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Molecular mechanisms of the high pressure inactivation of beer spoiling *Lactobacillus plantarum*

Helge Martin Ulmer

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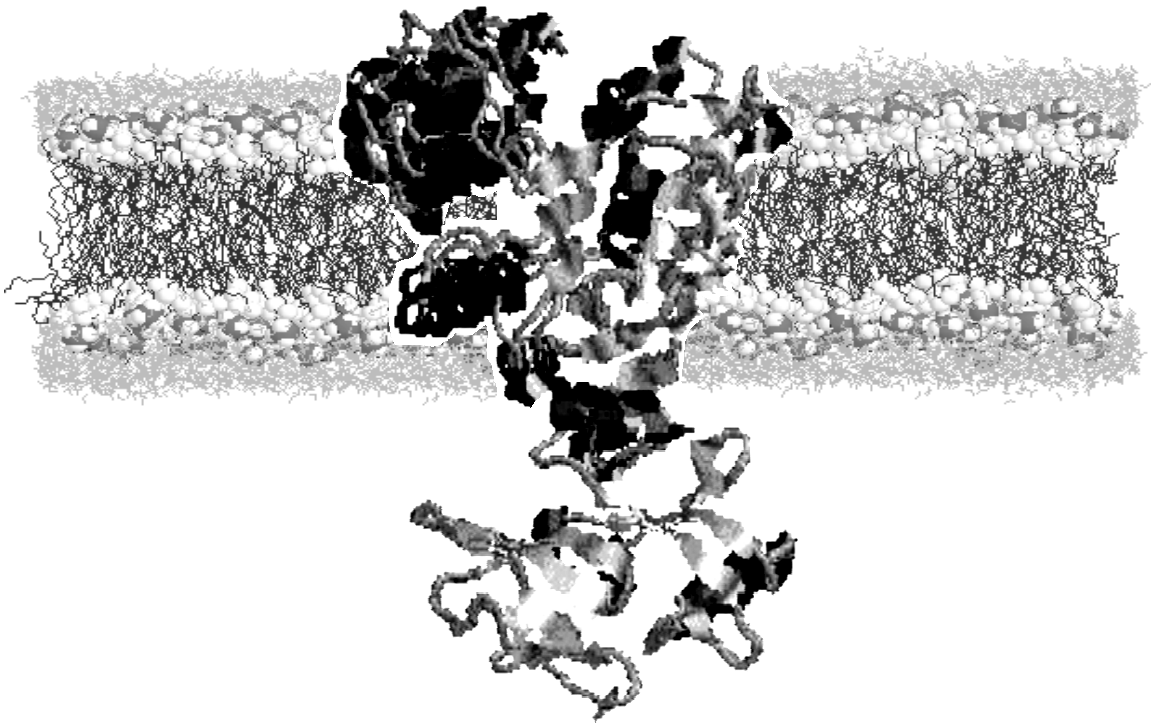

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Zusammenfassung

Molecular mechanisms of the high pressure inactivation of beer spoiling *Lactobacillus plantarum*

Hohe hydrostatische Drücke eignen sich für die Pasteurisation von Lebensmitteln. Enzyme und Proteine werden durch Hochdruck inaktiviert oder denaturiert. Dadurch wird die Grundlage der Mikroorganismen für Stoffwechsel zerstört. Zusätzlich werden durch Hochdruck die cytoplasmatischen Membranen der Mikroorganismen beeinflusst. Diese bilden die Barriere zwischen Innen- und Außenraum der Zellen. Durch Beeinflussung dieser Grenzschicht werden Mikroorganismen empfindlich in ihrem Stoffaustausch mit der Umgebung gestört und gegebenenfalls abgetötet. Das Zusammenwirken dieser letalen Einflüsse von Hochdruck auf Lebensmittelverderber blieb jedoch ungeklärt.

In dieser Arbeit wurde der Einfluss von Hochdruck auf lebende Zellen des Bierschädlings *Lactobacillus plantarum* TMW 1.460 identifiziert und anhand der gewonnenen Erkenntnisse, der Wirkort und –mechanismus von Hochdruckbehandlungen festgestellt. Darauf aufbauend gelang es die Effektivität der Hochdruckbehandlung in Bier unter Berücksichtigung des wachstumshemmenden Hürdenkonzeptes zu optimieren. Die in Bier vorkommenden, wachstumsbestimmenden Faktoren sind: das geringe Angebot an Wuchs- und Nährstoffen, der Alkohol- und Hopfensäuregehalt, der niedrige pH-Wert und das mit Kohlendioxid angereicherte anaerobe Milieu.

Lactobacillus plantarum TMW 1.460, aus verdorbenem Bier isoliert, hat die Fähigkeit, unter diesen Bedingungen zu wachsen. Hierzu besitzt er einen Resistenzmechanismus, der ihn gegen den Gehalt an iso-α-Säuren aus Hopfen unempfindlich macht. Diese Resistenz basiert vor allem auf dem membranintegrierten Enzym HorA, das mittels Konformationsänderungen und unter ATP-Verbrauch die Hopfensäuren aus der Zellmembran transportiert. HorA ist ein multi-drug-resistance Enzym, das den Transport verschiedener Stoffgruppen katalysiert. Dies ermöglichte die Entwicklung eines Färbeverfahrens, mit dessen Hilfe die Enzymaktivität des Hopfentransporters nachgewiesen werden konnte.

Untersuchungen der Zellen nach unterschiedlichen Druckhaltezeiten zeigten, dass zuerst die Lebensfähigkeit der Zellen und dann die Funktionsvoraussetzungen für das Transportenzym, nämlich Membranintegrität und Stoffwechselaktivität, durch Druck zerstört werden. Der Aktivitätsverlust des Transporters wurde jedoch vor dem Absterben der Zellen festgestellt. So konnte erstmals eine Reaktion der Zellen auf Hochdruck vor der eigentlichen Zellabtötung gemessen werden.

In weiteren Versuchsreihen wurden der Alkoholgehalt und der Hopfensäuregehalt während der Hochdruckbehandlung auf die Absterbekinetik der Zellen untersucht. Der für Bier relevante Alkoholgehalt führte zur Erhöhung der Absterbegeschwindigkeit der Zellen. Hopfen zeigte keine Verstärkung des Hochdruckeinflusses. Anschließende Lagerung in gehopftem Modellbier bestätigte die Beobachtung, dass Hochdruck spezifisch die Hopfenresistenz zerstört. Druckbehandelte Zellen wurden während der Lagerung durch die Hopfensäuren innerhalb von 24 Stunden abgetötet. Ein zusätzlicher Einfluss von gelöstem Kohlendioxid auf die Abtötungseffektivität von Hochdruck konnte in den für Bier wichtigen Konzentrationen nicht gefunden werden. Das Vorhandensein von Stickstoff und Sauerstoff in hohen Konzentrationen wirkte sich ebenfalls nicht unterstützend auf die Hochdruckpasteurisation aus. *L. plantarum* wurde erst durch Einsatz von flüssigem Kohlendioxid bei Drücken von 12 MPa abgetötet. Dabei zeigte sich, dass flüssiges Kohlendioxid als Extraktionsmittel wirkt und Phospholipide aus der Cytoplasmamembran der Zellen löst. Diese Extraktion von Membranbestandteilen führt zur Abtötung der Zellen.

Um den Mechanismus des Hochdrucks auf die Hopfenresistenzinaktivierung aufzuklären, wurde das System Membranenzym HorA Membran genauer untersucht. Mit Hilfe von FT-IR- und *in-situ* Fluoreszenzmessungen wurde der Phasenübergang der cytoplasmatischen Membran von *L. plantarum* in Abhängigkeit von Druck und Temperatur beobachtet. Durch Veränderung der Membranzusammensetzung konnte eine Temperaturverschiebung des Phasenüberganges von flüssig-kristallin zu gelartig herbeigeführt werden. Eine Verschiebung zu niedrigeren Temperaturen bewirkte eine Zunahme der Drucksensibilität des Enzyms HorA. Diese Zunahme korrelierte auch mit dem Absterben der Zellen. Damit war der

Hochdruckeinfluss auf die Inaktivierung des Enzyms HorA anhand des Verhaltens der Cytoplasmamembranen von *L. plantarum* im Druck-Temperatur-Diagramm nachzuvollziehen.

Die entwickelten Untersuchungsmethoden zu Membran- und Stoffwechseleigenschaften wurden zur Charakterisierung der Sterilisationsmethode „pulsed-electric-fields“ herangezogen. Zum Unterschied der Hochdruckbehandlung treten bei „pulsed-electric-fields“ in linearer Abhängigkeit der Feldstärke und des Gesamtenergieeintrags die Schäden an Stoffwechselmechanismen und Membranintegrität vor der Zelltötung ein. Damit konnte der Zusammenhang zwischen Wirkungsmechanismus auf Membranen und Abtötungseffekt der Zellen aufgeklärt werden.

Durch Berücksichtigung der Ergebnisse aus den dargestellten Hochdruckversuchen konnte das zur Sterilisation von Bier benötigte Druckniveau von 600 MPa auf 200 MPa reduziert werden. Die Phasenübergänge der Membran und die damit verbundenen irreversiblen Membranenzyminaktivierungen sind somit ein Schlüsselmechanismus zur Zellabtötung.

Introduction

Physical food sterilisation by non-thermal treatments gains interest in the food industry in areas where traditional physical and chemical processes (e.g. heating or addition of preservatives) imply a number of disadvantages. These are mainly changes of nutritional and sensory characteristics combined with long process times and unknown chemical reactions. Alternatively, ultra sonic, pulsed electric fields, and high pressure processes can widely avoid these embarrassing effects without waiving product shelf-life (1). In this context high pressure is a promising perspective, because this treatment affects food spoilers at low temperatures and thus saves sensorial and nutritional quality determining ingredients (2).

High pressure generation. High pressure is generated according to the physical principle: **P**ressure is equivalent to **F**orce per **A**rea. If a pressure generator is coupled to a force transmission, possessing two working areas of different sizes, particular high pressure levels can be achieved according to the ratio of these sizes. For example: pressure of 10 MPa generated from a hydraulic pump and pressing on an area of 100 cm^2 is converted by force transmission in a pressure of 1000 MPa if the connected area is 10 cm^2. The generateable pressure level is limited by material constants.

High pressure principles. Two physical principles govern pressure application. (i) The isostatic principle provides instantaneous and uniform pressure in the treatment chamber. Thus food materials can be treated independent of shape, size, and structure (Figure 1). Treatment of liquids and foods with high contents of water needs small energy compared to heating because of low compressibility but high specific heat capacity of water. Moreover, liquid foods can be used as pressure transmitting media themselves, which renders packing and unpacking for the high pressure process unnecessary.

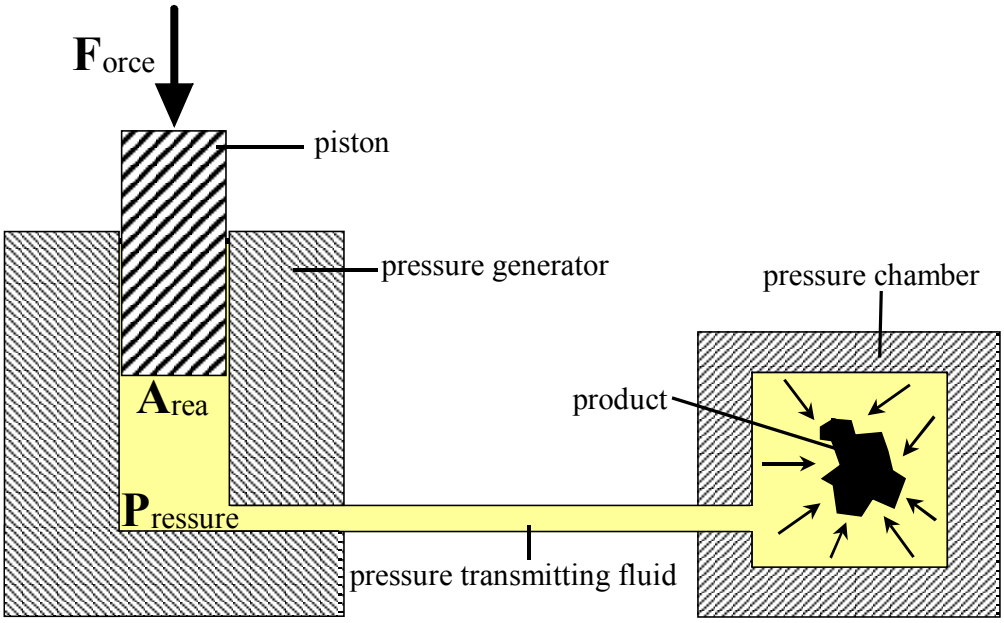

Figure 1 Schematic picture of high pressure generation and product treatment.

(ii) The microscopic ordering principle implies rearrangement of molecules and atoms during pressure application. At constant temperature the degree of order on molecular level is increased (3). Most ordering effects of high pressure are on non-covalent bonds between molecules. Therefore, molecules are sensitive to high pressure application, whose tertiary structure and biological functionality is determined by defined non-covalent interactions. This is the reason for elevated sensitivity against high pressure of enzymes and cytoplasmic membranes. High pressure disturbs the structure of these complex molecules working on their hydrogen, ionic and hydrophobic bonds. The loss of functionality leads to irritations of living cells and finally to cell death, if interferences are strong enough. The exact mechanisms of cell inactivation through high pressure are not yet elucidated.

High pressure and proteins. Reactions of proteins during pressure treatment led to the conclusion that up to 300 MPa molecular cavities are compressed (4, 5). Water molecules

enter the interior of the protein, leading to loss of hydrophobic contacts until a denaturated state is reached (6, 7).

