et al.* (8) reported increased thermotolerance of a piezotolerant mutant of *S. cerevisiae* compared to the wt. It is possible that a number of similar cellular properties underlie pressure and heat resistance, since both high pressure and heat destabilise the quaternary structure of proteins (9). This is supported by the findings that *E. coli* wt cells showed an induced expression of 55 proteins upon exposure to a pressure upshift to 55 MPa, many of which are also induced by heat shock (26). A correlation between pressure resistance and resistance to organic acids has previously been established for a variety of strains from different species (2), while increased oxygen tolerance was observed for a piezotolerant mutant of *S. cerevisiae* (8). Although it is not clear what mechanisms underlie the increased resistance of the piezotolerant strain to the different stresses, it could possibly be attributed to altered expression levels of proteins involved in (general) stress response. Furthermore, the piezotolerance of strain AK01 and the wt was increased in the stationary phase compared with the exponential phase of growth, which might be related to the increased expression of genes involved in stationary phase stress survival (13).

Important in relation to food processing is our observation that reductions in viable numbers of the wt and the AK01 strain were lower upon pressurisation in milk than in buffer. A lower sensitivity of cells to HHP treatment in milk and other food matrixes has previously been observed (18, 23, 17), and has been attributed to the protective effect of food components like sugars, free amino acids and vitamins (17). On the other hand, it has been shown that HHP treatment combined with other preservation factors has a synergistic effect

on the inactivation of microorganisms. We recently demonstrated that combined treatment with HHP and the essential oil compound carvacrol had a strong synergistic effects (10), while other authors demonstrated an effective control of pressure resistant foodborne microbes by combined treatment with HHP and acid (2).

The piezotolerance observed for AK01 is quite significant considering that pressures in the range of 300-600 MPa are selected for pasteurisation purposes (17) and that this resistance could increase in certain cases due to protective effects of some food constituents (17, 23). The application and optimisation of combined processing in food systems therefore seems to be required to ensure effective inactivation of pressure resistant strains in foods. Furthermore, the occurrence of piezotolerant isolates urges for further investigation of the mechanisms underlying HHP resistance of microorganisms. Studying in more detail the mechanisms involved in HHP resistance of microorganisms might resolve problems caused by phenomena like tailing and contribute in the design of safe, accurate and feasible HHP treatments.

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CHAPTER 5

A single amino acid deletion in the glycine-rich region of CtsR affects motility, piezotolerance, and virulence, of *Listeria monocytogenes* Scott A

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ABSTRACT

A spontaneous high hydrostatic pressure tolerant –or piezotolerant- mutant of *Listeria monocytogenes* ScottA, named AK01, was previously isolated and characterised (Karatzas and Bennik, in press). In this study, we conclusively linked several phenotypic characteristics of this mutant, such as high piezotolerance, immobility, and reduced virulence, to a single amino acid deletion in the highly conserved glycine-rich region of CtsR (CtsRΔGly). CtsR (class three stress gene repressor) negatively regulates the expression of Class III heat shock genes (*clpP*, *clpE* and the *clpC* operon), that prevent the accumulation of misfolded proteins. In the mutant strain AK01, expressing the CtsRΔGly protein, we observed upregulation of *clpP*, which demonstrates the involvement of Class III heat shock genes in increased survival upon HHP treatment and other stresses. The mutant strain furthermore lost its motile character, and importantly, showed attenuated virulence. The deletion of three base pairs in the region of *ctsR* that normally encodes four glycines is seemingly not an isolated incident, and suggests a functional role for this mutation in *L. monocytogenes* in surviving stress conditions that do not allow for adaptation of normal cells, but require genetic diversity.

INTRODUCTION

Listeria monocytogenes is a Gram positive facultative anaerobic pathogenic bacterium that can be present in a variety of foods from animal or plant origin (Brackett, 1988; Lovett, 1989). It can cause a severe food-borne illness called listeriosis, due to the ability of this organism to invade and multiply within the host cells (Gaillard *et al.*, 1987; Tilney and Portnoy, 1989). Since this disease is predominantly acquired after consumption of contaminated foods, effective elimination of *L. monocytogenes* is essential.

Inactivation of bacteria by High Hydrostatic Pressure (HHP) treatment is a relatively novel preservation technique (Knorr *et al.*, 1998; Smelt, 1998). HHP has

detrimental effects on cellular processes, resulting from perturbation of membranes and membrane-associated processes, to disruption of macromolecular quaternary structures (e.g. protein denaturation) (Yayanos and Pollard, 1969; Cheftel, 1995; Palou *et al.*, 1999). In general, bacterial growth is inhibited at pressures above 20 MPa, while growth halts at higher pressures, resulting from cessation of DNA-, protein- and RNA-synthesis, which was shown to occur at 50, 58, and 77 MPa, respectively, in *Escherichia coli* (Yayanos and Pollard, 1969). Cell death of non-piezophilic vegetative bacteria normally occurs at pressures above 200 MPa (Yayanos and Pollard, 1969).

Typical pressures used to inactivate vegetative bacterial cells range from 300 to 700 MPa (Metrick *et al.*, 1989; Patterson *et al.*, 1995; Smelt 1998; Palou *et al.*, 1999). These pressures are applied using an abrupt pressure upshift, and do not allow for bacterial growth and adaptation. Several authors have reported survival of a fraction of a bacterial population upon exposure to hydrostatic pressures that are normally lethal, but it is not known what mechanisms underlie this increased tolerance (Metrick *et al.*, 1989; Hauben *et al.*, 1997). Recently, we isolated *L. monocytogenes* strain AK01 from a wild type (wt) *L. monocytogenes* ScottA population upon HHP treatment. This strain showed ~1000-fold higher viability than the wt upon exposure to 350 MPa. Furthermore, it showed altered morphological characteristics, such as elongation of cells and lack of flagella, and increased resistance to heat, acid, and hydrogen peroxide (Karatzas and Bennik, in press).

This study points out a crucial role for the Class three stress gene Regulator (CtsR) in the observed phenotypic characteristics of *L. monocytogenes* AK01, including the increased tolerance to HHP. The CtsR protein is a negative regulator of the Class III heat shock genes, and is believed to act as a dimer, while the McsA or McsB proteins are believed to play a role in its activity (Derré *et al*., 2000; Krüger *et al*., 2001). CtsR contains domains that are highly conserved amongst low G + C containing Gram positive bacteria, namely, a presumed dimerisation domain and a highly conserved Helix Turn Helix (HTH) domain in the N-terminal region. The central region contains a conserved glycine-rich domain, while the C-terminus is less well conserved (Derré *et al*., 2000). CtsR is the product of the first gene of the *clpC* operon, that furthermore comprises *mcsA* and *mcsB*, and *clpC* (Nair *et al*., 2000; Krüger *et al*., 2001). CtsR negatively controls expression of the Class III heat shock genes *clpP*, *clpE*, and of the *clpC* operon by binding specifically to a direct heptanucleotide repeat in their promoter regions (Derré *et al.*, 1999; Nair *et al.*, 2000). Thereby, it has an autoregulatory function. ClpC and ClpE have ATPase activity and belong to the 100 kDa heat shock protein (HSP100) Clp family of highly conserved molecular chaperones (Schirmer *et al*., 1996), and the serine protease ClpP is a proteolytic subunit. Clp ATPases regulate ATP-dependent proteolysis and also play a role as molecular chaperones in protein folding and assembly (Wawrzynow *et al.*, 1996). CtsR and Class III heat shock genes are involved in one of the three classes of known heat shock regulatory mechanisms (Class IV is less well defined), and are highly conserved among Gram positive bacteria (Derré *et al.*, 1999, 2000).

In *L. monocytogenes* the Clp ATPases are required for stress survival and intracellular growth (Rouquette *et al*., 1996; Gaillot *et al.*, 2000; Nair *et al*., 2000). The majority of the virulence genes in are regulated by the pleiotropic regulator PrfA

(Cossart and Lecuit, 1998; Kreft and Vazquez-Boland, 2001). In addition, a PrfA-independent virulence protein, SvpA (surface virulence-associated protein), that is controlled by MecA, ClpP and ClpC was recently identified (Borezée *et al*., 2001). There is evidence that there is crosstalk between PrfA and Class III heat shock genes (Ripio *et al*., 1998), but so far, it is not clear how these interactions take place.

The aim of this study was to elucidate the mechanisms underlying the increased tolerance of *L. monocytogenes* AK01 to HHP treatment and other stresses. As a starting point, we analysed the protein expression of wt *L. monocytogenes* ScottA and strain AK01 using 2D gel electrophoresis. Since these data indicated that CtsR might play a role in stress resistance of this mutant, the *ctsR* gene was further analysed. To conclusively link the mutation in the *ctsR* gene of AK01 with the observed phenotype, we replaced wt *ctsR* with the mutated *ctsR* gene (CtsRΔGly) in a wt background and analysed the properties of the constructed mutant. Given the previously demonstrated role of CtsR in virulence, we evaluated the virulence properties of the spontaneous and constructed mutants in comparison to the wt ScottA strain. This study provides increased understanding of mechanisms in *L. monocytogenes* that allow for increased survival upon exposure to various stresses.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Wt *L. monocytogenes* ScottA (Department of Food Science, Wageningen Agricultural University, The Netherlands), *L. monocytogenes* ScottA AK01 (Karatzas and Bennik in press), *L. monocytogenes* ScottA MB01, MB06 and MB18 (this study; see below) and *Escherichia coli* DH5α were used in this study. Strain *L. monocytogenes* LO28 and LO28(*ctsR*Σ*aph*A3), lacking the *ctsR* gene, were kindly provided by Dr. Shamilla Nair (INSERM, Faculté de Médicine Necker, Paris, France). Strains were routinely grown in Brain Heart Infusion (BHI) broth (Oxoid, Hampshire, England). Erythromycin (Em) (Sigma-Aldrich Chemie, Steinheim, Germany) was used at 5 µg ml^{-1} for *L. monocytogenes* and 300 µg ml^{-1} for *E. coli*.

L. monocytogenes cultures were incubated overnight at 30^{o}C, and a 0.3 % (v/v) inoculum was then added to 100 ml of BHI broth. Cultures were incubated under shaking (160 rpm), and cells were harvested by centrifugation (10,000x g, 10 min) at mid exponential phase (OD_{660} ~0.3 for the wt strain and MB01 or ~0.2 for strain AK01, MB06 and MB18).

Protein analysis using two-dimensional gel electrophoresis (2D-E)

Stock cultures of the wt strain and strain AK01 were grown and harvested as described above. The pellet was suspended in water to an OD_{660} of 10. Proteins were extracted and 2D electrophoresis was performed as described by Wouters *et al*. (1999) and O'Farrell (1975). Proteins that were clearly differentially expressed in mutant AK01 and the wt were isolated by blotting of the proteins in the gel onto membranes, stained with Coomassie blue, and cutting out of the spots. The N-terminal sequence of these proteins was determined (Utrecht University, The Netherlands)

Northern blot analysis

Cultures were grown to mid exponential phase of growth at 30°C (see above) and total RNA was extracted using an RNAeasy kit (Qiagen, Hilden, Germany). The RNA was resuspended in DEPC-treated water, and quantified by measuring the A_{260}. A total of 5 µg of RNA was denatured by incubation for 30 min at 65°C in the presence of glyoxal (1M) and DMSO (50%) and run in a 1% agarose gel. The RNA was subsequently transferred to Nytran membranes using a Turboblotter set-up, and cross-linked to the membrane by UV irradiation. Hybridisation with 32P-labelled DNA fragments was performed at 42°C using Ultrahyb solution (Ambion, Austin, USA), followed by visualization using autoradiography. Probes were obtained by labelling PCR-amplified genes using Klenow-fragment by standard techniques (Sambrook *et al*., 1989).