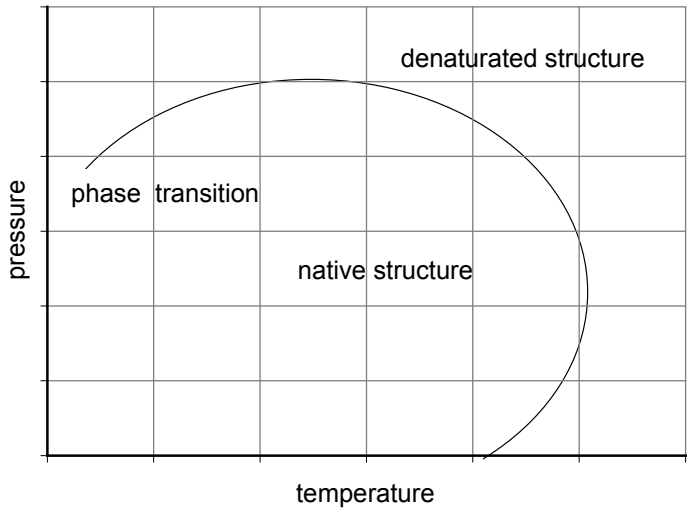

Figure 2 Schematic diagram of pressure and temperature dependent denaturation and inactivation of proteins and microorganisms, respectively (8).

The phase and inactivation diagram of proteins and microorganisms, respectively, shows, that at high temperature, pressure stabilizes against temperature mediated inactivation (Figure 2). At low temperatures, an increment of temperature increases the baro-tolerance against denaturation. Denaturation of enzymes based on irreversible changes in secondary and tertiary protein structure is considered to be responsible for deficits in providing the cell with essential substances. This finally leads to cell inactivation. In contrast to denaturation of enzymes, cell inactivation is found at pressure levels lower than 300 MPa. Consequently, additional effects of high pressure on survival mechanisms of cells are expected.

High pressure and phospholipid bilayers. In this context the cytoplasmic membrane is mentioned as main target for high pressure (9). Pressure up shift induces phase transitions in phospholipid bilayers from the liquid-crystalline to gel phase. This is characterized by an increased rigidity and reduced conformational degrees of freedom for the acyl chains according to the microscopic ordering principle. These phase state transitions occur far below

300 MPa depending on composition of membrane (phospholipid headgroups, acyl length and saturation) and reaction temperature (10). In contrast to proteins, membrane phase transitions are reversible, thus the gel phase is lost after decompression and fluid-crystalline phase is re-established (3, 11). The schematic simulation picture shows the behaviour of a model membrane after phase transition (Figure 3).

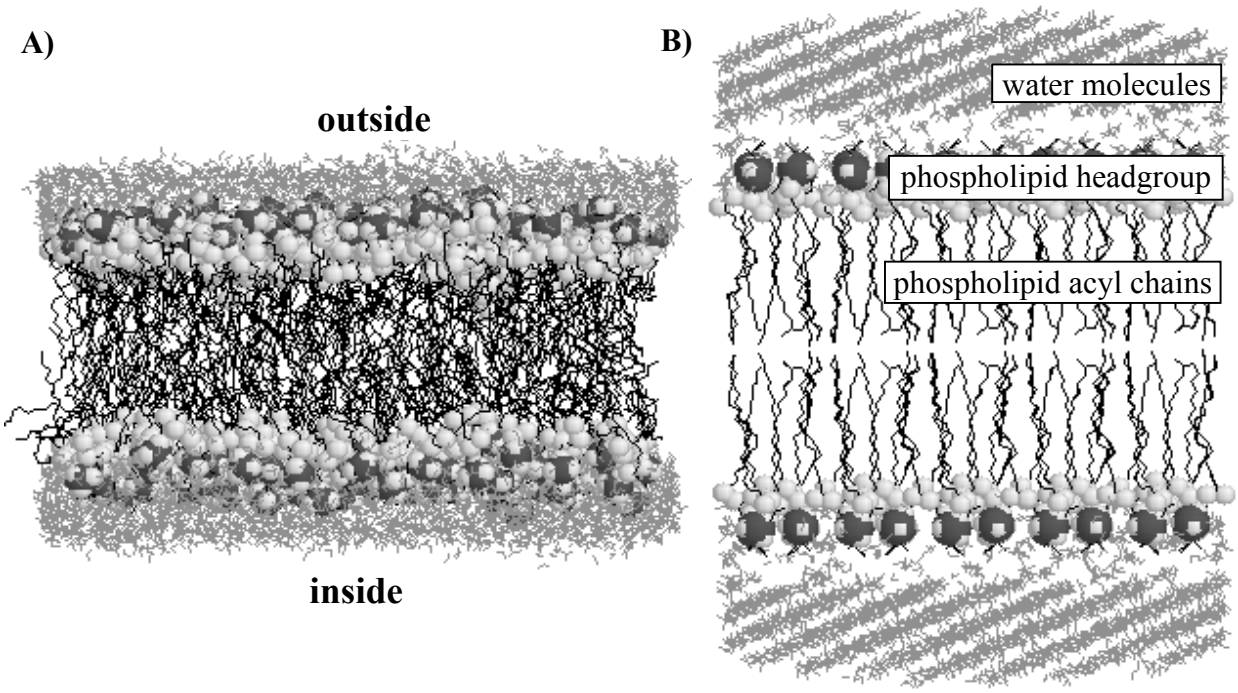

Figure 3. Schematic phospholipid bilayer structure in liquid-crystal phase A) and gel phase B) with water molecules as solvent.

Figure 3.A represents the liquid-crystal phase of a model bilayer. The acyl chains are mainly disordered and the headgroups are strictly together. Water molecules penetrate into the phosphate headgroup area. Figure 3. B shows the gel phase of the bilayer. Acyl chains got stretched according to molecular ordering principle and increase the thickness of the bilayer. Water molecules removed from the phosphate and choline headgroup area, expressing a hydrophobic effect taking place. The reason for this water repelling is not yet understood (12).

High pressure and cytoplasmic membranes. Basically, cytoplasmic membranes of cells are phospholipid bilayers with transmembrane and membrane bound enzymes. The mixture of different phospholipids and the integration of enzymes affect the thermodynamical behaviour of cytoplasmic membranes being pressurized. This complex behaviour of cytoplasmic membranes under pressure is not yet explored. Information of one-component phospholipid membranes is available about thermodynamical changes by inserted proteins. It was shown that the conformation of gramicidin, a low molecular weight peptide antibiotic, is modulated by phase transitions of the lipid matrix. Vice versa, incorporation of the peptide leads to significant changes of the structure and phase behaviour of the lipid bilayer system (13). Phase transition of cytoplasmic membranes therefore seems to be a complex process resulting in a wide coexistence of liquid-crystalline and gel formations under high pressure application. These strong interrelations between cytoplasmic membranes and incorporated or membrane-bound enzymes mainly determine functionality of the cell and its ability of surviving.

High pressure and membrane transporters. The phenomena of affecting activity of membrane transport enzymes by high pressure was reported by the loss of acid resistance of *Escherichia coli.* This suggests the inactivation of transport systems responsible for pH homeostasis (14, 15). In addition to that, high pressure inactivation of the membrane bound F_0F_1ATP-ase in *Lactobacillus plantarum* was reported by Wouters et al. (16). The effect of the membrane and its thermodynamical behaviour during high pressure application on enzyme inactivation and irreversible destruction is not yet explored sufficiently.

Resistance mechanism. General resistance mechanisms against cell affecting compounds are enzymatic inactivation or degradation, changing of the target, prevention of entry by cell surface alterations and, active extrusion of drugs. The latter mechanism will lower the cyto-plasmic drug concentration and increases drug resistance. Active drug extrusion was first recognized in multi-drug-resistance (MDR) tumor cells. Many anticarcinogenic drugs,

including alkaloids, anthracyclines, actinomycin, and cytotoxic compounds (colchicine, rhodamine and ethidium bromide), are extruded from these types of cells and complicate chemotherapy of cancer patients. The functional relationship and overlap in substrate specificity between different MDR transporters of both pro- and eukaryotic origin suggests a general mechanism of drug extrusion. Although MDR substrates can be structurally very different, they share several physical properties as high hydrophobicity, an amphiphilic nature, and a net positive charge. Due to these physical properties the compounds readily intercalate into the phospholipid bilayer. MDR transporters bind substrates to the binding pocket in the plane of the membrane and actively remove hydrophobic drugs from the inner to the outer membrane leaflet from where they diffuse into medium (17). The characteristic function of these compounds is the penetration of cell wall and location in the plane of cyctoplasmic membranes according to hydrophilic and hydrophobic areas. The toxic mechanisms are mainly based on (i) pore formation and (ii) ionophoric effects dissipating transmembrane gradients. (i) Pore formation is observed with Nisin, a polypetide of 34 amino acids, by initially binding at the lipid surface of the membrane and destabilisation of the lipid dynamics (18). The second effect (ii) is well known for several weak acid food preservatives, such as benzoic acid or sorbic acid, and plant materials, as hop acids (19). Due to the pH difference between interior and exterior of cells and the particular pKa value of these substances they are associated at low pH, penetrate cytoplasmic membranes and dissociate to protons and anions inside the cell. This lowers the internal pH. The loss of the pH gradient leads to cell starvation (20).

ATP-dependent resistance of Lactobacilli. Lactobacilli encounter various hydrophobic toxic compounds in their natural habitat, produced by plants or competitors. The first detected prokaryotic ABC (ATP-binding-cassette) transporter being able to transport multiple drugs was the lactococcal LmrA protein described by Bolhuis, *et al.* (17). LmrA possesses one

transmembrane domain, consisting of six hydrophobic transmembrane spanning α-helical segments. The nucleotide binding domain is located at the cytoplasmic side of the membrane and contains the highly conserved ABC signature. Amphiphilic compounds are extruded out of the cell involving a catalytic cycle of structural rearrangements induced by ATP binding and hydrolysis (21). In addition to LmrA from *Lactococcus lactis* a member of the ABC superfamily transporters and homologous to the mammalian MDR transporters, in *Lactobacillus brevis* was discovered by Sami *et al.* (22). This transporter HorA (hop-resistance-enzyme A) shares 53% sequence identity with LmrA of *Lactococcus lactis* and confers resistance of *Lactobacillus brevis* to hop-iso-alpha-acids in beer (23). The hop bitter acids derived from the flowers of *Humulus lupulus* are responsible for the tasty bitterness of beer and confer protection against bacterial spoilage. They pertain to the preserving hurdle system of beer as low pH and nutrition level, content of carbon dioxide and ethanol, and low temperatures during storage and transport. Experiments with trans-iso-humulones and *Lactobacillus brevis* showed, that protons are trapped at one side of the cytoplasmic membrane and exchanged for divalent cations on the other side. This suggests that iso-humulones are carriers of ions dissipating the transmembrane pH gradient resulting in cell starvation for non-resistant cells. Compared with other weak acid preservatives, as benzoic or sorbic acid, hop compounds inhibit bacterial growth three orders of magnitude more efficient. An explanation for this powerful preservation may be a occurring flip-flop-effect in the membrane combined with a re-association and dissociation of the molecules (24). The resistance of lactic acid bacteria against hop compounds is discussed in literature. Hayashi et al. (25) reported, that hop inducible transport enzyme HitA may play a role in making the bacteria resistant to iso-alpha-acids. The hop mediated lack of divalent cations is reduced by transport of ions into cytoplasm. This may reduce hop mediated stress. Sakamoto *et al.* (23) described the ability of the HorA enzyme to transport the iso-alpha-acids out of the membrane. This ability was proven by competitive transport functionality tests of the

fluorescence dye ethidium bromide, Hoechst 33342 and hop acids emphasising the character of HorA as a multi drug transporter (23). The possibility to measure the acivity of membrane enzymes with fluorescent dyes and consequently characterizing membrane enzyme's behaviour under high pressure opened a new way to the exploration of pascalisation effects on living systems.

Current limitations in high pressure research. Most experiments of microorganism inactivation with high pressure dealt with the *ex-situ* observation of loss of viability. The impact of high pressure on the particular cell components and the importance of these damages for cell surviving has hardly been examined. Sigmoid inactivation curves led to the conclusion, that a reversible metastable state is reached, before irreversible cell inactivation takes place. Therefore, a two step reaction passing through an intermediate metastable state for high pressure inactivation kinetics of microorganisms was postulated (26). This metastable state could be defined as sublethal injury of cells, where cell damages occur, which do not lead to immediate cell death. In optimal circumstances the cell repair mechanisms may overcome this condition and make injuries reversible. Cells are irreversibly inactivated if injury is severe enough and circumstances are adverse. The sublethal injury has however not yet been tracked to the molecular level.

Aim of this study. It was aim of this study to measure high pressure mediated reversible and irreversible damages and assort sublethal injury to cellular components or pathways. Results concerning sublethal injuries should open new possibilities to interpret high pressure mediated cell death. For this reason an adequate system consisting of cells and a habitat with different possibilities to measure and to distinguish sublethal injuries had to be found. Based on the hurdle preservation system of beer diverse stresses on beer spoiling bacteria are executed. Measurements of high pressure mediated cell death, sublethal or lethal injuries, and the capability of injured cells to recuperate under stresses present in beer should provide

information on high pressure cell damage. Therefore, in this study a model beer system was considered as a suitable system of investigating such effects because stressing parameters, as pH, ethanol, hop compounds, etc. are adjustable. The non-pathogenic and beer spoiling bacterium *Lactobacillus plantarum* TMW 1.460 was used as suitable model organism which possesses a membrane coupled transport system mediating resistance against membrane soluble and cell toxic compounds.