Materials and Recombinant DNA Techniques

Chromosomal DNA isolation, electrophoresis, hybridisation and amplification by PCR was performed according to standard protocols (Sambrook *et al.,* 1989). Restriction endonucleases and T4 DNA ligase were obtained from Roche (Mannheim,Germany). [α32P]dCTP was obtained from Amersham Biosciences (Buckinghamshire, UK). Plasmid DNA preparation, gel extraction of DNA fragments, and purification of DNA amplified by polymerase chain reaction (PCR) were performed using a QIAquick kit (Qiagen, Hilden, Germany). Oligonucleotides were obtained from Eurogentec (Seraing, Belgium). PCR reactions were performed using PCR High Fidelity (Roche), containing proofreading enzyme activities, according to instructions of the manufacturer. Sequencing of PCR fragments or cloned PCR fragments was performed by Eurogentec or Baseclear (Leiden, The Netherlands).

Sequencing of the* flaA, flaR, cheY, clpC, clpP *and* ctsR *genes in wt* L. monocytogenes *and strain AK01.

The nucleotide sequences of selected genes and their promoter regions were determined in the wt *L. monocytogenes* and the mutant strain AK01. Selected DNA fragments were PCR amplified using chromosomal DNA of the two strains as template, and the following primers were used for amplification of the genes: flaAdir and flaArev for *flaA*; flaRdir and flaRrev for *flaR*; CheYdir and CheYrev for *cheY*; ClpCdir and ClpCrev for *clpC*; ClpPdir and ClpPrev for *clpP*; CtsRsal1 and CtsReco1 for *ctsR*. The nucleotide sequences of these primers are given in Table 1.

Construction of L. monocytogenes MB06 and MB18 by allelic replacement of wt* ctsR *with mutant* ctsR (CtsRΔGly) *in* L. monocytogenes *Scott A

Strain *L. monocytogenes* AK01 carries a *ctsR* gene with a 3 basepair (bp) deletion in a triplet GTG repeat region that encodes four glycines between codon 60 (Arg) and codon 65 (Tyr) (Fig. 3). The mutant CtsR protein is designated CtsRΔGly. The gene encoding CtsRΔGly was transferred to a *L. monocytogenes* ScottA background by allelic exchange of the wt *ctsR* gene with the mutant *ctsR* gene of *L. monocytogenes* AK01. This procedure was performed using plasmid pAUL-A, containing a thermosensitive replication origin (Chakraborty *et al*., 1992), as descibed in detail by Schäferkordt *et al.* (1998). In short, a DNA fragment encompassing the mutant *ctsR*

gene of strain AK01 and ~800 bp of its 5' and 3' flanking regions was PCR amplified using genomic DNA of *L. monocytogenes* AK01 as template, and primers CtsRecofw and CtsRbamrv (Table 1). This fragment was digested with *Eco*RI and *Bam*HI, gel purified, and ligated to pAUL-A that had been digested with *Eco*RI and *Bam*HI. Transformations were performed using *E. coli* strain DH5α, and cells were plated onto BHI plates containing 300 µg/ml Em, followed by incubation at the permissive temperature (28-30°C). A plasmid with the correct insert (determined by DNA sequencing) was introduced into *L. monocytogenes* ScottA by electroporation, and transformants were selected for Em resistance (5 µg ml-1) at 28-30°C. The plasmid was integrated into the chromosomal target by culturing at the non-permissive temperature of 42°C. Spontaneous excision of the plasmid was achieved by culturing at the permissive temperature in the absence of Em. Subsequent loss of the plasmid was accomplished by incubation at the non-permissive temperature in the absence of Em.

A PCR based strategy was employed to distinguish *L. monocytogenes* strains containing the wt *ctsR* gene and the mutant *ctsR* gene. The forward primer Detmut1d (Table 1) was designed 91 bp upstream from codon 60. The reverse primer Detmut1r (Table 1) encompassed codon 61 (2 basepairs) to 70, and had a single base pair mismatch in the second last nucleotide at the 3' end (…ACCGC-3' instead of …ACCAC-3'), adjacent to the *Mae*II site (ACGT) present in codon 59 and 60 (Fig. 3). PCR amplification rendered a 118 bp DNA fragment containing an intact *Mae*II restriction site for the wt *ctsR* gene, while a 115 bp DNA fragment lacking the *Mae*II site was obtained for the mutant *ctsR* gene. PCR reactions were performed on genomic DNA of 19 isolates. Purified PCR products were incubated with *Mae*II (Roche), and visualised with EtBr on a 5% agarose gel (Nusieve, FMC, Rockland, USA). Two mutant clones, designated MB06 and MB18, were identified using this screening method, and sequencing of their complete *ctsR* genes confirmed the 3 bp deletion between codon 60 and 65 in absence of other mutations in the *ctsR* gene compared with wt *ctsR*. Strain MB01 contained the wt *ctsR* gene after the double crossover procedure, and was used as a control.

High Hydrostatic Pressure treatment

Mid exponential phase cultures of *L. monocytogenes* (see above) were subjected to High Hydrostatic Pressure (HHP) treatment of 350 MPa for 20 min at 20 °C as previously described (Karatzas and Bennik, in press). The viable numbers of *L. monocytogenes* were determined in triplicate before and after pressure treatment by plating decimal dilutions of samples onto BHI agar (1,2 % w/v agar). Plates were incubated at 30^{o}C for 5 days.

Motility tests

The motility of *L. monocytogenes* strains was tested as described previously (Karatzas and Bennik, in press). In short, semi-solid Motility Test medium containing 0.4% w/v agar, 10 g l^{-1} peptone (Oxoid, Hampshire, England), 5 g l^{-1} NaCl (Merck, Darmstadt, Germany), 3 g l^{-1} Beef Extract (Becton Dickinson, Sparks, USA), 0.05 g l^{-1} 2.3.5-Triphenyltetrazolium chloride (Sigma-Aldrich Chemie, Steinheim, Germany), was inoculated with *L. monocytogenes* by stabbing. After 5 days of incubation at 30°C,

isolates that were motile, and therefore able to swarm, showed a red cloudy pattern as a result of reduction of 2,3,5-Triphenyltetrazolium chloride to formazan caused by bacterial metabolism.

Mouse virulence assays

L. monocytogenes ScottA, the piezotolerant isolate *L. monocytogenes* AK01, and *L. monocytogenes* MB18 (CtsRΔGly) were analysed for virulence in a murine model of infection. Female Balb/c mice were inoculated intraperitoneously with a $4.5 * 10^5$ cfu inoculum in 200 µl PBS. Numbers of bacteria surviving in mouse spleens were determined for the first three days post-infection.

RESULTS

The piezotolerant strain AK01 shows reduced levels of FlaA protein and* flaA *mRNA, and increased levels of ClpP protein and* clpP *mRNA

To identify putative differences in protein expression between the High Hydrostatic Pressure tolerant isolate *L. monocytogenes* AK01 (Karatzas and Bennik, in press) and wt *L. monocytogenes* ScottA, we performed 2D gel electrophoresis of the proteins extracted from mid-exponential phase cultures. Compared with the wt, cells of strain AK01 showed increased amounts of ClpP, which was identified by N-terminal amino acid sequencing (Fig. 1). The heat shock protein ClpP is a serine protease involved in stress response, intracellular parasitism and virulence (Gaillot *et al*., 2000). Cells of AK01 showed decreased amounts of two proteins, which were both identified as flagellin (Fig. 1), which is the structural protein of the flagellum and it is encoded by the gene *flaA* (Dons *et al.*, 1992). The presence of two spots identified as flagellin (FlaA) on the 2D gel can be explained by post-translational modification, e.g. glycosylation, as described for flagellar proteins from several bacteria (Dons *et al.*, 1992, Peel *et al.*, 1988).

Northern analysis furthermore demonstrated 13-fold higher *clpP* mRNA levels and 10-fold lower *flaA* mRNA levels in strain AK01 than in the wt (Fig. 2). The nucleotide sequences of the promoter regions and coding regions of *flaA* and *clpP* were analysed to establish whether putative mutations were present that could account for the different expression levels in AK01 and the wt. This analysis demonstrated that the nucleotide sequences of these genes and their promoter regions were identical in both strains. Interestingly, Northern analysis showed a significant 30-fold increase in the levels of *ctsR* mRNA in strain AK01 compared with the wt (Fig 2).

***Sequencing of* clpP, flaA, flaR, cheY, clpC *and* ctsR *genes reveals a 3bp deletion in* ctsR**

The reduced levels of FlaA protein and *flaA* mRNA, and the increased levels of ClpP protein and *clpP* mRNA in strain AK01 compared with the wt suggested different transcription regulation of the *clpP* and *flaA* genes in the two strains. A number of genes have previously been shown to be involved in stress resistance, motility, and regulation of FlaA or ClpP proteins in *L. monocytogenes*, namely, *cheY* (Michel *et al.*, 1998), *flaR* (Sanchez-Campillio *et al.*, 1995), and *clpC* (Nair *et al.*, 2000, Rouquette *et al.*, 1998), *clpE* (Nair *et al.*, 1999), *prfA* (Michel *et al.*, 1998), *cheY* (Flanary *et al.*,

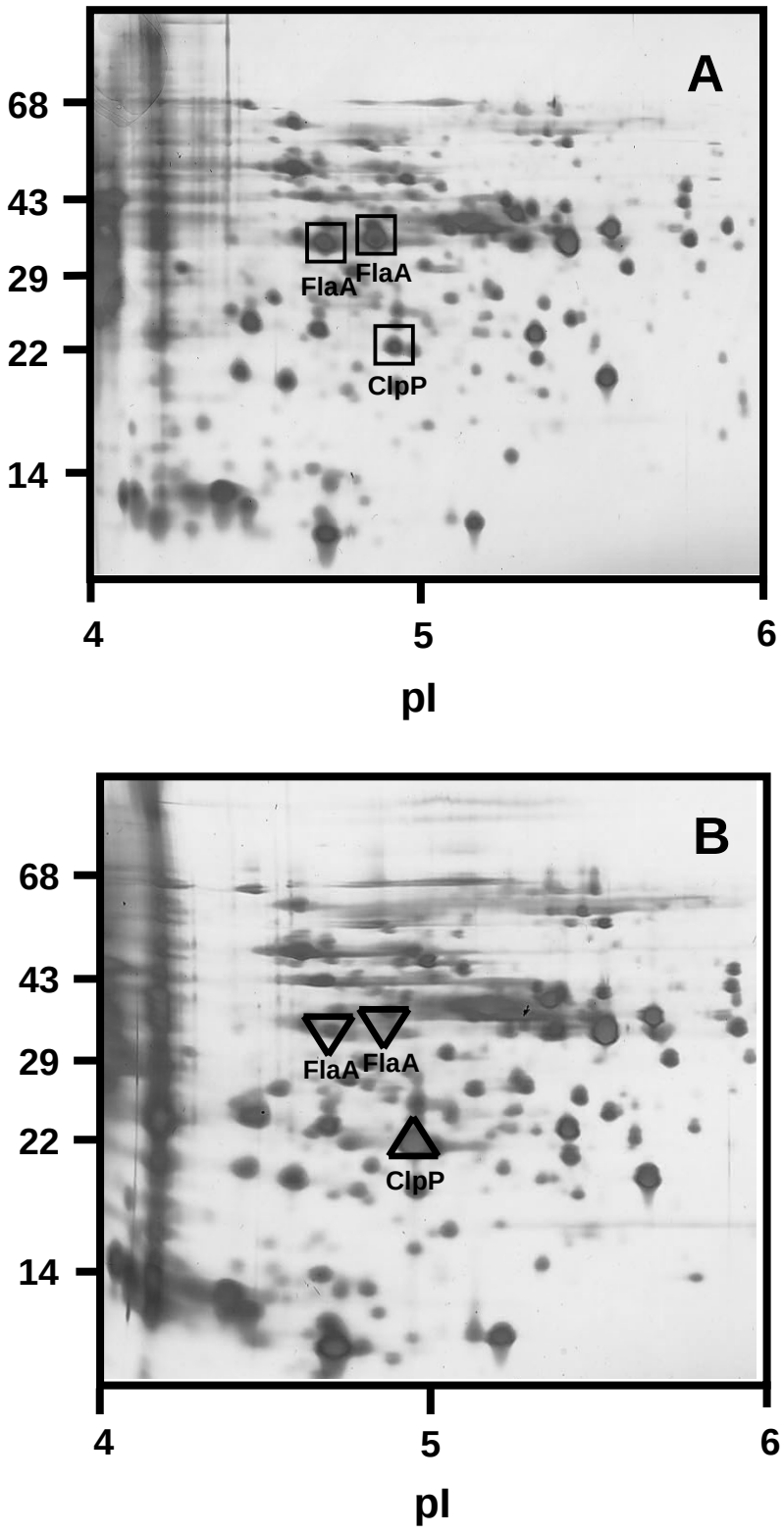

Fig 1. 2D protein gel electrophoresis of *Listeria monocytogenes* ScottA wild type (A), and the piezotolerant isolate *L. monocytogenes* AK01 (B). Cells were grown in BHI with shaking at 30^{o}C, and harvested at mid exponential phase. Gel analyses were repeated three times to confirm reproducibility, and representative results are shown. The symbols indicate upregulation (Δ) and downregulation (∇) of a protein.

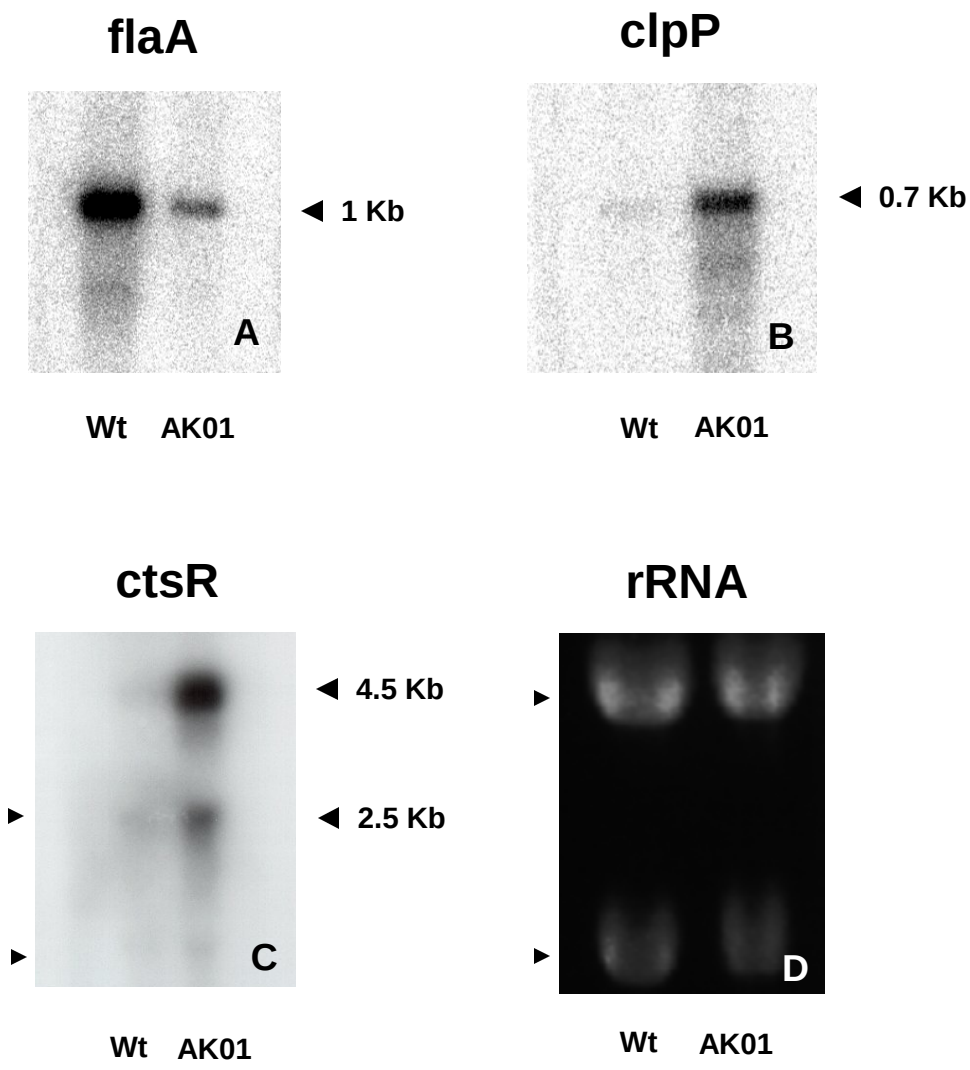

Fig 2. Gene expression in wt *L. monocytogenes* ScottA and *L. monocytogenes* Scott A AK01. A) Northern blot of *flaA* mRNA. B) Northern blot of *clpP* mRNA C) Northern blot of *ctsR* mRNA D) ribosomal RNA bands visualized by ethidium bromide staining confirming that equivalent amounts of RNA were loaded. Total RNA was extracted from mid-exponential cultures of *L. monocytogenes* ScottA (wild type) or mutant AK01 grown at 30°C. Autoradiograms were exposed for ~12 h. Small arrows indicate the position of the bands of rRNA.

1999) and *ctsR* (Nair *et al.*, 2000). The nucleotide sequences of these genes and their ~200 bp upstream regions were identical for strain AK01 and the wt strain, except for *ctsR*. The *ctsR* gene of AK01 showed a 3 basepair (bp) deletion between codon 60 and 65. This region encodes 4 glycines in wt CtsR protein, and only 3 glycines in the mutated CtsR protein of AK01, designated CtsRΔGly (Fig. 3).

Allelic replacement of* ctsR *with mutant* ctsRΔGly *renders the HHP tolerant phenotype

To investigate whether the 3 base-pair deletion in the *ctsR* gene of strain *L. monocytogenes* AK01 was exclusively responsible for the observed High Hydrostatic Pressure tolerant phenotype, we constructed strains *L. monocytogenes* MB06 and

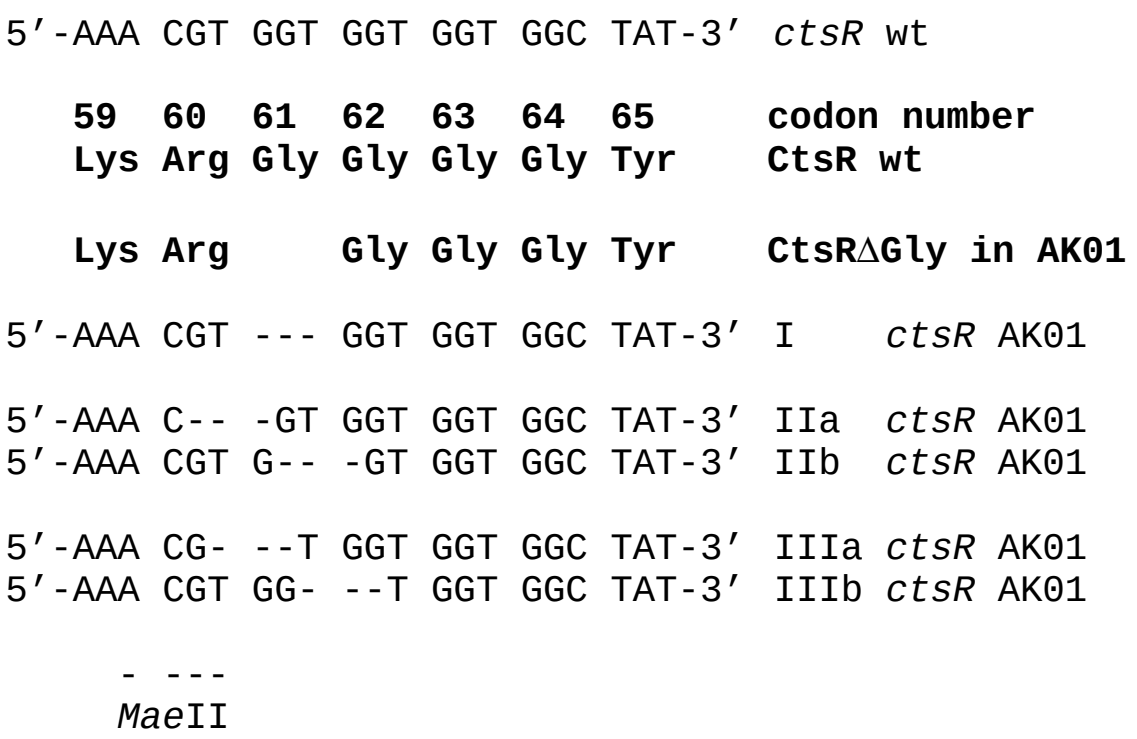

Fig 3. The CtsR protein of *L. monocytogenes* AK01, designated CtsRΔGly, contains 3 glycines versus 4 glycines in the wt CtsR protein of *L. monocytogenes* ScottA, as a result of (*I*) a GGT in frame deletion in codon 61, 62, or 63; (*II*) a GTG deletion in the –2 frame of codon 61, 62, 63, or 64 (a), or the +1 frame of codon 60, 61, 62, or 63 (b); (*III*) a TGG deletion in the –1 frame of codon 61,62,63, or 64 (a), or the +2 frame of codon 60,61, 62, or 63 (b).

MB18. Both strains carried the mutant *ctsR* gene, encoding CtsRΔGly, in a wt background after allelic replacement of the native *ctsR* gene with the mutant *ctsR* gene from strain AK01. Upon exposure to HHP treatment, strains MB06 and MB18 showed 2 to 3 log lower reductions in viable numbers than wt *L. monocytogenes* ScottA (Fig. 4). These reductions were similar to those observed for the piezotolerant strain AK01. HHP treatment of strain MB01, in which allelic replacement did not take place and served as a control, resulted in reductions in viable numbers that were similar to the wt.

L. monocytogenes *expressing the CtsRΔGly protein is non motile*

Strains *L. monocytogenes* MB06 and MB18, expressing the CtsRΔGly protein, did not swarm in semi-solid motility test medium, whereas the wt and the control strain MB01 clearly showed diffuse growth in the test tubes. The non motile character of strains MB06 and MB18 was indistinguishable from that of the piezotolerant strain AK01 previously observed (Karatzas and Bennik, in press). The non motile phenotype of AK01 is in agreement with the reduced FlaA expression observed in the 2D gel electrophoresis analysis.