Approach used in this work to investigate high pressure mediated cell injury. The first experiments of this thesis proving the applicability of high pressure at moderate levels as preservating method on beer led to the conclusion, that *Lactobacillus plantarum* loses HorA activity before cell viability. The destruction of metabolic activity and membrane integrity took place after cell viability loss and were consequently not responsible for HorA inactivation (Chapter I). Due to these results a further aim was to study the synergistic effects between the beer preservating hurdle system and efficacy of high pressure treatment on inactivation of microorganisms. Hop acids combined with different ethanol concentrations were tested under varying pressure levels. This elucidated their supplementary effects on cell inactivation. The loss of hop resistance after high pressure treatments was detected in storage experiments (Chapter II). It was excluded, that high concentrations of solubilized gases, especially carbon dioxide, did intensify high pressure cell inactivation. It has to be pointed out, that the concentration of carbon dioxide dissolved in beer (0.5 w/w %) did not affect *L. plantarum*. The application of liquid carbon dioxide kills microorganisms extracting cytoplasmic membrane compounds. The gaschromatographic measurements of extraction were done by D. Burger in the institute for Instrumentelle Analytik/Umweltanalytik of Prof. H. Engelhardt at the Universität des Saarlandes (Chapter III). Enzymatic inactivation of HorA took place before cell inactivation. This indicated an additional effect involving cell structures highly affected by high pressure. It was observed by in-situ measurements, that

cytoplasmic membranes changed their phase state before effects on HorA were detected. This correlated with the composition of the acyl chains, HorA and cell inactivation. A pressure-temperature-phase diagram for *L. plantarum* membranes was established to describe membrane triggered effects of high pressure on cells (Chapter IV). Cytoplasmic membranes are also the main target of pulsed electric fields, an additional non-thermal food preservation method. The effects of pulsed electric fields on the cytoplasmic membranes of *L. plantarum* in model beer were examined with the established measurement methods. Pulsed electric fields initiated ruptured membranes and caused cells death according to the total energy input. This result is in contrast with high pressure, where membrane integrity is destroyed after cell viability loss (Chapter V; 27).

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Chapter I: Effects of High Pressure on Survival and Metabolic Activity of *Lactobacillus plantarum* TMW1.460

ABSTRACT

The application of high pressure (HP) for food preservation requires insight into mechanisms of HP-mediated cell injury and death. The HP-inactivation in model beer of *Lactobacillus plantarum* TMW1.460, a beer spoiling organism, was investigated at pressures ranging from 200 to 600 MPa. Surviving cells were characterized by determination of (i) cell viability and sublethal injury, (ii) membrane permeability to the fluorescent dyes propidium iodide (PI) and ethidium bromide (EB), (iii) metabolic activity with tetrazolium salts, and (iv) the activity of HorA, an ABC-type multi-drug-resistance transporter conferring resistance to hop compounds. HP-inactivation curves exhibited a lag time, an exponential inactivation phase, and pronounced tailing caused by a barotolerant fraction of the population, about 1 in 10^6 cells. During exponential inactivation, more than 99,99% of cells were sublethally injured, however, no sublethal injury was detected in the barotolerant fraction of the culture. Sublethally injured cells were metabolically active and loss of metabolic activity corresponded to the decrease of cell viability. Membrane damage measured by PI occurred later than cell death, indicating that dye exclusion may be used as a fail safe method for rapid characterization of HP inactivation. An increase of membrane permeability to EB, and a reduction of HorA activity was observed prior to the loss of cell viability, indicating loss of hop resistance of pressurized cells. Even “mild” HP treatments thus abolished the ability of cells to survive under adverse conditions.

INTRODUCTION

Treatment of food with a high pressure (HP) of 200 – 800 MPa is a novel process in food technology employed to change functional food properties, to selectively affect the activity of food enzymes, to improve food texture, and to eliminate microorganisms. The application of hydrostatic pressures is especially promising to achieve preservation of minimally processed foods, as a pressure treatment does not compromise the sensorial food quality to the same extent as thermal treatments with a comparable germ reducing effect. Nevertheless, it is mandatory to achieve food preservation by "mild pressure" treatment in order to minimize quality deterioration, and to reduce equipment and energy costs. Information on the mechanisms of HP mediated inactivation of micro-organisms will facilitate the deliberate choice of the parameters pressurization temperature, pH, pressure level and holding time, and allow the use of synergistic interactions between HP and other preservative principles.

Proteins and membranes are considered to be the primary target for the pressure-induced inactivation of bacteria. Pressures of 150 to 250 MPa have been shown to induce dissociation of ribosomes in *Escherichia coli* (25). Wouters et al. (46) found no morphological changes in the cytoplasmic membrane upon lethal pressure treatment of *Lactobacillus plantarum*, however, the membrane permeability was increased and the ability to maintain a Δ pH across the membrane was impaired. Pressure induced leakage of sodium and calcium ions was observed in *Saccharomyces cerevisiae* (27). Effects of high pressure treatment on membrane potential and membrane bound transport systems may result from phase transitions in the membrane (23). Adaptation of barophilic deep sea bacteria to high pressure involved a shift of membrane lipid composition from saturated to unsaturated fatty acids (47). Both yeasts and bacteria have been found to exhibit a maximum of barotolerance at ambient temperature (12, 38). Ter Steeg et al. (42) observed an increased efficacy of HP treatment if the pressurization temperature was reduced or if the incubation temperature of the preculture was increased, i.e.

under conditions where the liquid crystalline state of cytoplasmic membrane during growth of the organisms is altered to a more rigid, semicrystalline state during pressurization.

Comparison of cells counts of pressurized samples on selective and non-selective media shows that a large proportion of a given population is sublethally injured prior to cell death, i.e. pressure treated cells fail to survive and multiply in harsh environments tolerated by untreated cells. The validity of this approach for Gram-negative organisms was shown by use of selective media probing the permeability barrier of the outer membrane by bile salts (14, 18, 19). Many foods must be considered as "selective media" for micro-organisms where growth or survival requires specific resistance mechanisms e.g. acid tolerance, osmo-tolerance, or resistance to inhibitory compounds. Sublethal injury of HP-treated cells may therefore indicate the inability to survive during food storage, however, mechanisms accounting for this effect have so far not been elucidated.

As a model system to study the kinetics and mechanisms of HP inactivation of lactic acid bacteria (LAB), we choose a beer spoiling organism, *Lactobacillus plantarum* TMW1.460. Beer is a highly selective medium for growth of micro-organisms because of the content of hop bitter compounds, its low pH, and the high content of ethanol and carbon dioxide. The mechanisms that allow beer spoiling bacteria to overcome these hurdles have been characterized in the past years. The major bactericidal components in beer are hop bitter compounds – colupulone, humulone, trans-isohumulone and trans-humulinic acid - which dissipate the transmembrane pH gradient (35, 36). Beer spoiling LAB have been shown to possess a plasmid encoded hop resistance mechanism, HorA (32, 33). HorA mediates ATP-dependent transport of hop bitter compounds and has high homology to other bacterial ABC type multidrug transporters as well as mammalian multidrug resistance proteins (33, 44). Sami et al. (31) screened 95 lactobacilli on the presence of the gene coding for the hop efflux

pump, *horA*, and found that this resistance mechanism is a prerequisite for their growth in beer.

The objective of this study was to characterize the HP-treated cells not only to obtain information on cell viability but also to determine sublethal injury. A number of assays recently proposed for the rapid determination of cell viability (2, 5, 43) was adapted to *Lactobacillus plantarum* TMW1.460 in order to determine whether the loss of metabolic activity, membrane integrity, or the failure to maintain hop resistance accounts for sublethal injury of pressurized cells.

MATERIALS AND METHODS

Strains and culture conditions. *Saccharomyces cerevisiae ssp. uvarum* TMW 3.001, a commercially available brewer's yeast (TU München, Lehrstuhl Technologie der Brauerei II, Freising, Germany), was cultured at ambient temperature on malt extract medium (12% w/w malt extract, Ireks, Kulmbach, Germany, sterilized at 121°C, 21 min). *Lactobacillus plantarum* TMW1.460, an organism previously isolated from spoiled beer, was cultivated using model beer (MB), MRS-agar (7), or MRS agar containing 4% NaCl (MRS-NaCl) at 30°C. MB was prepared by inoculating malt extract medium with *S. cerevisiae* TMW 3.001 to a cell count of about 5×10^6 cells ml^{-1}. The mash was fermented for 140 h at 10°C, autoclaved, and the yeast was removed by centrifugation (20 min and 20,000 x g, 0°C). The clear supernatant was collected, residual ethanol and CO_2 were removed in a rotary evaporator under vacuum, and the weight loss was compensated with H_2O_{demin}. The pH was adjusted to 4.0, and the medium was sterilized at 121 °C for 21 min.

High pressure treatment. An overnight culture of *L. plantarum* TMW1.460 in MB was subcultured with 1% inoculum. Late stationary cells were harvested by centrifugation and resuspended in an equal volume of MB. This cell suspension was transferred to 2 ml Eppendorf reaction tubes (ERT), sealed with silicon stoppers avoiding enclosure of air, and stored on ice until pressurization. The high pressure inactivation kinetics of *L. plantarum* was investigated in HP-autoclaves precooled to 15°C. Compression and decompression rates were 200 MPa min^{-1}. Upon pressurization, samples were stored on ice until further analysis as described below. For each HP inactivation kinetics, untreated cultures and cultures sterilized by treatment with 800 MPa for 10 min were used for preparation of "calibration samples" containing 100, 50, and 0% viable cells.

Determination of plate counts. Cell counts were determined on MRS agar and MRS-NaCl agar for determination of viable and sublethally injured cells. Appropriate dilutions were

plated using a spiral plater (IUL, Königswinter, Germany) and plates were incubated at 30°C for 2 days under controlled atmosphere (76% N_2, 20% CO_2, 4 % O_2). Cell counts of overnight cultures of *L. plantarum* in MB were 4.27±2.09 x 10^8 CFU ml^{-1} on either MRS or MRS-NaCl agar (mean of 24 determinations). The cell counts on MRS are referred to as ”viable cells” and the difference between cell counts on MRS and MRS-NaCl is referred to as ”sublethally injured cells”.

Determination of metabolic activity. Cells from 100µl sample were harvested by centrifugation at 0°C, 10 000 x g for 10 min, resuspended in 100µl phosphate buffer with glucose (PBG, 50 mM H_2KPO_4, 0.1 g l^{-1} $MgSO_4$ x 7 H_2O, 0.05 g l^{-1} $MnSO_4$ x H_2O, and 4 g l^{-1} glucose, pH 6.5), and transferred to microtiterplates. To this cell suspension was added a stock solution of 4 mmol l^{-1} 2-(-iodophenyl)-3-(*p*-nitrophenyl)5-phenyltetrazolium chloride (INT, Molecular Probes, Eugene, OR, USA) in PBG to a final concentration of 2 mmol l^{-1} INT. The kinetics of reduction of the colorless INT to the red formazan dye was followed by measuring absorption at 590 nm in an spectrafluor microtiter plate reader (Tecan, Grödig, Austria) in 1 min intervals for 60 min at 30°C. The initial rate of INT reduction was used for calculation of the metabolic activity of the cells. A calibration curve was established for each inactivation kinetics using the “calibration samples” described above and the results are reported as % metabolic activity.

Determination of HorA activity. Ethidium bromide (EB) is a substrate for HorA conferring hop resistance and related MDR transport systems of LAB (3, 33). Therefore, an assay for HorA activity was developed using EB as substrate. EB stock solutions were prepared by solving 40 µmol l^{-1} EB in PBG and PB0 (50 mM H_2KPO_4, 0.1 g l^{-1} MgSO4 x 7 H_2O, and 0.05 g l^{-1} $MnSO_4$ x H_2O, pH 6.5). Cells were harvested from 1 ml sample by centrifugation (10 min at 15°C, 6000 x g) and resuspended in 1 ml PB0. Each sample was divided in two aliquots. One aliquot received 200 µl of cell suspension and 200 µl of EB stock solution in

PB0 to obtain a EB concentration 20 µmol l^{-1}. The other aliquot received 200 µl EB stock solution in PBG to obtain the same buffer and EB concentrations, and additionally 2 g l^{-1} glucose as energy source. Samples were mixed and incubated at 30°C for 0, 20, 45, 100 and 140 min (assay validation) or 1.5 h (characterization of HP-treated samples) in the dark. After incubation cells were harvested and resuspended in 200 µl PB0 and transferred to black microtiterplates. The fluorescence of this cell suspension was measured using a spectraflour microtiter plate reader (λ_{Ex} = 485 nm, λ_{Em}=595 nm). The difference between EB fluorescence of starved and energized cells was considered to indicate HorA activity.

Determination of membrane integrity. The integrity of the cytoplasmic membrane of HP-treated cells was determined using two different dye exclusion assays.

(i) The BacLight® Live/Dead Kit (Molecular Probes, Eugene, OR, USA) was used according to the instructions of the supplier. In short, cells from 100 µl samples were harvested by centrifugation, resuspended in an equal volume of PB0 and transferred onto black microtiterplates. To each sample, 100µl dye solution (33.4 µmol l^{-1} Syto9 and 200 µmol l^{-1} PI in PB0) was added and the plates were incubated for 15 min at ambient temperature. The ratio of Syto9 and PI fluorescence was used to calculate the % of viable cells in the sample. The applicability of the BacLight® kit to *L. plantarum* was verified with cells killed by heat (80°C for 10 min), or HP treatment (800 MPa, 10 min). A calibration curve was established for each inactivation kinetics using the "calibration samples" described above and the results are reported as % intact membranes.

(ii) The determination of HorA activity described above required incubation of cells of *L. plantarum* with EB in the presence and absence of an energy source, glucose. In the absence of glucose (i.e. in the absence of a functional EB efflux system), EB uptake was considered to depend solely on the barrier properties of the cytoplasmic membrane. The fluorescence intensity of samples stained with EB in PB0 (see HorA assay described above)

thus provides further information on the membrane integrity of pressurized cultures of *L. plantarum*. The samples with known content of viable and dead cells were stained with EB in the absence of glucose as described above, and the resulting calibration curve was used to calculate % intact membranes in the HP-treated samples.