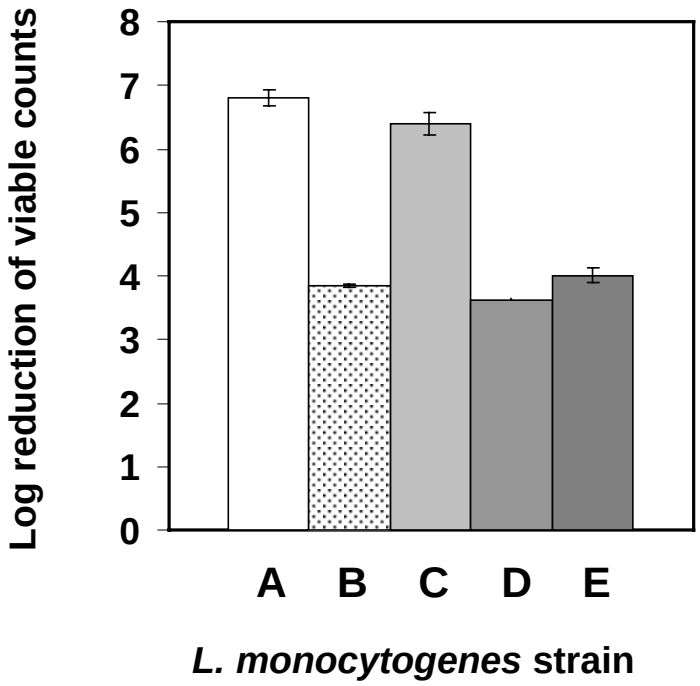

Fig 4. Log reduction in the viable counts of wild type *Listeria monocytogenes* ScottA (A), of AK01 (B), strain MB01 containing the wt *ctsR* gene after double crossover in wt background (C), or MB06, MB18 containing the mutant *ctsRΔGly* gene after double crossover (C, D) after HHP treatment at 350 MPa, at 20^{o}C for 20 min. Cells were grown in BHI broth at 30^{o}C under shaking, and values are means of triplicate measurements. Bars represent the S.D. (n=3).

L. monocytogenes *expressing the CtsRΔGly protein is less virulent than the wt*

We compared the virulence properties of wt *L. monocytogenes* ScottA with those of the piezotolerant strains AK01 and MB18, carrying the gene encoding CtsRΔGly, by analysing the kinetics of bacterial growth in the spleens of mice infected by the intraperitoneal route. Wt *L. monocytogenes* ScottA was detected at relatively high levels in the spleens of infected mice at 24 hours post inoculation. The wt strain was subsequently capable of significant growth in infected mice over the following 48 hours, reaching levels of 3.4 * 10^6 bacteria per organ by day 3 post infection. In contrast, the *L. monocytogenes* piezotolerant mutants AK01 and MB18 were detected at low levels in the spleens of infected mice 24 hours following inoculation, suggesting an inability to survive the initial stages of murine infection. The mutant was incapable of significant growth in the spleens of infected mice but did persist in infected spleens over the three days of the study (Fig 5).

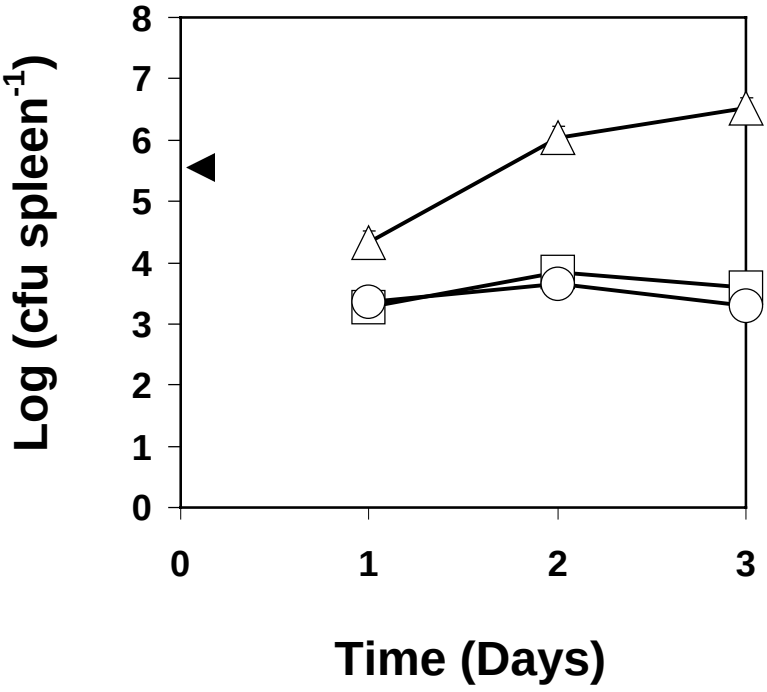

Fig 5. Colony forming units (cfu) of *Listeria monocytogenes* ScottA (Δ), AK01 (□) and MB18 (O) in the spleens of infected Balb/c mice through time (days). Arrow indicates infectious dose used through the experiment (log cfu = 5.65) by intraperitoneal route.

Table 1. Nucleotide sequences of primers used in this study

Primer name	Nucleotide sequence
CheY dir	5‘-ATTACAAATAGGGCGCAGAG-3‘
CheY rev	5‘-GAAGCTTCTTCTATAAACAG-3‘
ClpC dir	5‘-CGATTTGGTGTAGAATTAGG-3‘
ClpC rev	5‘-GACGCGAATCATGATTTTTC-3‘
ClpP dir	5‘-CGCTTCAGACTTTATCGTTTGACC-3‘
ClpP rev	5‘-AATACTAGTGTATACATTCTATGG-3‘
CtsRsal1	5‘-GAGAGCGTCGACCGTAGCACAATTCTCGCAT-3‘
CtsReco1	5‘-AAGCTTGAATTCGCCAATGGTAGTTGGGGGC-3‘
CtsRecofw	5‘-GATAAAGAATTCCCCGGGGATAACAGGCTTATC-3‘
CtsRbamrv	5‘-TCCTCAGGATCCAGAACCGCACCATATTCACTC-3‘
Detmut1d	5‘-AGATAAGTTTGAATGTGTACCTTC-3‘
Detmut1r	5‘-AATAATCCTAATATAGCCACCACCGC-3‘
FlaA dir	5‘-CGTAAAAACGTTGATAATAAGCCG-3‘
FlaA rev	5‘-GGGGCTAAGGGTAAACAATGTTCG-3‘
FlaR dir	5‘-TCGCGGTAAGCTAAAAGATG-3‘
FlaR rev	5‘-GAAGTAATACGTATTATCGC-3‘

DISCUSSION

This study demonstrated that increased tolerance of *L. monocytogenes* to high hydrostatic pressure resulted from a single amino acid deletion in the highly conserved glycine region of CtsR. Moreover, the loss of this residue was responsible for reduced virulence and loss of motility of *L. monocytogenes.*

The deletion of the glycine residue in CtsR significantly impairs repression of genes that are under the negative control of this regulator, as indicated by induced transcription of *clpP* and *ctsR*, which are part of the *clpC* operon. Clp proteases of *Bacillus subtilis* were shown to be directly involved in degradation of misfolded proteins (Krüger *et al*., 2000). Thereby, increased expression of the Clp proteins prevents the accumulation of proteins that are misfolded by heat, HHP or other stresses, and that might be toxic to the cell (Gaillot *et al*., 2000). To our knowledge, this is the first study that demonstrates the involvement of Class III heat shock genes in increased survival of HHP treatment, and it indicates that one of the survival mechanisms is directly related to the ability of the cells to cope with misfolded proteins. These findings are in line with previous studies, which showed that increased resistance to HHP coincided with increased heat resistance in various microorganisms (Alpas *et al*., 2000; Iwahashi *et al*., 1993; Karatzas and Bennik, in press). Furthermore, Welch *et al.* (1993) observed a transient induction of proteins upon exposure of *E. coli* to relatively low, non-lethal, hydrostatic pressures (55 MPa). Many of the observed pressure-inducible proteins were known heat shock or cold shock proteins. Given these observations, it is highly likely that heat shock genes other than those belonging to Class III can mediate increased survival to HHP treatment.

There is evidence that proper folding of CtsR is mediated by the ClpC chaperone protein (Derré *et al*., 2000), and that the highly conserved glycine-rich region is essential for CtsR to adopt its proper conformation and act as an effective repressor. Derré *et al*. (1999) previously postulated the hypothesis that the glycine rich region in CtsR acts as a heat sensor, with intrinsic temperature sensitivity. This could involve a conformational change leading to an inactive form of the repressor and derepression of the target genes. A mutant with an in frame deletion of 14 codons encompassing the region encoding the glycines showed normal expression of the mutant protein, however, its repressor functions were abolished (Derré *et al*., 2000). Derepression of *clpP* was also observed in the strain AK01, expressing the CtsRΔGly protein. This suggests that the glycine-residue deletion causes a conformational change in CtsR that affects its binding to DNA. We have compelling evidence that the occurrence of this specific 3 base pair deletion in the highly conserved glycine region is not an isolated incident in *L. monocytogenes*. In three independent experiments, we obtained over 30 HHP tolerant isolates of *L. monocytogenes* and determined the nucleotide sequence of their *ctsR* genes. The majority of the isolates showed the 3 basepair deletion in the GTG repeat region that encodes the glycine region of CtsR. The frequency of mutants containing this deletion in *ctsR* was unexpectedly high (0.04%) (Karatzas, Valdramidis and Bennik, unpublished), indicating that a fraction of the wt population has intrinsic different properties, resulting from genetic variation. Many bacteria can generate genetic variation at individual loci, called contingency

loci, many of which are controlled by simple DNA repeats that accumulate reversible, *rec*-independent mutations at high frequency (Moxon *et al*., 1994; Bayliss *et al*., 2001). Such a strategy can allow for survival under stress conditions that do not allow for adaptation of individual cells, but require genetic diversity to survive (Bayliss *et al*., 2001).

Interestingly, strains expressing the CtsRΔGly protein were non-motile. In *Listeria*, mobility has been shown to be under the control of the PrfA regulator (Michel *et al*., 1998). This study demonstrated that transcriptional regulation of *flaA*, encoding the structural protein of flagella, is dependent on CtsR. Since a CtsR binding site in absent in the promoter region of this gene, this is more likely an indirect effect.

Importantly, strains expressing CtsRΔGly were less virulent than the wt strain. These mutant strains persisted in the mouse spleens, and caused enlargement of the spleen, but did not show an increase in their numbers in the host tissue during three days. Whereas most of the virulence genes in *L. monocytogenes* are controlled by PrfA (Cossart and Lecuit, 1998; Kreft and Vazquez-Boland, 2001), recent studies have pointed out a role for CtsR and the *clp* genes in virulence. Gaillot *et al*. (2000) showed that deletion of *clpP* gave rise to reduced virulence, likely as a result from reduced activity of listeriolysin O, the major virulence factor implicated in bacterial escape from phagosomes of macrophages, while complementation with *clpP* restored virulence. In addition, attenuated virulence was observed upon inactivation of ClpC and ClpE (Rouquette *et al*., 1996; Nair *et al*., 1999), and overproduction of CtsR (Nair *et al*., 2000). However, a *ΔctsR* mutant did not show increased virulence (Nair *et al*., 2000). Despite increased ClpP expression and upregulation of the *clpC* operon, we observed reduced virulence of strains AK01 and MB18, expressing the CtsRΔGly protein. It is possible that a recently identified surface virulence-associated protein (SvpA) (Borezée *et al*., 2001) accounts for the observed attenuated virulence in our mutant strain. These authors demonstrated that SvpA is PrfA-independent and essential in the virulence of *L. monocytogenes*. Increased levels of ClpP and ClpC downregulated SvpA, leading to reduced virulence (Borezée *et al*., 2001). It seems that CtsR and the Clp proteins have a dual and highly fine-tuned role in virulence, and can mediate increased or attenuated virulence. The use of attenuated invasive bacteria such as *L. monocytogenes* as vaccines or vaccine delivery systems has been shown experimentally to be a delicate balance between virulence and immunogenicity (Guzman *et al*., 1997). Thus attenuated bacteria should infect and persist for long enough to provoke protective immunity without causing symptoms of disease (Gahan and Collins 1995; Koenig *et al*., 1982). Further investigation of the role of stress response systems in the virulence of *L. monocytogenes* will increase our understanding of the virulence of this organism and may contribute to the rational design of attenuated bacterial vaccines and vaccine delivery systems.