RESULTS

Assay validation for determination of metabolic activity with tetrazolium salts. The use of tetrazolium salts has become a standard laboratory methods to determine metabolic activity and viability of micro-organisms. The assay is based on the ability of metabolically active cells reduce INT to insoluble red formazan. However, the feasibility of this method to characterize metabolic activity of lactobacilli has so far not been demonstrated, and previous investigators have used the microscope to determine deposition of formazan crystals in individual cells (43, 45). To develop a miniaturized assay for the rapid analysis of a large amount of samples, we evaluated whether the initial rate of formazan production by cell suspensions is a suitable means to estimate their metabolic activity. Mixtures of heat killed cells of *L. plantarum* with viable cells were incubated with INT and the rate of formazan formation was determined. The increase of absorption at 595 nm is shown in Figure 1.

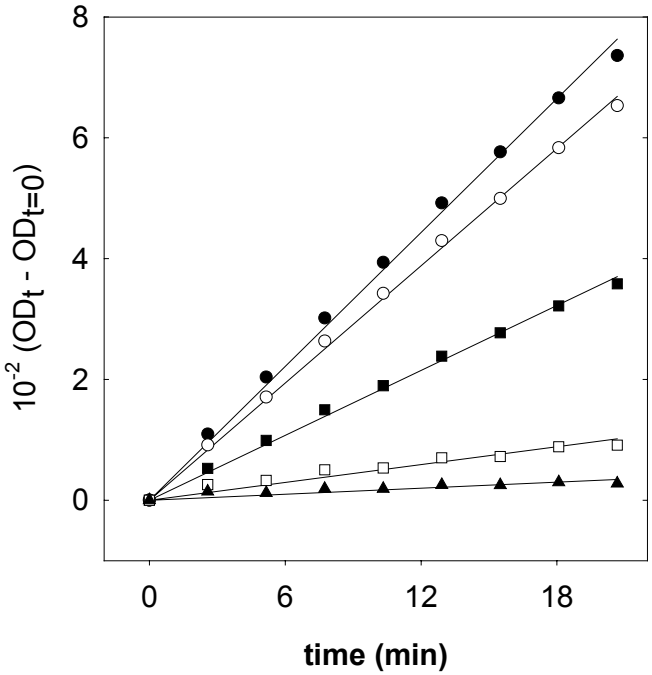

Figure 1. Formazan formation by cultures of *L. plantarum* TMW1.460 containing 100% (●), 90% (❍), 50% (■), 10% (❑), and 0 % (▲) viable cells. Lines represent curves obtained by linear regression.

A linear rate of INT reduction was observed during the first 20 – 30 minutes of incubation, and the INT reduction rate was calculated from these data by linear regression. The INT reduction rate correlated well to the content of viable cells in the culture (r^2 = 0.9985). This assay was therefore used for characterization of HP-treated cultures, and a r^2 of 0.95 or greater was obtained for all calibration curves determined with HP-experiments (n=12).

Assay validation for determination of HorA activity. To demonstrate that *L. plantarum* TMW1.460, a highly hop resistant beer isolate, has a functional HorA efflux system, and to develop a HorA activity assay, the hop resistance was evaluated on the genetical and physiological level. Using *horA* targeted primers and PCR conditions as described by Sami et al. (31), we obtained a PCR product of the expected size, 345 bp (data not shown) using chomosomal DNA from *L. plantarum* TMW1.460 as template. Sequencing of this PCR product revealed identity to the corresponding *horA* fragment of *L. brevis* but for one conservative base pair exchange (data not shown, 31, 32). This demonstrates that *L. plantarum* TMW1.460 carries a *horA* gene. HorA activity of *L. plantarum* TMW1.460 was investigated using EB as substrate. Energy dependent EB efflux by *L. plantarum* was assessed by incubation of cells with EB in the presence or absence of an energy source, glucose, and monitoring of EB uptake over time. The results are shown next page in Figure 2. In the presence of glucose, cells were able to maintain an internal low EB concentration, whereas starved cells accumulated EB. This points to the presence of a energy dependent EB efflux system which was attributed to HorA activity. Based on the kinetics of EB accumulation, an incubation time of 1.5 h was chosen for determination of HorA activity in pressurized cells.

For assay calibration, mixtures of viable cells with cells killed by either HP treatment (800MPa, 10 min) or heat (80°C, 10 min) were used. In Figure 3 next page is shown the correlation of EB fluorescence in the presence and absence of glucose to the content of viable cells in the culture.

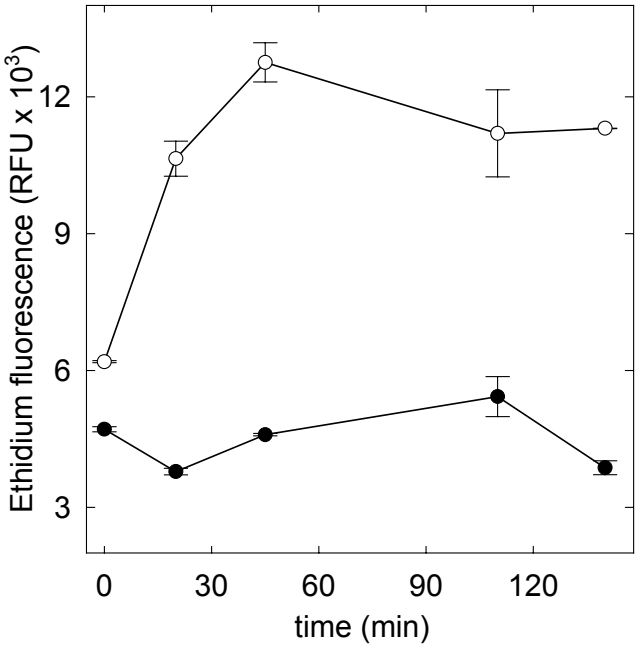

Figure 2. Kinetics of EB diffusion into starved (❍) and energized (●) cells of *L. plantarum* TMW1.460. EB influx was determined by measuring the fluorescence of cells harvested after incubation times of 0 to 145 min. Symbols represent means ± standard deviation of two independent experiments.

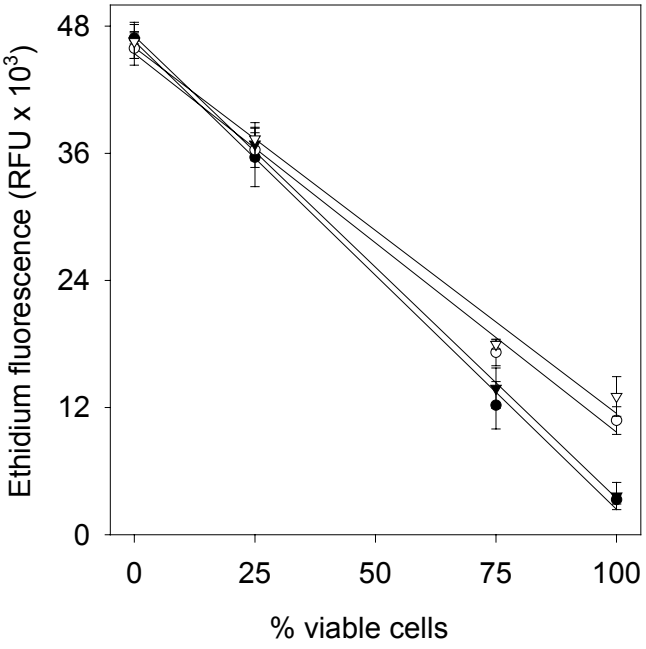

Figure 3. Correlation of EB fluorescence of starved (open symbols) and energized (black symbols) to the content of viable cells in the sample. Samples were prepared by mixing an untreated cultures with cultures sterilized by heat (80°C, 10 min, ●) or high pressure (800 MPa, 10 min, ▼). Symbols represent means ± standard deviation of three independent experiments.

Dead cells exhibited high EB fluorescence and an increasing content of viable cells resulted in a decrease of EB-fluorescence, as in viable cells EB influx occurs by diffusion through the lipid bilayer only. For samples containing viable cells, EB fluorescence in the presence of glucose was lower than in the absence of glucose, in accordance with HorA activity of viable, energized cells. Cultures sterilized either by heat or HP-treatment exhibited the same behavior in the assay. It must be emphasized that the killing of cells by severe heat (80°C, 10 min) or HP (800 MPa, 10 min) treatments results in simultaneous disruption of membrane integrity, inactivation of glycolytic enzymes, and inactivation of HorA activity. However, during pressurization not all of these three components required for EB transport across the membrane are necessarily inactivated at the same time. Therefore the interpretation of the assay results with respect to HorA activity requires additional information on metabolic activity and membrane integrity.

HP inactivation of *L. plantarum*: sublethal injury and cell death. *L. plantarum* TMW1.460 was subjected to HP-treatment at 200, 400, 500, and 600 MPa and cell inactivation was monitored over time by plating on MRS and MRS-NaCl agar. The results are shown in Figure 4A-4D. Cell counts of untreated cells on MRS and MRS-NaCl agars were not different ($P < 0.001$); therefore, failure of *L. plantarum* to grow on MRS-NaCl indicates sublethal injury. Because samples could not be taken during compression and decompression, the x-axis on Figure 4 indicates the pressure holding time.

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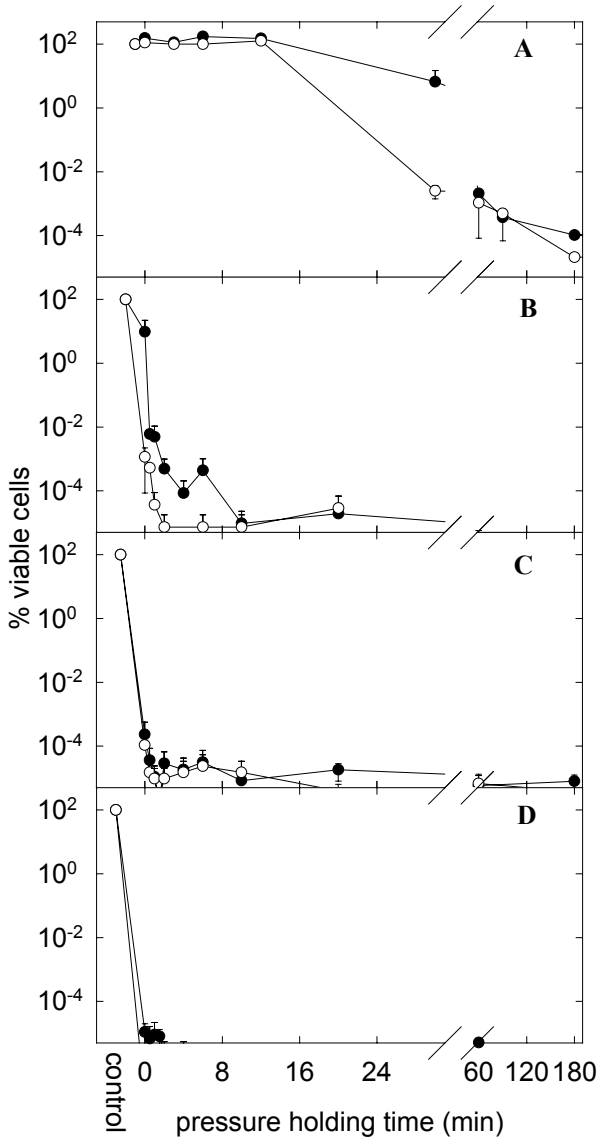

Figure 4. Kinetics of inactivation of *L. plantarum* TMW1.460 incubated at 200 MPa (A, n=4), 400 MPa (B, n=3), 500 MPa (C, n=3) and 600 MPa (D, n=2). Shown is the viable cell count on MRS (non-selective agar, ●) and MRS-NaCl (selective agar, ❍) compared to that of untreated cultures. The cell count of control cultures was 4.27 ± 2.09 x 10^8 on either agar and the detection limit was 120 CFU ml^{-1}. Symbols represent means ± standard deviation of n independent experiments.

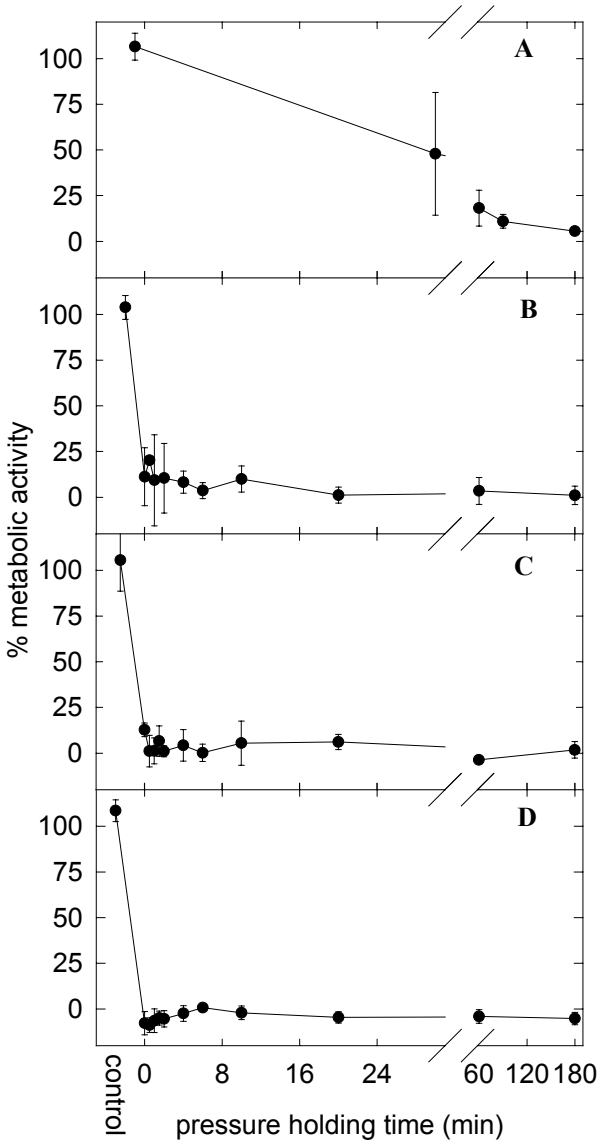

Figure 5. Kinetics of inactivation of *L. plantarum* TMW1.460 incubated at 200 MPa (A, n=4), 400 MPa (B, n=3), 500 MPa (C, n=3) and 600 MPa (D, n=2). Shown is the INT reduction rata compared to the INT reduction rate of untreated cells. Symbols represent means ± standard deviation of n independent experiments.

As shown in Fig. 4A, the inactivation kinetics of *L. plantarum* exhibited an sigmoid shape and were characterized by a lag time, an exponential inactivation phase, and a pronounced tailing where no or little further inactivation took place. During lag time, no loss of viability was observed. During the exponential inactivation phase, cells were sublethally injured prior to irreversible cell damage as demonstrated by the observation that more than 99.99% of viable cells lost their ability to grow on selective media. At longer pressure holding times (more than 120, 12, or 2 min at 200 , 400 or 500 MPa, respectively) sublethal injury was no longer observed. Apparently, a fraction of the population, about one in 10^6 cells, was highly resistant to pressure, independent whether the pressure treatment was performed at 200, 400, or 500 MPa. To evaluate whether this tailing of pressure inactivation curves stems from mutants or reflects phenotypic diversity of the population, survivors of HP treatments were isolated, subcultured once, and their pressure tolerance was determined. If this selection cycle was carried out four times, no increase in barotolerance was observed (data not shown).

This characteristic shape of the inactivation curves was not observed at pressures of 400 and 500 MPa as the lag time and part of the exponential inactivation occurred already during the pressure ramps during which no samples were taken. However, a barotolerant fraction of the population exhibiting no sublethal injury was also observed. At 600 MPa, virtually all cells were killed during pressure ramps and the fraction of barotolerant cells was at or below the detection level (120 cfu ml^{-1}).

HP inactivation of *L. plantarum*: loss of metabolic activity. Cell counts demonstrated sub-lethal injury during HP-treatment of *L. plantarum*. To evaluate whether this phenomenon is related to the loss of metabolic activity, the metabolic activity of pressurized cells was determined with tetrazolium chloride. The results are shown in Figure 5A-D. Whereas metabolic activity could be detected after 60 min pressure holding time at 200 MPA, the pressure ramp to achieve 600 MPa sufficed to completely inactivate the cultures. Sublethally injured cells

exhibited metabolic activity comparable to the untreated controls, but the loss of metabolic activity was highly correlated to cell death (r^2 = 0.92). However, metabolic activity above baseline level (4% of untreated cultures) was observed upon pressurization of cells at 200, 400 or 500 MPa even if more than 99.99% of the cells were killed, indicating that the loss of metabolic activity is unlikely to be a primary cause for cell death.

HP inactivation of *L. plantarum*: loss of membrane integrity. HP-inactivation kinetics were further characterized by two different dye-exclusion assays based on EB and PI permeation into cells. The results are shown in Fig. 6. Curves obtained with PI exhibited the same sigmoid shape as observed with plate counts (Fig. 4) and staining with INT (Fig. 5), however, loss of PI exclusion was observed only after the cells were dead and lost any metabolic activity. This observation was highly significant at pressure levels of 200, 400, and 500 MPa, where 25 – 50% membrane integrity was observed after HP-treatments resulting in reduction of viable counts by 4 – 6 orders of magnitude.

EB was used as second probe for determination of membrane integrity. As opposed to PI, EB also penetrated intact membranes. Remarkably, effects of HP-treatment on the permeability of cells to EB differed from the results obtained with PI. As compared to PI, longer pressurization timed were required to obtain 0% membrane integrity as determined by EB. However, the inactivation kinetics at 200 MPa indicates that membrane damage occurs prior to cell death. EB diffusion into HP-treated cells was facilitated already after 4 – 20 min of pressure holding time, resulting in 50 – 75% membrane integrity, although cells were not stained with PI and neither sublethal injury nor loss of viability were detectable by plate counts. Accordingly, pressurization at 400 MPa resulted in a rapid drop of EB membrane integrity to about 50% within 1 min followed by a much slower decrease to 0% within 3 hours. Incubation of cells with EB in the absence of an energy source excluded that ATP or membrane potential dependent transport mechanisms such as HorA affected EB uptake.

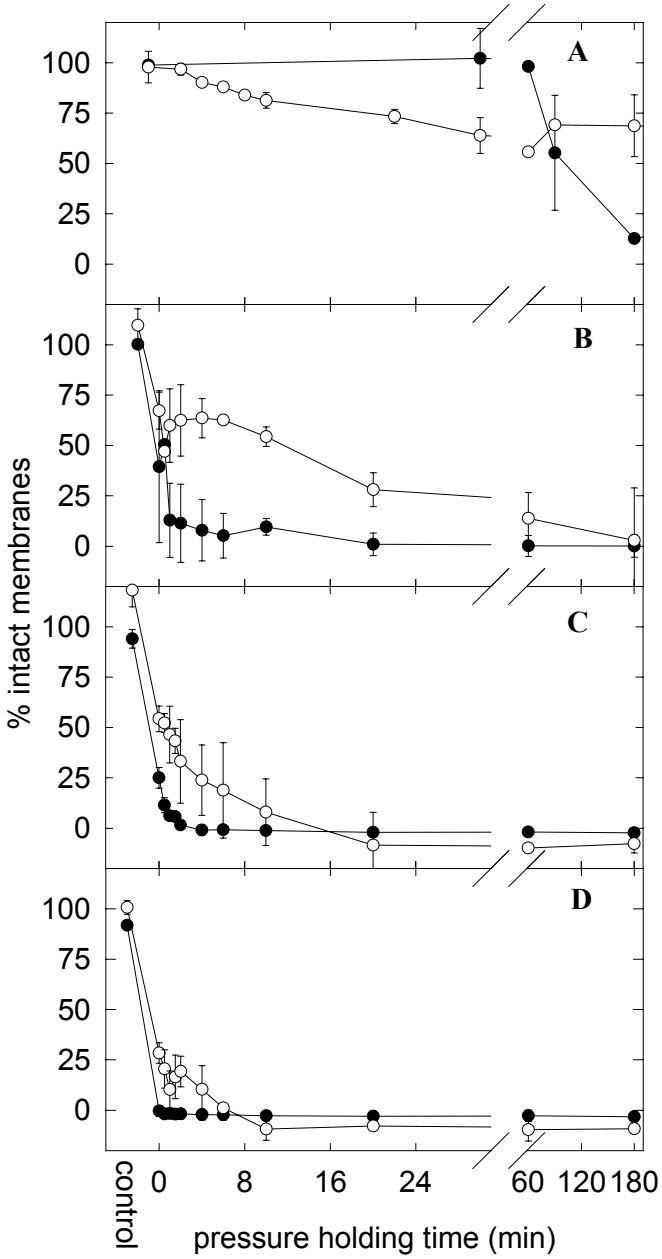

Figure 6. Kinetics of inactivation of *L. plantarum* TMW1.460 incubated at 200 MPa (A, n=4), 400 MPa (B, n=3), 500 MPa (C, n=3) and 600 MPa (D, n=2). Shown is the membrane permeability determined with the dyes propidium iodide (●) or ethidium bromide (❍) and compared to the membrane permeability of untreated cells. Symbols represent means ± standard deviation of n independent experiments.

HorA activity of pressurized cells. HorA activity is a prerequisite for growth of LAB in the presence of hop bitter compounds, and is therefore crucial for the beer spoiling capability of LAB. Therefore, in addition to membrane integrity and metabolic activity, the HorA activity of pressurized cells was estimated by determination of EB diffusion into starved and energized cells. The results for HP-treatments at 200 and 600 MPa are shown in Figure 7.

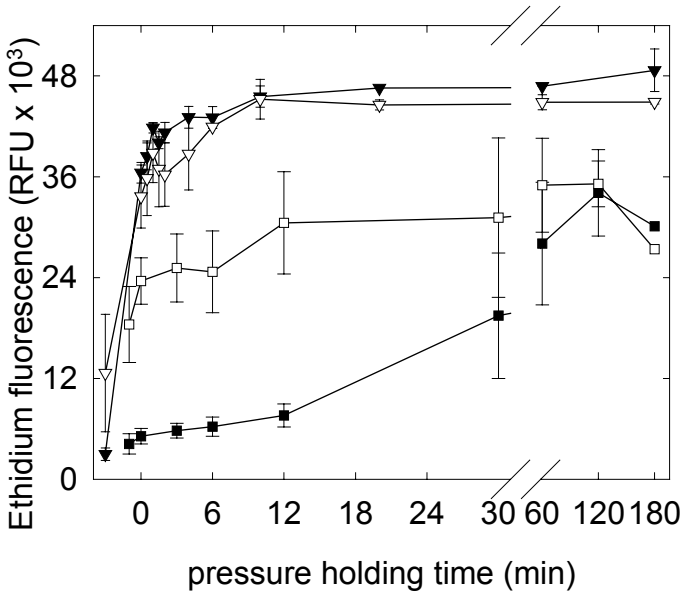

Figure 7. HorA activity of *L. plantarum* TMW1.460 after pressure treatment at 200 MPa (■, n=4) and 600 MPa (▼, n=2). Shown is the EB-fluorescence of cells incubated in phosphate buffer with 20 µmol l^{-1} EB for 90 min in the presence (black symbols) and absence (open symbols) of glucose. Symbols represent means ± standard deviation of n independent experiments.

As described above, control cultures (no HP treatment) exhibited a large difference in EB influx depending on the presence of glucose, indicating HorA activity (Fig 2 and Fig 3). Pressurization with 600 MPa resulted in complete loss of metabolic activity and membrane integrity (compare to Fig. 5 and 6), accordingly, EB uptake into cells was facilitated and reached the level of cells killed by heat (80°C, 10 min) after 6 – 10 min of pressurization time. A significant effect of glucose on EB uptake was not observed. Cells pressurized at 200

MPa for 0 to 12 min were able to maintain an internal low EB concentration although EB diffusion across the membrane was facilitated. During these first 12 min, no loss of cell viability or sublethal injury was observed by plate counts (Fig 3). After 30 and 60 min pressure holding time, the cultures lost their ability to exclude EB even in the presence of glucose, indicating loss of hop resistance. This observation that viable but sublethally injured cells exhibited reduced HorA activity was more evident if data were corrected for the systematic difference (± 2 log difference) between HP-inactivation kinetics carried out on different days.

DISCUSSION

In this study the pressure-mediated inactivation of *L. plantarum* was investigated and surviving cells were characterized with respect to viability, metabolic activity, membrane integrity, and functionality of hop resistance mechanisms. Based on this methodology, a rationale could be provided for the observation that mild pressurization of micro-organisms strongly reduces the ability of microorganisms to survive in adverse environments. It was furthermore demonstrated that assays developed for characterization of HP-treated cells are suitable methods for rapid determination of cell viability and activity.

The calculation of thermal death time data is based on the observation that killing of bacteria and endospores is accurately described by first order kinetics. First order kinetics have also been used to calculate pressure death time data for yeasts and ascospores of yeasts (12, 26, 48), and bacteria (10, 11, 38). However, for *Bacillus* species (16, 28) and for *Escherichia coli* (15) it was shown that pressure inactivation curves may exhibit a pronounced shoulder and tailing. Our results corroborate these observations and thus provide further evidence that first order kinetics does not adequately describe pressure death time data.

Tailing of pressure inactivation curves obtained with spores of *Bacillus subtilis* was thought to reflect a heterogeneous distribution of barotolerance in the population (28) based on genotypic or phenotypic diversity. Hauben et al. (13) have isolated barotolerant mutants of *Escherichia coli*. 18 selection cycles were required to obtain mutant strains that tolerated pressure treatments resulting in greater 8 log cycles inactivation of the wild type strain. (13). We were unable to isolate baroresistant mutants after 4 selection cycles, indicating that tailing of pressure inactivation reflected phenotypic diversity within the population rather than the presence of baroresistant mutants.

The shoulder or lag time of pressure inactivation curves was explained by phenotypic heterogeneity in the population and a mathematical model that used a Weibull-distributed kinetics for the transition from “stable” to “metastable” state during pressure treatments fitted experimental death-time data well (16). If pressure treatment of bacteria indeed is a two stage inactivation process leading to “metastable” or sublethally injured cells, it should be possible to identify physiological changes occurring during the lag-time of pressure death-time curves. A number of investigators have proposed selective media for enumeration of sublethally injured *Escherichia coli*, *Salmonella* spec., *Staphylococcus aureus,* and *Listeria monocytogenes* after pressure treatments (8, 14, 18, 19). As selective agent for lactobacilli, the use of 0.6% NaCl was proposed (41) and the failure of lactobacilli to grow on MRS-NaCl was thought to indicate membrane damage (40). We used 4% NaCl to inhibit growth of sublethally injured cells upon HP-treatments and observed a shorter lag-time (200 MPa) or no lag time at all (400 MPa) in pressure-death time curves characterized with MRS-NaCl agar. These data thus provide further physiological proof for a two-stage inactivation process during pressurization micro-organisms. However, as MRS-NaCl cell counts obtained from treatments with 200 MPa still exhibited lag-times, the selective medium used in this study failed to indicate cell damage occurring early during inactivation.

The determination of sublethal injury in HP-treated populations may be of major interest for food preservation as sublethal injury may eliminate the ability of a culture to grow during food storage. Indeed, pressurized cells of *Escherichia coli* failed to survive in fruit juices under conditions tolerated by untreated cells (8, 21) although only a minor part of the population was actually killed by the HP treatment.