In conclusion, CtsR and the Clp proteins play an important role in resistance to HHP treatment and other stresses, mobility, and virulence. These proteins are therefore essential for maintaining fitness in diverse and changing environments, apparently not only by adaptation through gene regulation, but also by genetic variation resulting in intrinsically different phenotypes.

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CHAPTER 6

General Discussion

Combined processing

The microbial stability and safety of most traditional and novel foods is based on a combination of several preservative factors that inactivate microorganisms present in the food, or do not allow for their growth. The combination of preservative factors –or hurdles- allows for gentle but efficient preservation of foods. In general, hurdle technology can lead to less robust treatments of foods compared to traditional preservation methods, and offers more fresh and more nutritious products that are safe for consumption (Leistner, 1999). For the advanced application of hurdle technology, a continuously increasing number of preservative factors have become available (Leistner, 1999). Different hurdles can be applied simultaneously or at different stages throughout the production of the food, and can have additive or synergistic antimicrobial effects. In addition to the classical preservation factors like heat, acids, preservatives, and the use of competitive microorganisms such as lactic acid bacteria, more than 50 hurdles with potential use in foods have been identified and described, and the list is by no means complete (Leistner, 1999). Two of the lately investigated food preservation methods are the use of essential oils and High Hydrostatic Pressure (HHP). In this thesis, the antimicrobial potential of these preservation methods to inactivate the foodborne pathogen *L. monocytogenes* was studied. We demonstrated that volatiles present in plant-derived essential oils could enhance the antimicrobial effect of mild heat and High Hydrostatic Pressure (HHP) treatments against the foodborne pathogen *L. monocytogenes*. Since variable pressure resistance of cells within a population might hinder successful application of HHP, we furthermore studied properties of a *L. monocytogenes* isolate with increased HHP tolerance, which was obtained from a pure wild type culture.

Combined action of essential oils and mild heat

Plant-derived essential oils are known for their antimicrobial properties, which are attributed to the hydrophobic nature of some substances they contain (Sikkema *et al.*, 1994). It was demonstrated that these compounds in combination with a mild heat treatment can be used to inactivate *L. monocytogenes*. The combined action of the plant-derived volatile S-carvone and mild heat treatment (30 min at 45^{o}C) reduced the viability of exponential phase cells of *L. monocytogenes* that were grown at low temperature (8°C). Similar results were obtained with several combinations of mild heat treatment and other plant-derived volatiles, e.g. carvacrol, cinnamaldehyde, thymol, or decanal. Such a combined effect proved to be synergistic, as neither individual treatments with all concentrations of volatiles tested, nor exposure to 45°C for 30 min, resulted in a loss in viability. This synergism was probably caused by the simultaneous effect of both hurdles on a common cellular target, possibly the membrane. This is supported by the observation that the same combined treatments were ineffective in reducing the viable numbers of cultures of *L. monocytogenes* that were grown at 35 or 45^{o}C. Cultures grown at these temperatures have lower membrane fluidity at 45°C than cultures grown at 8°C. Lower membrane fluidity allows lower amounts of these compounds to

accumulate in the cellular membrane and thus results in reduced antimicrobial effects caused by these compounds (Sikkema *et al.*, 1995).

Combined process of essential oils and High Hydrostatic Pressure

Furthermore, we studied the potential of essential oil compounds in combination with HHP with the aim of reducing the intensity of HHP treatments. This could be achieved by combined processing, as HHP treatment in the presence of carvacrol or thymol demonstrated synergistic antimicrobial activity. This synergism is believed to result from effects of both treatments on the cellular membrane (Sikkema *et al.*, 1995; Cheftel, 1995). HHP greatly affects membranes with a relatively low fluidity (MacDonald, 1992), and in line with this, we found that the synergistic antimicrobial effect of HHP and essential oil compounds on cells that were grown at 8°C was higher during treatment at 1^{o}C, where cells have lower membrane fluidity, than at 8°C or 20^{o}C. We furthermore observed that treatment with HHP, subsequently followed by treatment with carvacrol, resulted in similar reductions in the viable numbers compared with simultaneous treatment, while HHP treatment alone, or carvacrol treatment alone, showed higher survival rates. This indicates that HHP treatment renders sub-lethally injured cells, and that carvacrol does not allow for recovery of these cells. The combined effect of HHP with carvacrol was also achieved in semi-skimmed milk demonstrating the potential use of this combination in foods. In line with our observations, Adegoke *et al.* (1997) have previously shown that the plant volatiles α-terpinene and (R)-(+)-limonene enhanced the antimicrobial effects of HHP by preventing the recovery of pressure-injured *Saccharomyces cerevisiae* cells. These findings show that essential oil compounds can play an important role in minimally processed foods, and can be used in the concept of hurdle technology to reduce the intensity of heat treatment, HHP and possibly other individual hurdles (Pol and Smid, 1999).

***A mechanism contributing to piezotolerance of* L. monocytogenes**

One of the novel techniques with possible applications in combined processing is HHP. An important problem for successful application of HHP is the increased piezotolerance of individual cells within a population (Palou *et al.*, 1999). This could be the result of bacterial heterogeneity depending on the phase of growth, genetic heterogeneity within a population, or stress response (Palou *et al*., 1999). Previously, it was shown that HHP tolerant strains could be isolated from an *E. coli* culture upon exposure to HHP (Hauben *et al*., 1997). In this thesis, we have shown that spontaneous mutants with increased piezotolerance can be present in populations of *L. monocytogenes*. We isolated a HHP tolerant strain of *L. monocytogenes* after a single pressurisation treatment. This mutant, named AK01, showed a range of altered phenotypic characteristics compared to the wild type, namely, reduced maximum specific growth rates at different temperatures, lack of flagella, and elongation of cells. The piezotolerance of AK01 was increased showing survival more than 2 logs higher than that of wild type *L. monocytogenes* over a broad range of pressures (150-500 MPa), for exponential and stationary phase cells, while both strains showed higher piezotolerance in the stationary than in the exponential phase of growth. In semi-skimmed milk, exponential phase cells of both strains showed lower reductions upon pressurisation than in ACES buffer, but again, AK01 was more piezotolerant than the wild type. AK01 also demonstrated a multiple resistant phenotype against heat, acid and H_2O_2, while maximum NaCl growth concentration was identical. In line with that there are several reports linking increased HHP between different

strains or species with increased heat resistance (Metrick *et al*.1989; Iwahashi *et al*., 1997; Smelt, 1998; Alpas *et al*., 2000). In an attempt to identify the cause for all these altered characteristics we determined the membrane fluidity of AK01 and the wildtype, which proved to be identical. This finding was in line with the observation of Hauben *et al*. (1997) that the increased HHP resistance of the *E. coli* mutants could not be linked to altered membrane properties. In this thesis, we present conclusive evidence that the increased resistance of strain AK01 to HHP and heat was the result of a mutation in the *ctsR* (class three stress gene repressor) gene. To our knowledge, this is the first time that increased piezotolerance is directly linked to an altered genotype.

CtsR and Class III heat shock genes

In *L. monocytogenes* and in *B. subtilis*, the expression of heat shock genes involves at least four different regulatory mechanisms. Class I heat shock genes encode classical heat shock chaperones, such as GroES, GroEL and DnaK, which are controlled by the HrcA repressor. Class II genes encode general stress proteins, the transcription of which is dependent on the alternative sigma factor σ^B. Class III heat shock genes encoding ClpP and two heat shock protein (Hsp) 100 Clp ATPases, ClpC and ClpE, form part of the CtsR stress response regulon. These genes are negatively controlled by CtsR, the product of the first gene of the *clpC* operon. Class IV includes stress response genes, whose expression is independent on HrcA, σ^B or ctsR, and whose regulatory mechanisms remain unidentified (Derré *et al.*, 1999; Nair *et al*., 2000). The expression of CtsR, the negative regulator of Class III heat shock genes, is thermoregulated. At low temperatures (< 30°C) its upregulation prevents production of unnecessary stress proteins unless another stress is present, and it thereby contributes to the overall cell economy (Nair *et al.*, 2000). CtsR and the Clp proteins not only play a role in heat shock response of the cell, but also in virulence of *L. monocytogenes* (Rouquette *et al.*, 1996; Rouquette *et al.*, 1998; Nair *et al.*, 1999; Gaillot *et al.*, 2000; Nair *et al.*, 2000). At 37^{o}C, which corresponds to the temperature of the infected host (human), CtsR synthesis is low, resulting in high expression of Class III heat shock proteins that protect the cell against the defence mechanisms of the infected host at the initial stages of infection in the phagosomal compartment (Ripio *et al.*, 1998; Nair *et al.*, 2000). Subsequently, low amounts of CtsR lead to negative autoregulation (Roquette *et al*., 1996; Nair *et al.*, 2000) resulting in higher concentrations of CtsR in the cell. This inhibits the expression of unnecessary ClpC, ClpP and ClpE, while at this stage the cell is safe in the cytoplasm of the infected host (Ripio *et al.*, 1998; Nair *et al.*, 2000). In addition to CtsR, also PrfA, which is overexpressed at 37^{o}C, downregulates ClpC (Ripio *et al*., 1998; Chakraborty *et al*., 1992). At 42^{o}C *ctsR* transcription and the transcription of Class III heat shock genes is moderately upregulated in exponential growth as their regulation by CtsR is inactive at that temperature (Roquette *et al*., 1996).

The role of ctsR in stress survival and virulence

As apparent from the above, CtsR plays an important role in the stress survival and virulence of *L. monocytogenes*. CtsR contains a helix-turn-helix DNA motif in position 26 (RSEIADKFECVPSQINYVIN), that allows for its binding to the promoter regions of ClpP, ClpC and ClpE, and competes or interferes with the RNA polymerase Eσ^A binding sites (Nair *et al*., 2000; Roquette *et al*., 1996). In addition to the HTH region, a glycine-rich region in the central region of CtsR seems to be modulating binding to the promoter regions (Derré *et al*., 2000). In the piezotolerant strain AK01 of *L. monocytogenes*, a single glycine deletion in this

region was responsible for increased piezotolerance, reduced motility, and attenuated virulence. Increased transcription of *clpP* and production of ClpP was demonstrated in strain AK01, and in addition, the expression of the *clpC* operon was increased as indicated by elevated levels of *ctsR* mRNA. Hereby, we obtained evidence that increased amounts of Class III heat shock proteins are responsible for the increased piezotolerance and probably the multiple stress resistance of strain AK01 to heat, acid and hydrogen peroxide. Welch *et al.* (1993) has previously indicated that heat shock proteins might play a significant role in piezotolerance. Importantly, strain AK01 showed reduced virulence compared with the wild type. A recent study by Borezée *et al.* (2001) indicated that increased levels of ClpP and ClpC downregulate the surface virulence-associated protein SvpA, which is essential for virulence. Possibly, AK01 is able to perform the initial stages of infection resulting from increased amounts of Class III heat shock proteins, but is unable to grow and infest the host at a later stage, keeping its numbers steady in the spleens of infected mice for 3 days. This could be caused by downregulation of SvpA or other virulence factors, the inability to shut down production of Class III heat shock genes in the latter stages of infection, or other functions related to growth, which was slower for strain AK01 than for the wt in BHI broth. The fact that a mutation in a gene related to the expression of heat shock proteins causes such a broad alteration in phenotypic characteristics indicates the existence of a fine-tuned but complex network of information that is mediated by gene expression and protein production. At a first glance, this complex network involves unrelated characteristics, like stress response, virulence, and motility. However, regulation of these characteristics can be vital in environments like the cytoplasm, where motility and stress resistance genes are shut down, while virulence-mediating genes are upregulated (Michel *et al.*, 1998). Nevertheless, because of the complexity of this network, not all of its functions and interactions are known, and further studies are needed to reveal the missing links and pathways governing the CtsR and Clp protein-mediated behaviour of *L. monocytogenes*.