The staining of cells with tetrazolium salts was proposed as rapid method for determination of cell viability (9, 43, 45). Tetrazolium reduction to formazan by lactobacilli depends on the

activity of NADH-dependent enzymes such as NADH oxidase / peroxidase activities or specific NADH dehydrogenases that are present in lactic acid bacteria (4, 20, 24, 30, 37, 39). Reduction of tetrazolium salts by lactobacilli thus requires NADH-generation by an ongoing carbohydrate metabolism, and staining of cells with INT does not only rely on the activity of a single enzyme system, i.e. NADH reducing enzymes, but may be considered as an indicator for the overall metabolic activity of lactobacilli. Accordingly, we observed a loss of acidification capacity of pressurized cells of *L. plantarum* concomitant with loss of capability for INT reduction (data not shown). The observation that a loss of metabolic activity of HP-treated LAB is linked to cell death rather than sublethal injury does conform with literature data obtained with different methodology. Measuring the intracellular ATP pool and F_0F_1ATP-ase activity of HP-treated *L. plantarum* revealed that ATP-generating glycolytic enzymes retained activity after treatments resulting in 60 – 95% reduction of viable cell counts (46) and that severe pressure treatments (0.4% survival) were required to completely inhibit ATP-generating enzymes (46).

The assessment of membrane integrity by dye exclusion assays has been proposed by several authors to determine the efficacy of germ killing processes, including HP treatments (1, 2, 5). Bunthof et al. (5) used PI staining for characterization of *Lactococcus lactis* stressed with freeze-thaw treatment, bile salts, low pH, and heat treatment. Whereas PI exclusion by *Lactococcus lactis* corresponded to plate counts for most stress treatments, membranes of heat killed cells were impermeable to PI (5). The observation that HP-treated cells are not recoverable by plate counts but are not stained with PI was interpreted as indication for the presence of living, but metabolically inactive cells (1). Our data confirm that failure to grow on non-selective media precedes membrane permeability to PI. Membrane damage was observed with the PI assay only for treatments resulting in greater 5 log reductions of viable cell counts. Thus, membrane impermeability to PI alone is an inappropriate indicator for cell

viability as proposed by Arroyo et al. (1) but may serve as “fail safe” method to evaluate the effect of pressure treatments.

Ethidium bromide penetrates cells with intact membranes and may therefore serve as indicator of transient membrane perturbation. *Lactococcus lactis* and beer spoiling LAB are known to possess transport systems mediating pmf or ATP dependent EB efflux, respectively, against a concentration gradient (3, 33). Therefore, to take into both the changes in membrane permeability to EB, and the inactivation of efflux systems, we performed EB transport assays with energized and starved cells. Membrane damage and inactivation of membrane-bound transport systems was identified as an important event of pressure inactivation of *Lactobacillus plantarum*, *Salmonella typhimurium*, and *Saccharomyces cerevisiae* (27, 29, 42, 46). Remarkably, EB uptake by starved cells revealed that the permeability of cells to EB was increased by pressurization. The kinetic resolution of HP-inactivation of *L. plantarum* at 200 MPa indicates that membrane damage is be an early event during pressurization of micro-organisms as it was observed even upon pressure treatments that did not affect viability or induce sublethal injury. We expect that membrane response to pressure may be nearly universal as it was observed that pressures as low as 100 – 200 MPa results in destruction of lysosomal membranes in bovine muscle tissue (17).

In addition to increased EB permeability of HP treated cells, we observed a reduction of HorA activity prior to cell death. As the metabolic activity in these cells was largely unaffected by pressurization, this finding indicates inactivation of HorA. Modifications of lipid-enzyme interactions are considered to affect the activity of membrane bound enzymes in eucaryotic cells. Membrane-bound Na^+, Mg^{2+}-ATPase of *Acholeplasma laidlawii* B is active only in association with liquid-crystalline lipids and inactivation occurs when its boundary lipids undergo a phase transition (34). Phase transitions in the lipids associated with ATP-ase

were considered to determine its activity at pressures ranging from 30 – 100 MPa (22, 23). Inhibition of Na,K-ATPase by pressure was directly correlated to increased membrane order (6). Extrapolating these findings with eucaryotic ATP-ase to corresponding bacterial enzymes suggests that phase transitions of membrane lipids may contribute to the pressure inactivation of membrane bound transport systems such as HorA (this study) or F_1F_0ATPase (46) in addition to pressure mediated protein denaturation or subunit dissociation. Membrane damage and subsequent HorA inactivation thus results in sublethally injured, hop-sensitive cells that fail to survive or even grow during beer storage. We could thus demonstrate that relatively "mild" pressure treatments are suitable in applications with the aim to prevent or delay growth of spoilage bacteria rather than the sterilization of foods.

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Chapter II: High pressure inactivation of *Lactobacillus plantarum* in a model beer system

Abstract

The effects of hop extract and ethanol on growth, high pressure inactivation, and survival of *Lactobacillus plantarum* were determined in model beer. Corresponding to the beer spoiling ability of this strain, levels of hops and ethanol typical for beer did not inhibit growth. Pressure death time curves determined at 300 MPa were described by an empirical model taking into account the sigmoid shape of survivor curves, sublethal injury, and the presence of baroresistant cells. Ethanol (5 and 10%) enhanced pressure effects on *L. plantarum* whereas hop extract (50 and 100 ppm) was less effective. In contrast, hop extracts killed pressurized cells during subsequent storage in beer but ethanol did not.

Introduction

Consumer demand for minimally processed foods and the reduced use of preservatives have fostered recent research to guarantee shelf life and safe food supply by means of novel processes. The application of hydrostatic pressure ranging from 100 – 1000 MPa allows the preservation of foods without altering food quality to the same extend as thermal treatments with a comparable preservation effect (Palou et al., 1999). High pressure processes thus allow the preservation of heat-sensitive, "fresh" food products that can not be introduced on the market otherwise due to their short shelf life. It is desirable to achieve the preservation effect with "mild" pressure application. (i) Alterations in food texture, aroma, and enzyme activities may occur at pressures above 200 – 400 MPa, well in the range of pressures required for the killing of microorganisms (Palou et al., 1999, Cheftel, 1995). (ii) Capital costs amount to a substantial share of overall costs of HP processed foods and equipment costs decrease if the machinery is designed for mild pressure application (Palou et. al., 1999, Grant et al., 2000). It has therefore been suggested to apply high pressure in combination with further preservative principles in the context of a hurdle concept.

Several combination treatments have been evaluated, including combined pressure-temperature application (Patterson and Kilpatrick, 1998, Kalchayanand et al., 1998), pressurization in the presence of bacteriocins (Roberts and Hoover, 1996, Kalchayanand et al., 1992, Morgan et al., 2000), and organic acids (Carlez et al., 1992, Ogawa et al., 1990). Microbial resistance to high pressure application is highest at ambient temperature (Sonoike et al., 1992). High pressure killing of *Escherichia coli* has been found to be more effective at acidic pH (Garcia-Graells et al., 1998) whereas inactivation of yeasts appears to be much less affected by acidic pH values (Ogawa et al., 1990). It is furthermore well established that food ingredients may exert a baroprotective effect. Low water activity decreases the efficacy of

high pressure processes (Palou et al., 1999), furthermore, a protective effect of divalent cations for high pressure killing of *Escherichia coli* has been reported (Hauben et al., 1998). Pressure induced bacterial stress negatively affects the ability of microorganisms to survive during food storage under adverse conditions. Sublethal injury caused by pressure application was demonstrated for *Escherichia coli* as well as Gram-positive pathogens (Kalchayanand et al.,1998, Hauben et al., 1996). It was demonstrated that sublethal pressure treatment of *Escherichia coli* reduced the ability of these organisms to survive in pressure treated fruit juices and that the tolerance of pressurized *E. coli* to cold storage depended on the pH of the juice (Linton et al., 1999, Garcia-Graells et al., 1998). However, sites of injury that may be responsible for reduced tolerance to acid or other environmental stressors have so far not been identified. Ulmer et al. (2000) have investigated high pressure effects on survival, metabolic activity, membrane integrity, and hop resistance of *L. plantarum* TMW 1.460 in model beer. This physiological characterization of pressure treated cells indicated that mild pressure treatments with little effect on cell viability increased the permeability of the cytoplasmic membrane, and inactivated specific hop resistance mechanisms. These results lead to the hypothesis that membrane-active solvents and hop bitter acids affect the barotolerance of *L. plantarum* and that pressure treatment eliminates the tolerance of pressure treated cells towards hop bitter acids (Ulmer et al., 2000). It was therefore the aim of the present study to test this hypothesis by evaluation of the influence of parameters hop extract and ethanol concentrations on the high pressure inactivation of *L. plantarum* TMW1.460 as well as the survival of pressure treated cells during beer storage.

Material and Methods

Microorganisms, media, and culture conditions

Lactobacillus plantarum TMW1.460, a organism previously isolated from spoiled beer, was maintained in MRS broth (de Man et al., 1960) containing 30% glycerol at –80°C. The organism was cultivated at 30°C in model beer (MB). Model beer was prepared by inoculating sterile wort (distilled water containing 12% malt extract, Ireks, Kulmbach, Germany) with the commercially available brewer's yeast *Saccharomyces cerevisiae ssp. uvarum* TMW 3.001 (TU München, Lehrstuhl Technologie der Brauerei II, Freising, Germany) to a cell count of about 5 x 10^6 cfu ml^{-1}. This mash was fermented for 140 h at 10°C, heat sterilized, and the yeast was removed by centrifugation. The broth was concentrated in a rotary evaporator, thus removing residual ethanol and carbon dioxide. The pH was adjusted with 2 N HCl to 4.00 unless otherwise stated and the broth was heat sterilized (121°C, 20 min). The weight loss resulting from evaporation and autoclaving was compensated with sterile demineralized water to obtain model beer, or with sterile water, ethanol and isomerized hop extract containing 22% iso-α-acids (HHV, Mainburg, Hallertau, Germany) to obtain model beer with specified content of ethanol and hop extract.

For enumeration of cells, a non-selective medium, MRS-agar, and a selective medium, MRS agar containing 4% NaCl (MRS-NaCl), were employed. Plates were incubated for 48 h under controlled atmosphere (76% N_2, 20% CO_2 and 4% O_2) at 30°C. To determine the level of sodium chloride addition to MRS agar for enumeration of sublethally injured cells, the NaCl content of MRS broth was adjusted to levels ranging from 0 to 8%, inoculated with *L. plantarum* TMW 1.460 and the optical density of the cultures was monitored after 24 and 48 h. Addition of 4% NaCl inhibited growth after 24 h, but not after 48 h. Cell counts of untreated cells of *L. plantarum* on MRS and MRS-NaCl agar were virtually identical. Failure

of viable cells to grow on MRS-NaCl agar thus indicates sublethal injury and cell counts on MRS are referred to as "viable cells" and the difference between cell counts on MRS and MRS-NaCl is referred to as "sublethally injured cells".

Determination of growth rates.

Growth rates of *L. plantarum* TMW1.460 were determined in model beer containing various amounts of ethanol and hop extract. Ethanol concentrations ranging from 0 to 18% were prepared by serial 2/3 fold dilutions of model beer with model beer containing 18 %, 12%, and 8% (w/w) ethanol (total of 24 different concentrations). Hop effects on growth of *L. plantarum* were determined in model beer containing various levels of isomerized hop extracts. To take into account the pH effects on the inhibitory effect of hop compounds, model beer was employed with the pH adjusted to 3.55, 4.00, 4.91, and 5.06 with 2N HCl or 2N NaOH. Model beer with hop extract concentrations ranging from 0 to 200 ppm (pH 5.06), 0 to 100 ppm (pH 4.91), 0 to 20 ppm (pH 4.00) and 0 to 10 ppm (pH 3.55) was prepared by serial 2/3 fold dilutions of model beer with model beer containing 450 and 300 ppm hop extract, 225 and 150 ppm hop extract, 45 and 30 ppm hop extract, and 22.5 and 15 ppm hop extract at pH values of 5.06, 4.91, 4.00, and 3.55, respectively. A total of 16 different hop concentrations was prepared for each pH. Standardized precultures of *L. plantarum* TMW1.460 were prepared by subculturing the organism twice for 24 h in model beer. The various media were inoculated to cell counts of 1 x 10^7 cfu ml^{-1}, and 150 µl of the cultures were transferred to 96 well microtiterplates for incubation at 30°C for 6 days. Media were overlaid with 100 µl sterile paraffin to avoid evaporation losses of water or ethanol during incubation. Growth of the organism was monitored by measuring the optical density of the medium at 590 nm in an automated microtiterplate reader (Spectrafluor, Tecan, Grödig, Austria). For each growth curve, the lag phase, maximum specific growth rate μ_{max}, and the

maximum population density were obtained by fitting the data sets optical density over time to the logistic growth curve as described previously (Gänzle et al., 1998).

High pressure treatments of *L. plantarum*

High pressure inactivation of *L. plantarum* TMW 1.460 was investigated using high pressure autoclaves (Dunze Hochdrucktechnik, Hamburg, Germany) operating with polyethylene glycol as pressure transmission fluid. The autoclaves were connected to a water bath for external temperature control and the internal temperature was measured by a Pt100 temperature sensor. The computer controlled compression and decompression rates were set to 200 MPa min^{-1}. All pressure experiments were performed at 300 MPa and 20°C and temperature changes during compression and decompression were less than 5°C. Standardized precultures of *L. plantarum* TMW 1.460 were prepared by subculturing the organism twice for 24 h in model beer. The cell counts of overnight cultures were 3.43 ±1.59 x 10^8 cfu ml^{-1}. Prior to HP experiments, cells were harvested by centrifugation (4500 x g, 4 °C, 15 min) and resuspended in model beer, model beer containing 5 or 10% (w/w) ethanol, or model beer containing 50 or 100 ppm hop extract. The samples were transferred to 2 ml Eppendorf reaction tubes, closed with silicon stoppers avoiding enclosure of air, and stored on ice until pressurization. Effects of high pressure treatment on cell viability and sublethal injury were determined by plating the organisms on selective and non-selective agar, MRS agar and MRS-NaCl agar, respectively.