Occurrence of HHP tolerant* L. monocytogenes *strains lacking a glycine in CtsR

In additional experiments that are not presented in this thesis, the occurrence of HHP resistant isolates of *L. monocytogenes* was investigated (Karatzas , Valdramidis and Bennik, data not shown). Therefore, three independent cultures of *L. monocytogenes*, that were started from single colonies, were subjected to a pressurization treatment. In total, over 30 HHP tolerant strains of *L. monocytogenes* were isolated, and the nucleotide sequences of their *ctsR* genes were determined. Identical to strain AK01, about ¾ of the isolates showed the 3 basepair deletion in the region that encodes the glycine rich domain of CtsR. The frequency of mutants in the wildtype population that contained this deletion in *ctsR* was unexpectedly high (0.04%) (Karatzas *et al.*, in preparation). This indicates that a fraction of the wt population has intrinsic different properties, resulting from genetic change, in this case a GTG nucleotide deletion in a 4x GTG repeat. Many bacteria can generate genetic variation at individual loci, called contingency loci, many of which are controlled by simple DNA repeats that accumulate reversible, *rec*-independent mutations at high frequency (Moxon *et al.*, 1994; Bayliss *et al.*, 2001). Such a strategy can allow for survival of unforeseen events that do not allow for adaptation of individual cells, but require genetic diversity to survive. The observed natural variation in the *ctsR* sequence of *L. monocytogenes* provides insight in a mechanisms that seems to be important for this organism to survive high pressure treatment, and possibly other adverse conditions. It explains, in part, the increased piezotolerance of a subpopulation of *L.*

monocytogenes. However, since not all piezotolerant strains contained the mutation in CtsR, other resistance mechanisms, possibly related to other heat shock responses or membrane stability, account for their increased survival to HHP.

Implications of the work described in this thesis

In this thesis, it is demonstrated that compounds of essential oil fractions of plants, such as S-carvone, carvacrol, thymol, cinnamaldehyde and decanal can contribute to combined minimal processing. These compounds can be employed to enhance the antibacterial effects of heat and HHP. Importantly, the above-mentioned combined treatments showed synergistic antimicrobial effects on cells of *L. monocytogenes*. In addition, their presence could offer a prolonged antibacterial effect in the product until the time of consumption. Application of essential oils in food products will be restricted because of their distinctive odour, but we showed that in specific cases their application is possible, and these substances can offer a valuable additional hurdle against microorganisms.

The piezotolerant strain *L. monocytogenes* AK01 demonstrated elevated resistance, not only to HHP, but also to heat, acid, and H_2O_2. The multiple stress resistance of this strain could potentially cause problems when higher viable numbers of this strain would remain in a food product after antimicrobial treatment with heat or acid. However, given the strongly attenuated virulence of strain AK01 compared with the wildtype, this risk is presumably low. Furthermore, with regard to HHP treatment, it is important to assess the pressure that will effectively kill the strains with increased piezotolerance. In the case of *L. monocytogenes* AK01, this was achieved at levels of 500 Mpa for exponential phase cells and 550 MPa for stationary phase cells , and if HHP would be applied as the sole antimicrobial treatment, this level would be required. The intensity of the treatment could be reduced using combined treatment with essential oils, and possibly other antimicrobial agents. However, since food constituents might have a protective antagonistic effect, combined treatments need to be fine-tuned for specific products (Palou *et al*., 1999; Smelt, 1998). Knowledge about microbial inactivation by combined processes and resistance mechanisms of microorganisms will help to develop safe standards for HHP treatments that can be applied in the food industry. In this respect, variability in piezotolerance of different species, different strains of a certain species, and even subpopulations of a culture need to be taken into account (Hauben *et al*., 1997; Metrick *et al*., 1989; Alpas *et al*., 2000).

In this thesis, we demonstrated that at least one of the mechanisms of HHP resistance of *L. monocytogenes* is related to the induced expression of heat shock proteins. This provided useful information concerning one of the primary sites of pressure damage and resistance, and suggests that proteins are one of the key targets of HHP treatment. The ability of the cell to cope with misfolded proteins resulting from pressure treatment was enhanced in the piezotolerant mutant. This was indicated by the increased amounts of Clp stress proteins that have proteolytic and chaperone activities, and eliminate toxic misfolded proteins (Gaillot *et al*., 2000; Krüger *et al*., 2000, 2001). Such a mechanism might also account for increased HHP tolerance in other species.

Class III heat shock proteins and CtsR, particularly its helix-turn-helix domain and the glycine-rich region in which we found a spontaneous mutation, are highly conserved among the Gram positive bacteria (Derré *et al.*, 1999; Derré *et al*., 2000). Therefore, it is possible that a similar mechanism of HHP tolerance also exists in other Gram positive bacteria. It was

furthermore demonstrated that CtsR and Class III heat shock genes play in important aspects related to stress resistance, motility and virulence of *L. monocytogenes* and possibly other Gram positive bacteria. Further studies are required to elucidate the complex interactions that are governed by these proteins, and their role in processes other than merely heat shock.

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SUMMARY

A variety of non-thermal mild preservation techniques have lately been introduced in food production, or are currently being investigated thoroughly for potential use in food processing. Two of these novel preservation techniques are the use of essential oil compounds isolated from plants and of High Hydrostatic Pressure (HHP) treatment. Applications of these preservation methods, alone or combined, have shown to reduce the viable numbers of microorganisms occurring in foods, and in addition, are able to preserve the nutritional and sensory characteristics of the product. The objectives of this thesis were to asses the antimicrobial potential of these combined processing techniques against the foodborne bacterium *Listeria monocytogenes*, which can cause serious disease with a relatively high mortality rate. In addition, to get more insight in the primary cellular targets that are affected upon HHP treatment of *L. monocytogenes*, we examined the mechanism underlying increased HHP tolerance of an isolate of this organism.

Chapter 1 gives a short introduction on *L. monocytogenes.* Furthermore, mild and novel preservation techniques in food processing are addressed, with special emphasis on the use of essential oil compounds from plants, and the use of High Hydrostatic Pressure (HHP). General characteristics and the mode of action of these preservation techniques are described, followed by resistance mechanisms that bacteria use to counteract their potentially lethal action. This chapter is concluded with the combined use of these preservation methods, and an outline of this thesis.

In **Chapter 2**, it is demonstrated that various plant-derived volatiles, namely, S-carvone, carvacrol, cinnamaldehyde, thymol, and decanal, have the ability to reduce the viable numbers of *L. monocytogenes* if used in combination with mild heat treatment, while these treatments alone were not affecting the viability of this pathogen. These findings show that essential oil compounds can play an important role in minimally processed foods, and can be used in the concept of Hurdle Technology to reduce the intensity of heat treatment or possibly other individual hurdles.

In **Chapter 3**, the combined antimicrobial action of carvacrol or thymol in combination with HHP was investigated at different temperatures. These treatments showed a synergistic action, with greater antimicrobial effects upon pressurisation at 1^{o}C than at 8°C or 20°C. Furthermore, addition of carvacrol to cells after exposure to sub-lethal HHP treatment showed reductions in viable numbers that were similar to those using simultaneous treatment with carvacrol and HHP. Synergism was also observed between carvacrol and HHP in semi-skimmed milk that was artificially contaminated with *L. monocytogenes*. The study demonstrates the synergistic antimicrobial effect of essential oils in combination with HHP. Together with the results described in Chapter 2, these data indicate the potential of essential oils to be employed in food processing if combined with other techniques.

A major problem that successful application of HHP treatment faces is the variable piezotolerance of bacterial species, strains within a species, and even cells within pure cultures that are not homogenous. In **Chapter 4**, we investigated the characteristics of a *L. monocytogenes* ScottA isolate, named AK01, that was derived from a wild type (wt) culture after a single pressurisation treatment. The survival of exponential and stationary phase cells of AK01 was at least 2 logs higher than that of the wt over a broad range of pressures (150-500 MPa), while both strains showed higher piezotolerance in the stationary than in the exponential

phase of growth. In semi-skimmed milk, exponential phase cells of both strains showed lower reductions upon pressurisation than in buffer, but again, AK01 was more piezotolerant than the wt. The piezotolerance of AK01 was retained for at least 40 generations in rich medium, suggesting a stable phenotype. Interestingly, cells of AK01 lacked flagella and were elongated, and this strain showed slightly lower maximum specific growth rates at 8°C, 22°C and 30°C than the wt. Moreover, the piezotolerant strain AK01 showed increased resistance to heat, acid, and H_2O_2 compared with the wt. The difference in HHP tolerance between the piezotolerant strain and the wt strain could not be attributed to differences in membrane fluidity, since strain AK01 and the wt had identical *in situ* lipid melting curves, as determined by Fourier Transfer Infrared spectroscopy.

In **Chapter 5**, the mechanisms underlying the altered phenotypic characteristics of AK01 are further examined. In this chapter, high piezotolerance, immobility, and reduced virulence were conclusively linked to a single amino acid deletion in the highly conserved glycine-rich region of the regulatory protein CtsR (Class three stress gene repressor). CtsR negatively regulates the expression of Class III heat shock genes (*clpP*, *clpE* and the *clpC* operon). The Clp proteins prevent the accumulation of misfolded proteins that might be toxic to the cell. In the mutant strain AK01, expressing the CtsRΔGly protein, we observed upregulation of *clpP*, which demonstrates the involvement of Class III heat shock genes in increased survival upon HHP treatment and other stresses. Replacement of the wt *ctsR* gene with the *ctsRΔGly* gene in a wt background resulted in a mutant strain that also lost its motile character, and importantly, also showed attenuated virulence. The deletion of three base pairs in the region of *ctsR* that normally encodes four glycines is seemingly not an isolated incident, since we detected the same mutation in the majority of other spontaneous piezotolerant isolates of *L. monocytogenes*. This suggests a functional role for this mutation in *L. monocytogenes* to survive unforeseen events that do not allow for adaptation of normal cells, but require genetic diversity.

In conclusion, this work demonstrated some of the potentials that novel techniques, like the use of essential oils and HHP, can offer alone or combined with other novel or traditional techniques, like heat treatment. We furthermore investigated risks that could emerge from the use of those novel techniques. The occurrence of the piezotolerant strain AK01 of *L. monocytogenes* demonstrated the existence of bacterial heterogeneity within a supposedly pure wt population with regard to HHP resistance. The variance of piezotolerance within a microbial population, but also between different strains, is an important parameter that determines successful application of HHP treatment. This will be an important aspect for risk assessment of HHP treated foods that are, or will become, available on the market. Tailored HHP treatments, possibly in combination with additional mild preservatives, will be required for different products, and increased knowledge about the effects of these treatments will enable the food industry to produce safe foods at minimal costs.

SAMENVATTING

Recentelijk zijn diverse milde niet-thermische conserveringstechnieken geïntroduceerd in de voedselverwerking, en andere worden momenteel onderzocht op hun mogelijke toepassing in verwerkingsprocessen van levenmiddelen. Twee van deze nieuwe conserveringstechnieken zijn de toevoeging van etherische plantenextracten (*essential oils*) en de toepassing van Hoge Hydrostatische Druk (*HHP)*. Het is gebleken dat door gebruik van deze conserveringsmethoden, afzonderlijk of in combinatie, een efficiënte reductie van micro-organismen kan worden bewerkstelligd, terwijl de nutritionele en sensorische kwaliteit van het product behouden blijft. Een belangrijke doelstelling van deze dissertatie was het beoordelen van de anti-microbiële werking van deze gecombineerde technieken op hun effectiviteit tegen de pathogene bacterie *Listeria monocytogenes*, welke de levensbedreigende ziekte listeriose kan veroorzaken. Deze bacterie kan worden aangetroffen in levensmiddelen die niet afdoende zijn behandeld of geconserveerd dan wel zijn nabesmet. Daarnaast is het mechanisme bestudeerd dat ten grondslag ligt aan verhoogde HHP tolerantie van een *L. monocytogenes* isolaat, om meer inzicht te krijgen in de primaire cellulaire respons van dit organisme welke een rol speelt bij resistentie tegen HHP behandeling.

In de introductie **(Hoofdstuk 1)** wordt kort ingegaan op de voedselpathogeen *L. monocytogenes*. Vervolgens komen nieuwe milde en niet-thermische conserveringstechnieken aan de orde, met nadruk op het gebruik van etherische plantenextracten en hoge hydrostatische druk. De algemene eigenschappen en werkingsmechanismen van deze technieken worden beschreven. Verder wordt ingegaan op de respons van bacteriën op deze behandelingen en hun overlevingsstrategieën Ten slotte wordt het gecombineerde gebruik van deze technieken besproken, en wordt een overzicht van de inhoud van dit proefschrift gegeven.

In **Hoofdstuk 2** wordt aangetoond dat afdoding van *L. monocytogenes* kan worden bewerkstelligd door gebruikmaking van relatief lage concentraties van vluchtige componenten geïsoleerd uit kruiden, namelijk S-carvon, cinnamaldehyde, thymol en decanal, in combinatie met een milde hittebehandeling. Afzonderlijke toepassing van deze verbindingen of milde hitte behandeling had echter geen antimicrobieel effect. De resultaten geven aan dat etherische oliën een belangrijke rol kunnen spelen binnen het concept van de Hordentechnologie. Een reductie van de intensiteit van bijvoorbeeld thermische behandelingen of andere conserveringsmethoden kan op een dergelijke wijze worden gerealiseerd voor minimaal behandelde levensmiddelen.

Het **Hoofdstuk 3** beschrijft het onderzoek naar de gecombineerde anti-microbiële activiteit van carvacrol en thymol in combinatie met HHP bij verschillende temperaturen. Deze behandelingen laten een synergistisch effect zien dat groter is bij 1°C dan bij 8 of 20°C. Bovendien bleek dat additie van carvacrol na een sub-letale HHP behandeling een vergelijkbare afdoding van *Listeria monocytogenes* tot gevolg had als een gelijktijdige behandeling met carvacrol onder HHP. Een synergistisch effect van carvacrol en HHP werd niet alleen waargenomen in een buffer, maar eveneens in halfvolle melk die kunstmatig gecontamineerd was met *L. monocytogenes*. Deze studie toont het synergistische effect van carvacrol en HHP aan. Evenals in Hoofdstuk 2 wordt hier de potentie van het gebruik van etherische oliën in combinatie met andere conserveringsmethoden aangetoond.

Een serieus probleem waar succesvolle toepassing van HHP mee te kampen heeft is de variatie in druk-tolerantie tussen micro-organismen. In **Hoofdstuk 4** worden de eigenschappen onderzocht van een *L. monocytogenes* isolaat (stam AK01) met hogere HHP tolerantie dan het wildtype. Stam AK01 werd geïsoleerd uit een wildtype cultuur na een enkelvoudige hoge druk behandeling. Cultures van stam AK01 in zowel het exponentiële als het stationaire groeistadium vertoonden overleving die ten minste twee log-eenheden hoger was dan die van het wildtype na blootstelling aan drukintensiteiten tussen 150 en 500 MPa. Zowel het wildtype als stam AK01 hadden een hogere druk-tolerantie in de stationaire groeifase dan in de exponentiële groeifase. Beide typen cellen vertoonden een verminderde reductie als gevolg van HHP behandeling na groei in halfvolle melk vergeleken met buffer, maar ook hier vertoonde AK01 een hogere druk-tolerantie dan het wild type. De druk-tolerantie van AK01 bleef behouden gedurende ten minste 70 generaties in een rijk medium, wat een stabiel fenotype suggereert. Opmerkelijk is dat de AK01 cellen geen flagellen hadden, langer dan normaal waren, en een licht gereduceerde maximale specifieke groeisnelheid hadden bij 8, 22 en 30°C vergeleken met het wild type. Bovendien vertoonde de druk-tolerante stam AK01 een hogere weerstand tegen hitte, zure condities en waterstofperoxide dan het wildtype. Het verschil in druk-tolerantie tussen AK01 en het wild type kon niet worden toegeschreven aan verschillen in membraanfluïditeit; beide stammen hadden een identieke *in situ* smeltcurve van hun lipiden, gemeten met FTIR.

In **Hoofdstuk 5** worden de mechanismen die ten grondslag liggen aan de gewijzigde fenotypische karakteristieken van AK01 verder onderzocht. Er wordt aangetoond dat hoge druk-tolerantie, immobiliteit en verlaagde virulentie van stam AK01 wordt veroorzaakt door deletie van één aminozuur (glycine) in het sterk geconserveerde glycine-rijke domein van het regulatoire eiwit CtsR (*Class three stress gene Repressor*). CtsR remt de expressie van de klasse III genen die worden geïnduceerd door een thermische schok (*clpP*, *clpE* en het *clpC* operon). De Clp eiwitten gaan de accumulatie van (gedeeltelijk) gedenatureerde eiwitten tegen, welke mogelijk toxisch zijn voor de cel. Bij de expressie van het gemuteerde CtsR in AK01 (dat een glycine mist) werd verhoogde expressie van het gen *clpP* waargenomen. Dit toonde aan dat de klasse III thermische schok genen betrokken zijn bij de verhoogde weerstand tegen HPP en andere stresscondities. Uitwisseling van het wildtype *ctsR* gen in een wildtype stam met het gemuteerde *ctsR* gen resulteerde in een mutant welke eveneens verminderde mobiliteit en virulentie vertoonde. De deletie van 3 baseparen in het domein van CtsR dat normaliter voor vier glycines codeert lijkt geen incident te zijn, aangezien we dezelfde mutatie konden aantonen in de meerderheid van spontane hoge druk-tolerante isolaten van *Listeria monocytogenes*. Deze bevindingen suggereren dat de mutatie in het CtsR eiwit van *Listeria monocytogenes* een functionele rol vervult in een overlevingsstrategie waarbij adaptatie niet mogelijke is, maar genetische variëteit een uitkomst biedt.

Samenvattend heeft deze dissertatie aangetoond dat nieuwe niet-thermische conserveringsmiddelen, zoals het gebruik van etherische oliën en hoge druk, effectief kunnen worden gebruikt om bacteriën af te doden, al dan niet in combinatie met nieuwe of traditionele methoden zoals hittebehandeling. Verder zijn een aantal risico's onderzocht die kunnen voortvloeien uit het gebruik van dit soort nieuwe technieken. De isolatie van een *Listeria monocytogenes* stam met verhoogde hoge druk tolerantie toonde aan dat er binnen een wildtype culture heterogeniteit bestaat aangaande hoge druk resistentie. De variatie in druktolerantie binnen een microbiële populatie, maar ook tussen verschillende micro-organismen, is een belangrijke parameter welke mede bepalend is voor de succesvolle

toepassing van hoge druk. Dit is een belangrijk aandachtspunt in risico evaluatie van hoge druk behandelde levensmiddelen die op de markt worden gebracht. HHP behandelingen, mogelijk in combinatie met aanvullende milde conserveringstechnieken, dienen te zijn toegespitst op specifieke producten. Uiteindelijk zal kennis omtrent de effecten van deze behandelingen op bacteriën de voedselindustrie beter in staat stellen veilige levensmiddelen te produceren tegen zo laag mogelijke kosten.

ΠΕΡΙΛΗΨΗ

Μία ποικιλία από μη θερμικές ήπιες τεχνικές συντήρησης έχει εισαχθεί τελευταία στην παραγωγή τροφίμων, ή είναι υπό έρευνα για πιθανή χρήση τους στην παρασκευή τροφίμων. Δύο από αυτές τις νέες τεχνικές συντήρησης είναι η χρήση, Υψηλής Υδροστατικής Πίεσης (Υ.Υ.Π.), και συστατικών από φυτικά αιθέρια έλαια. Εφαρμογή αυτών των μεθόδων συντήρησης, μεμονωμένα ή σε συνδυασμό, μπορεί να μειώσει τούς αριθμούς των μικροοργανισμών που συναντώνται στα τρόφιμα, και επιπλέον μπορεί να διατηρήσει τη διατροφική αξία και τα οργανοληπτικά χαρακτηριστικά του προϊόντος. Οι στόχοι αυτής της διδακτορικής διατριβής ήταν να εκτιμήσει τις αντιμικροβιακές ιδιότητες αυτών τών συνδυασμών τεχνικών επεξεργασίας εναντίον του αναπτυσσόμενου στα τρόφιμα βακτηρίου *Listeria monocytogenes,* που μπορεί να προκαλέσει σοβαρότατη ασθένεια με υψηλή θνησιμότητα. Επιπλέον, στην προσπάθειά μας γιά εξιχνίαση των κυτταρικών δομών τής *L. monocytogenes* που πρωταρχικά επηρρεάζονται από την Υ.Υ.Π., διερευνήσαμε τον μηχανισμό που είναι υπεύθυνος γιά την αυξημένη πιεζοαντοχή ενός στελέχους του οργανισμού αυτού.

Στο **κεφάλαιο 1** δίνεται μία σύντομη εισαγωγή γύρω από την *L. monocytogenes*. Επιπλέον γίνεται αναφορά σε νέες ήπιες μεθόδους επεξεργασίας τροφίμων με ιδιαίτερη έμφαση στη χρήση, συστατικών από φυτικά αιθέρια έλαια, και της Υψηλής Υδροστατικής Πίεσης (Υ.Υ.Π.). Επίσης περιγράφονται, τα γενικά χαρακτηριστικά και ο τρόπος δράσης των παραπάνω μεθόδων επεξεργασίας, αλλά και μηχανισμοί αντίστασης που χρησιμοποιούν τα βακτήρια για να ανταπεξέλθουν της πιθανά θανατηφόρου δράσης τους. Το κεφάλαιο ολοκληρώνεται με την παρουσίαση της συνδυασμένης χρήσης των παραπάνω μεθόδων, και με μία γενική περιγραφή της δομής αυτής της διδακτορικής διατριβής.