Survival of pressure treated *L. plantarum* in beer and model beer.

Cells of *L. plantarum* TMW 1.460 were pressure treated at 300 MPa for 5 min as described above and diluted 1:10 with model beer or model beer containing ethanol (final concentration 5%), hop extract (final concentration 50 ppm hop extract), or model beer containing ethanol

and hop extract (final concentration 50 ppm hop extract and 5% ethanol). The same media were inoculated likewise with untreated cells of the same culture. The inoculated model beer and model beer containing various levels of ethanol and hop extracts was stored at 10°C and plate counts were determined during 72 h. For comparison, this experiment was also performed with beer obtained at a local supermarket.

Results

Effect of hop extract and ethanol on growth of *L. plantarum* TMW1.460

The levels of ethanol and hop extract employed in pressure inactivation experiments were chosen to (i) cover the concentration levels usually found in beer, and (ii) include levels of inhibitory compounds that reflect stress conditions for our model organism, *L. plantarum* TMW1.460, and thus result in considerable reduction of growth rate or complete growth inhibition. We therefore determined the effect of hop extract and ethanol on growth of *L. plantarum* in model beer. The growth rate of this organism at ethanol concentrations ranging from 0 to 12% (w/w) are shown in Figure 1.

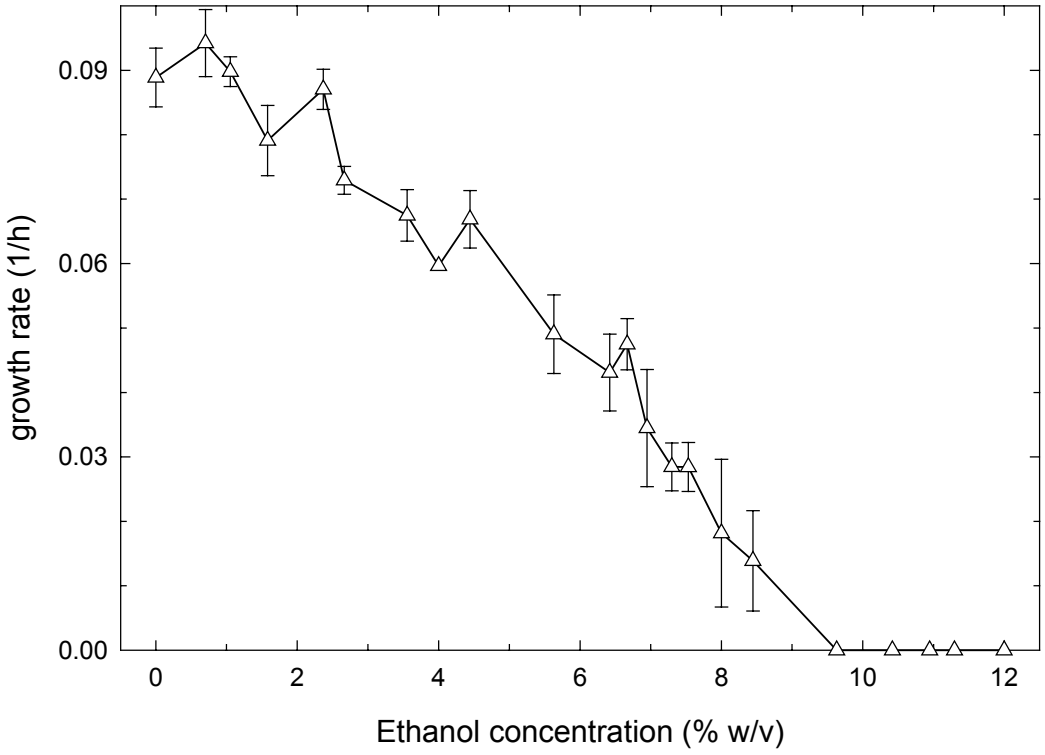

Figure 1. Effect of ethanol on the growth rate μ_{max} of *L. plantarum* TMW1.460 in model beer. Shown are the mean ± standard deviation of two independent experiments.

Levels of 3% ethanol were tolerated without significant effects on the growth rate and addition of 6% ethanol reduced the growth rate by 50%. Growth inhibition was observed at a level of 10% ethanol, well above ethanol levels usually encountered in beer. As model beer is a rather unfavorable medium for growth of *L. plantarum* because of its low pH (4.0) and limited substrate availability, it is conceivable that this organisms tolerates higher concentrations if all other environmental parameters are at their optimum value for growth. The ethanol tolerance of *L. plantarum* TMW1.460 is in the same range as that reported for *L. sanfranciscensis*, growth of which was inhibited by 8% ethanol (Gänzle et al., 1998) but well below that reported for strains of *L. hilgardii* that are able to grow in fortified wine containing 18% ethanol (Couto et al., 1996). The response of lactic acid bacteria to a lethal ethanol challenge was reported to involve the synthesis of ethanol shock proteins (Laplace et al., 1999). In the contrary, the adaptation of strains of *L. hilgardii* able to ethanol concentrations as high as 18% did not require de novo synthesis of „ethanol tolerance“ proteins but involved an increased fluidity of the cytoplasmic membrane (Couto et al., 1996 and 1997).

The growth rates of *L. plantarum* in the presence of hop extracts at levels of 0 – 200 ppm and at pH values of 3.55 to 5.06 are shown in Figure 2. In the absence of hops, the acidification of the model beer from pH 5.06 to 4.0 and 3.55 resulted in a reduction of growth rates by about 30 and 60%, respectively. At pH 5.06 and 4.91, hop extract inhibited growth of *L. plantarum* only at levels of 200 ppm. In contrast, 10 and 20 ppm sufficed to inhibit growth of *L. plantarum* TMW1.460 at pH values of 3.55 and 4.00, respectively. Given a content of 0.52 mol l^{-1} of isohumulones in the hop extract used in our experiments, these MIC values are well in agreement with MIC values reported for other beer spoiling lactobacilli with high hop resistance (Sami et al., 1997a; Simpson and Fernandez, 1994; Simpson and Smith, 1992).

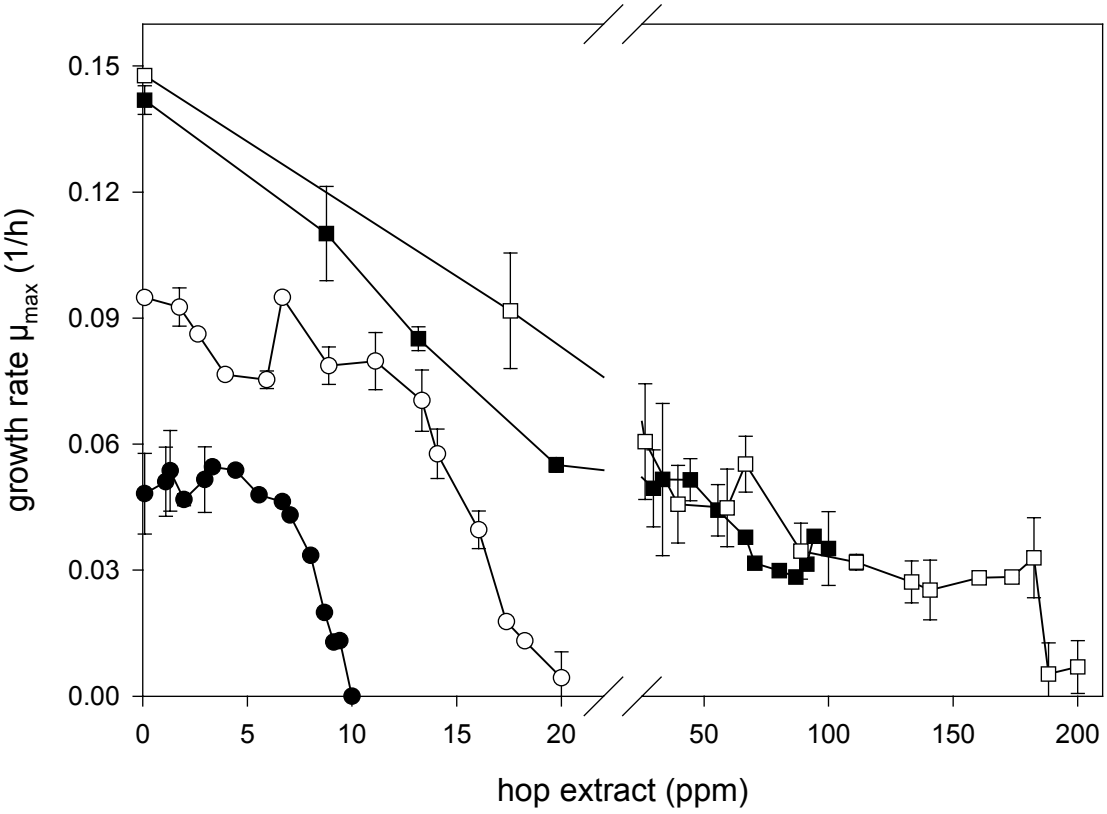

Figure 2. Effect of hop extract on the growth rate μ_{max} of *L. plantarum* TMW1.460 in model beer at pH 3.55 (●), 4.00 (❍), 4.91 (■) and 5.06 (❑). Shown are the mean ± standard deviation of two independent experiments.

Our data further corroborate the strong effect of pH on the activity of the major antimicrobially active compounds in hop extract, *trans*-isohumulone, *trans*-humulinic acid, and humulone. These acids have pK_a values ranging from 2.7 to 6.1 and the undissociated acid are 100 fold more active than the corresponding salt (Simpson and Smith, 1992). Re-plotting the data shown in Figure 2 using the content of undissociated hop bitter acids in the culture medium as x-axis reveals that growth rates measured at different pH values but comparable acid levels virtually coincide (plot not shown). The inhibitory activity of undissociated hop bitter acids depends on their ability to penetrate the cytoplasmic membrane of sensitive micro-organisms, thereby dissipating electrochemical gradients (Simpson, 1993; Simpson and Smith, 1992). Hop resistance of beer spoiling lactic acid bacteria depends on the

presence of a ABC-type multi drug resistance protein, HorA, mediating ATP dependent efflux of hop bitter compounds (Sami et al., 1997a and 1997b, Sami et al., 1998). It was previously verified that *L. plantarum* TMW1.460 carries a *horA* gene and exhibits HorA activity (Ulmer et al., 2000).

Effect of hop and ethanol on high pressure inactivation of *L. plantarum* TMW 1.460.

The effect of hop and ethanol on high pressure inactivation of *L. plantarum* TMW 1.460 was investigated at levels of 50 and 100 ppm hop extract and 5 and 10% ethanol. These ethanol concentrations correspond to levels resulting in 50 and 100 % growth inhibition, however, 50 and 100 ppm hop extract are considerably higher than the MIC at pH 4.0 (20 ppm). Preliminary experiments have shown that hop addition at the level of the MIC had no significant effect on pressure inactivation of *L. plantarum* (data not shown). It was verified that incubation of *L. plantarum* with the levels of hop extracts and ethanol employed in pressure experiments did not affect viability or sublethal injury at atmospheric pressure (data not shown). The pressure inactivation kinetics at the various conditions are shown in Figure 3A – 3E (page 12). Sigmoid inactivation kinetics characterized by a shoulder, an exponential inactivation phase, and slow inactivation of a barotolerant fraction of population were observed under all conditions, in agreement with kinetic data previously obtained with *L. plantarum* TMW 1.460 in model beer at 200, 400, and 600 MPa (Ulmer et al., 2000). The ratio of cell counts on MRS to cell counts on selective medium is depicted in the graphs to indicate the degree of sublethal injury in surviving cells. In all experiments, 90 to 99.9% of cells were sublethally injured prior to cell death and the highest proportion of sublethally injured cells was observed during the exponential inactivation phase. Addition of hop extract or ethanol accelerated HP inactivation of *L. plantarum*.