Το **κεφάλαιο 2** περιγράφεται η ικανότητα διαφόρων συστατικών από φυτικά αιθέρια έλαια, όπως η S-καρβόνη, η κινναμαλδεΰδη, η θυμόλη, και η δεκανάλη, να μειώνουν τους αριθμούς της *L. monocytogenes* εάν χρησιμοποιηθούν σε συνδυασμό με ήπια θερμική επεξεργασία, ενώ οι ποσότητες που χρησιμοποιήθηκαν δεν ήταν από μόνες τους δυνατές να επηρρεάσουν την επιβίωση του παθογόνου αυτού μικροοργανισμού. Αυτά τα στοιχεία δείχνουν ότι τα συστατικά των αιθερίων ελαίων μπορούν να διαδραματίσουν έναν σημαντικό ρόλο στην παρασκεύη των ήπια επεξεργασμένων τροφίμων (minimally processed foods), και μπορούν να χρησιμοποιηθούν στην Τεχνολογία Εμποδίων (Hurdle Technology) για να μειώσουν την ένταση της θερμικής επεξεργασίας ή πιθανώς άλλων μεμονωμένων επεξεργασιών.

Στο **κεφάλαιο 3** διερευνάται η συνδυασμένη αντιμικροβιακή δράση της καρβακρόλης και της θυμόλης με την Υ.Υ.Π. σε διάφορες θερμοκρασίες. Αυτές οι επεξεργασίες έδειξαν συνεργιστική δράση, με μέγιστη αντιμικροβιακή ικανότητα στους 1^{o}C παρά στους 8°C ή 20°C.

Επιπλέον, η προσθήκη καρβακρόλης στα κύτταρα μετά από την επίδραση υποθανάσιμων (sub-lethal) επεξεργασιών Υ.Υ.Π. παρουσίασε παρόμοια μείωση στους βιώσιμους αριθμούς με αυτήν που επιτεύχθηκε με την σύγχρονη εφαρμογή καρβακρόλης και Υ.Υ.Π.. Συνέργεια παρατηρήθηκε επίσης μεταξύ καρβακρόλης και Υ.Υ.Π. σε ημιαποβουτυρωμένο γάλα που προηγουμένως είχε εμβολιαστεί με *L. monocytogenes*. Η μελέτη παρουσιάζει την συνεργιστική αντιμικροβιακή δράση των αιθερίων ελαίων σε συνδυασμό με την Υ.Υ.Π.. Τα παραπάνω αποτελέσματα σε συνδυασμό με αυτά του κεφαλαίου 2 αναδεικνύουν την δυνατότητα χρήσης των αιθερίων ελαίων στην επεξεργασία τροφίμων σε συνδυασμό με άλλες τεχνικές.

Ένα σημαντικό πρόβλημα που η εμποδίζει την επιτυχή εφαρμογή επεξεργασίας με Υ.Υ.Π. είναι η αυξημένη ποικιλότητα που παρουσιάζει η πιεζοαντοχή των βακτηριακών

ειδών, των στελεχών ενός είδους, ακόμα και των κυττάρων μιας θεωρητικά καθαρής καλλιέργειας που όμως πρακτικά δεν είναι ομοιογενής. Στο **κεφάλαιο 4**, μελετούνται τα χαρακτηριστικά ενός στελέχους *L. monocytogenes* ScottA που ονομάσαμε AK01, και απομονώθηκε από καλλιέργεια του άγριου στελέχους μετά από μία μεμονομένη επεξεργαασία Υ.Υ.Π.. Η επιβίωση κυττάρων του AK01 από την λογαριθμική φάση και την φάση στασιμότητας ήταν τουλάχιστον 2 λογαριθμικούς κύκλους υψηλότερη από αυτήν του αγρίου στελέχους, σε μια σειρά από πιέσεις (150-500 MPa), καθώς τα δύο στελέχη (ScottA και AK01) παρουσίασαν μεγαλύτερη πιεζοαντοχή κατά την φάση στασιμότητας παρά κατά την λογαριθμική φάση ανάπτυξης. Στο ημιαποβουτηρωμένο γάλα, τα κύτταρα και των δύο στελεχών από την λογαριθμική φάση ανάπτυξης, παρουσίασαν χαμηλότερες μειώσεις των αριθμών τους σε σύγκριση αυτές που επιτεύχθηκαν σε εξισοροπιστικό διάλυμα (buffer), αλλά και πάλι το AK01 ήταν πιο πιεζοάντοχο από το άγριο στέλεχος. Η πιεζοαντοχή του AK01 διατηρήθηκε για τουλάχιστον 40 γενεές σε πλούσιο BHI παρουσιάζοντας έναν σταθερό φαινότυπο. Τα κύτταρα του AK01 ήταν επιμηκησμένα, χωρίς φλαγέλες, και το στέλεχος αυτό είχε αυξημένο ρυθμό ανάπτυξης σε σχέση με το άγριο στους 8°C, 22°C και 30°C. Επιπλέον το πιεζοάντοχο στέλεχος AK01, συγκρινόμενο με το άγριο στέλεχος, παρουσίασε αυξημένη αντοχή στην θέρμανση, στο οξύ, και στο H_2O_2. Η διαφορά στην πιεζοαντοχή ανάμεσα στα δύο στελέχη δεν οφειλόταν σε διαφορές στην ρευστότητα των μεμβρανών τους, καθώς και τα δύο στελέχη είχαν πανομοιότυπες *in situ* καμπύλες τήξης λιπιδίων, όπως αυτές προσδιορίστηκαν με Φασματοσκοπία Μετάθεσης Υπεριώδους κατά Fourier (Fourier Transfer Infrared spectroscopy).

Στο **κεφάλαιο 5**, μελετούνται οι μηχανισμοί που είναι υπεύθυνοι για τα αλλότρια φαινοτυπικά χαρακτηριστικά του AK01. Στο κεφάλαιο αυτό αποδεικνύεται ότι, η αυξημένη πιεζοαντοχή, η ακινησία, και η μειωμένη μολυσματικότητα του AK01 οφείλονται στην απώλεια ενός αμινοξέως από την εξαιρετικά συντηρημένη, και πλούσια σε γλυκίνη, περιοχή της ρυθμιστικής πρωτεΐνης CtsR (Class three stress gene repressor ή Αναστολέας των Γονιδίων της Τρίτης Κλάσης). Η CtsR ρυθμίζει αρνητικά την έκφραση των γονιδίων θερμικού σοκ της Κλάσης III (Class III heat shock genes) που αποτελείται από τα *clpP, clpE* και το *clpC* όπερον. Οι Clp πρωτεΐνες αποτρέπουν την συσσώρευση μετουσιωμένων πρωτεινών που μπορεί να είναι τοξικές για το κύτταρο. Στο μεταλλαγμένο στέλεχος AK01, που εκφράζει την CtsRΔGly πρωτεϊνη, παρατηρήσαμε θετική ρύθμιση (upregulation) του *clpP*, αναδεικνύοντας την εμπλοκή των γονιδίων θερμικού σοκ της Κλάσης III στην αυξημένη επιβίωση σε Υ.Υ.Π. και σε άλλους αντιμικροβιακούς παράγοντες. Αντικατάσταση του *ctsR* γονιδίου του άγριου στέλεχους με το *ctsRΔGly* γονίδιο του AK01, στο γονιδίωμα του αγρίου σελέχους, είχε σαν αποτέλεσμα το άγριο στέλεχος να χάσει την κινητικότητα του, και να παρουσιάσει μειωμένη μολυσματικότητα. Η απώλεια τριών ζευγών βάσεων νουκλεοτιδίων στην περιοχή του *ctsR* που κανονικά κωδικοποιεί για τέσσερεις γλυκίνες προφανώς δεν είναι ένα απομονωμένο περιστατικό, καθώς εντοπίσαμε την ίδια μετάλλαξη στην πλειονότητα των πιεζοάντοχων στελεχών *L. monocytogenes* που απομονώσαμε. Μπορούμε να συμπεράνουμε ότι αυτή η μετάλλαξη παίζει έναν σημαντικό ρόλο στην δυνατότητα της *L. monocytogenes* να επιβιώσει σε πιθανές αντίξοες συνθήκες που κανονικά δεν επιτρέπουν την επιβίωση της, και αυτό καθίσταται δυνατό μέσω μιας γενετικής ποικιλότητας.

Εν κατακλείδει, αυή η έρευνα παρουσίασε μερικές απο τις δυνατότητες που προσφέρουν νέες τεχνικές, όπως η χρήση αιθερίων ελαίων και Υ.Υ.Π., μεμονωμένα ή σε συνδυασμό με άλλες νέες η παραδοσιακές τεχνικές, όπως η θερμική επεξεργασία. Επιπλέον μελετήσαμε κινδύνους που θα μπορούσαν να προκύψουν από την χρήση των νέων αυτών τεχνικών. Η παρουσία του πιεζοάντοχου στελέχους AK01 του μικροοργανισμού *L.*

monocytogenes ανέδειξε την ύπαρξη ετερογένειας εντός ενος θεωρητικά καθαρού βακτηριακού πληθισμού όσον αφορά την πιεζοαντοχή τους. Η ποικιλότητα της πιεζοαντοχής εντός ενος μικροβιακού πληθισμού, αλλά και μεταξύ διαφορετικών στελεχών, είναι μία σημαντική παράμετρος για την επιτυχή εφαρμογή της επεξεργασίας με Υ.Υ.Π.. Αυτό είναι ένα σημαντικό ζήτημα που αφορά την εκτίμηση των κινδύνων στην παραγωγή των επεξεργασμένων με Υ.Υ.Π. τροφίμων που θα προσφερθούν προς κατανάλωση. Διάφορες επεξεργασίες με Υ.Υ.Π., πιθανώς σε συνδυασμό με ήπια συντηρητικά, απαιτούνται για διάφορα προϊόντα, όπως και γνώση σχετικά με την επίδραση των μεθόδων αυτών που τελικά θα βοηθήσει την βοιμηχανία να παράγει ασφαλή προϊόντα και να μειώσει το κόστος.

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CURRICULUM VITAE

Kimon Andreas Karatzas was born on the 22nd of November 1971 in Athens. He completed the basic education at the 2nd Public Lyceum of Charilaou (Thessaloniki) in 1989. In June 1990 he succeeded in countrywide Panhellenic contest and attended courses of the 1st year in the Department of Livestock Production, T.E.I. (Technological Educational Institute of Thessaloniki). Next year (June 1991) succeeded in Panhellenic contest and started attending courses at the Department of Agriculture, School of Geotechnical Sciences in Aristotlian University of Thessaloniki. In November 1996 he was awarded the degree of the Department of Agriculture, School of Geotechnical Sciences with spesialisation in Agricultural and Food Industries. During his thesis he studied the production of aromatic compounds by several species of Lactic acid bacteria under the supervision of prof. Dr. N. Tzanetakis. In March 1997 and in countrywide competition held by the State Scholarships Foundation of Greece he succeeded in taking the first place, and awarded the scholarship for post-graduate studies in Food Microbiology. Between October 1997 and December 2001 he completed his PhD thesis under the daily supervision of Dr. L.G.M. Gorris, Dr. E.P.W Kets the first two years and of Dr. M.H.J. Bennik the last two years, while the project was overall supervised by prof. Dr. ir. F.M. Rombouts. The first two years of his thesis were funded by the State Scholarships Foundation of Greece, and subsequently the project was adopted by WCFS (Wageningen Centre for Food Sciences) with project-leader Dr. T. Abee, for the last two years.

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Cover photograph: A cell of wild type *Listeria monocytogenes* Scott A. Dr. Jan Dijksterhuis, Felix Thiel, and Andrian van Aelst

Cover design: Kimon Andreas G. Karatzas and Denise Cysneiros