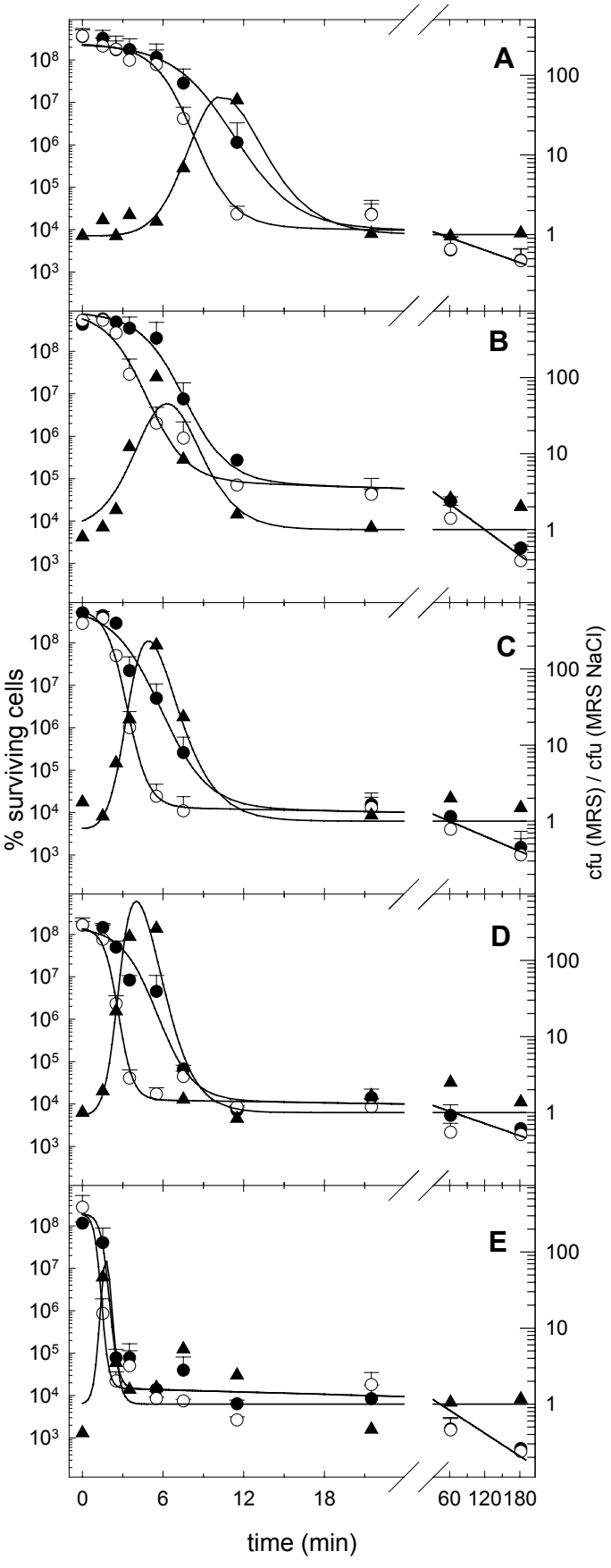

Figure 3. High pressure inactivation of *L. plantarum* TMW 1.460 at 300 MPa, 15°C in model beer containing various levels of hop extract and ethanol. A: control, n=5, B: 50 ppm hop extract, n=2, C: 100 ppm hop extract, n=2, D: 5% ethanol, n=2, and E: 10% ethanol n=2,. Shown are viable cell counts (●), uninjured cell counts (❍) and the ratio of viable to uninjured cells (▲). Error bars indicate the mean ± standard deviation of n independent experiments. Lines depict model predictions.

Whereas in the absence of additives about 10 min pressure holding time were required to reduce viable cell counts by 99% and uninjured cells by 99.99%, the same effect was obtained after 6 minutes and 1 minute in the presence of 100 ppm hop extracts and 10% ethanol, respectively. A barotolerant fraction of the population was observed that was killed much slower and exhibited no sublethal injury. Remarkably, whereas addition of hop extract and ethanol accelerated the inactivation of the major proportion of the population (reduction of viable counts from 10^8 to 10^4 cfu ml^{-1}), these additives had no effect on the size and inactivation rate of the barotolerant fraction of the population.

Calculation of pressure death-time kinetic parameters

In order to quantify the effects of hop extract and ethanol on the high pressure inactivation kinetics of *L. plantarum* TMW 1.460, a mathematical model was developed to describe the experimental data. First order kinetics are the standard model for calculation of thermal death time data and have furthermore been used do describe pressure inactivation of micro-organisms (Gervilla et al., 1999a and 1999b, Hashizume et al., 1995; Palou et al., 1997; Sonoike et al., 1992; Zook et al., 1999, Raso et al., 1998). However, shouldering or tailing as they have been observed previously for *E. coli* (Hauben et al. 1998), *Bacillus subtilis* (Heinz and Knorr 1996), *L. plantarum* TMW1.460 (Ulmer et al., 2000), and in this study are not adequately described by first order kinetics. Furthermore, the model employed should take into account the two physiological states of the microorganisms that could be distinguished, uninjured cells and sublethally injured cells. The Fermi equation was previously found to be suitable for the description of log transformed bacterial inactivation with the model parameters N (maximum log population decrease due to inactivation treatment), k (inactivation rate), and t_c (time required for 50% inactivation) (Peleg, 1996 and 1997). To take into account the fast and slow inactivation of the sensitive and resistant fractions of the

population, respectively, inactivation was modeled as sum of two kinetics, inactivation of barosensitive cells, N_s, and inactivation of barotolerant cells, N_r. As the number of data points obtained for the slow inactivation of the resistant N_r fraction of the population did not allow to distinguish whether log-linear or sigmoid kinetics occurred, a first order kinetics was used to describe inactivation of N_r. For description of inactivation of sensitive cells (N_s), two rate constants k_{s1} and k_{s2} and two 50% reduction times (t_{c1} and t_{c2}) were used to describe sublethal injury and cell death, respectively. Furthermore, model predictions concerning the ratio of viable cell counts to uninjured cells were compared to the experimental data. No sublethal injury was observed in the barotolerant fraction N_r, therefore, only one rate constant, k_r was used to describe death of barotolerant cells determined on either selective or non-selective media. The model equations can thus be written in the following form:

$$\log(y_1) = (N_r - k_r t) + \frac{N_s}{1 + e^{(k_{s1}(t_{c1} - t))}} \qquad [1],$$

$$\log(y_2) = (N_r - k_r t) + \frac{N_s}{1 + e^{(k_{s2}(t_{c2} - t))}}, \qquad [2] \qquad \text{and}$$

$$\log(y_3) = \log(\frac{y_1}{y_2}), \qquad [3],$$

where y_1, y_2, y_3 denote viable cells (cell counts on MRS), uninjured cell (cell counts on MRS-NaCl), and the ratio of viable to uninjured cells, respectively. N_s and N_r denote the pressure sensitive and baroresistant fractions of the population, respectively. The parameters k_{s1} and k_{s2} denote the two inactivation rates for the barosensitive fraction of the population (N_s), i.e. sublethal injury of uninjured cells and killing of viable cells, respectively, and t_{c1} and t_{c2} denote the respective 50% reduction times. k_r denoted the killing rate for the baroresistant fraction N_r. For optimized fitting of model parameters to experimental data, cell counts determined experimentally and the ratio of viable cell counts to uninjured cell counts were considered in the calculation of residual least squares for parameter optimization:

$$\sum_{k=1}^{3}\sum_{i=1}^{m}(y_{i,k}-\overline{y_k})^2 \xrightarrow{!} \min$$

where y_k refer to the three different physiological states taken into account by the model and y_i refer to individual data points. It must be emphasized that this model approach allows a merely empirical description of the experimental data and does consider mechanisms governing sublethal injury, resuscitation, and cell death.

The parameter estimates and correlation coefficients for the pressure death time kinetics of *L. plantarum* are shown in Table 1 and model predictions are depicted in Figure 3.

Table 1. Parameter estimates and correlation coefficients for pressure death time kinetics of *L. plantarum* in model beer.

additives / parameter estimates[a)]	none	hop extracts (50 ppm)	hop extracts (100 ppm)	ethanol (5%)	ethanol (10%)
N_s	4.3 ± 0.2	4.2 ± 0.2	4.6 ± 0.2	4.0 ± 0.2	4.1 ± 0.3
k_{s1} [min^{-1}]	0.68 ± 0.10	0.67 ± 0.10	1.26 ± 0.18	1.84 ± 0.35	3.8 ± 0.4
t_{c1} [min]	8.3 ± 0.3	4.8 ± 0.3	3.3 ± 0.1	2.7 ± 0.1	1.4 ± 0.1
k_{s2} [min^{-1}]	0.44 ± 0.08	0.61 ± 0.13	0.57 ± 0.07	0.76 ± 0.14	3.1 ± 0.8
t_{c2} [min]	11.3 ± 0.35	7.6 ± 0.4	5.8 ± 0.2	5.6 ± 0.3	2.0 ± 0.1
N_r	4.1 ± 0.1	4.2 ± 0.15	4.1 ± 0.1	4.1 ± 0.1	4.2 ± 0.1
k_r [10^{-3} x min^{-1}]	4.9 ± 1.2	6.5 ± 1.6	5.9 ± 1.2	4.8 ± 1.5	9.0 ± 2.7
r^2	0.9978	0.9940	0.9964	0.9901	0.9824
dof[b)]	23	23	22	23	23

[a)] shown are the parameter estimates ± standard error.

[b)] dof: degrees of freedom.

Model predictions for viable cells, uninjured cells, and sublethal injury (ratio of viable to uninjured cells) were generally within experimental error and the correlation coefficients

were between 0.982 and 0.998, indicating a good quality of fit. The time span between the 50% log reduction times relating to sublethal injury and cell death, t_{c1} and t_{c2}, respectively, indicated at all pressurization conditions the inactivation phase where the majority of cells are viable but sublethally injured. At the time t_{c2}, 99% of cells were dead and virtually all cells of the pressure sensitive population were sublethally injured. The parameter estimates for the size of the sensitive and resistant subpopulations, N_s and N_r, respectively, were independent of the additives hop extracts and ethanol. At all pressurization conditions, about 2 x 10^4 cfu ml^{-1} or 0.01% of the total population exhibited high pressure resistance. Furthermore, k_r values for killing of the resistant subpopulation N_r were independent of the presence of additives. These results indicate that hop compounds and ethanol affected neither the size nor the barotolerance of the the resistant subpopulation N_r. The addition of hop extracts enhanced pressure inactivation of *L. plantarum*, with more pronounced effects on the t_c values than on the killing rates k_{s1} and k_{s2}. Addition of ethanol resulted both in an increase of k_s values and a reduction of the t_c values. It is remarkable that ethanol added at the level of the MIC (10%) had a much stronger effect on pressure inactivation than hop concentrations exceeding the MIC by a factor of four (100 ppm).

Survival of pressure treated *L. plantarum* in model beer and beer.

The high pressure inactivation of *L. plantarum* TMW 1.460 demonstrated that pressure mediated killing of this organisms is a two step process involving an intermediate stage, sublethally injured cells (Figure 3). In order to whether sublethal injury correlates to a reduced potential of *L. plantarum* to spoil beer, the survival of pressure treated cells in beer and model beer was compared to untreated cells. The pressure treatments were performed in model beer (no addition of either hops or ethanol), and survival was monitored in model beer

and model beer containing 50 ppm hop extract, 5% ethanol, or both. The results are shown in Figure 4A – 4D.

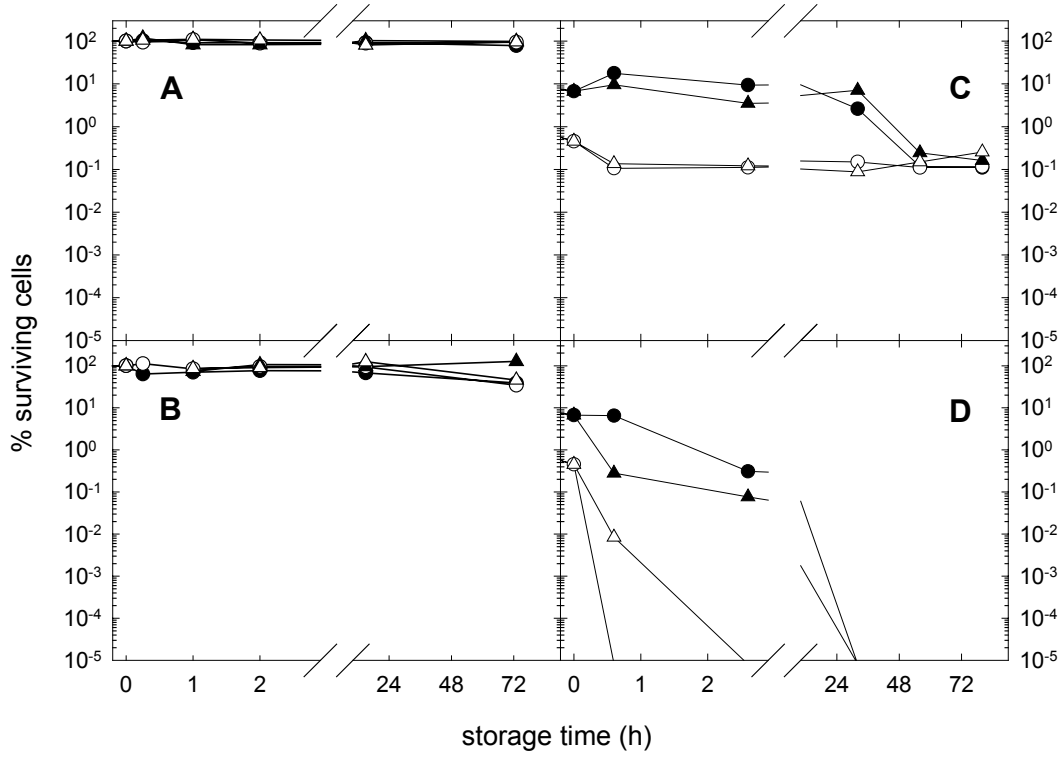

Figure 4. Survival of *L. plantarum* in model beer stored at 10°C in model beer containing various levels of hop extracts and ethanol. Untreated cell (A,B) or HP-treated cells (300 MPa, 5 min, C,D) were stored in model beer without hop extracts (A,C) or model beer containing 50 ppm hop extracts (B,C). Model beer contained 0 % (circles) or 5% ethanol (triangles). Closed symbols represent viable cell counts; open symbols represent uninjured cell counts. Lines dropping below the x-axis indicate cell counts below the detection limit (50 cfu ml^{-1}). Data represent results of four independent experiments.

Commercially available beer served additionally for comparison. In accordance with the origin of the strain from spoiled beer, untreated cells *L. plantarum* tolerated the levels of hop bitter acids and ethanol in beer without decrease of viable cell counts and sublethal injury was not observed during the storage time (Fig 4A and 4B). The pressure treatment employed, 300 MPa and 5 min pressure holding time, reduced the viable cell counts by 90% and rendered 90% of the surviving cells sublethally injured (Fig. 4C and D, 0 h storage time). A reduction

of viable cell counts was observed during storage of pressure treated cells in model beer or model beer containing 5% ethanol. Whereas uninjured cells remained unaffected by cold storage, sublethally injured cells were killed within 48 h of storage so that a difference between cell counts on the two media was no longer observed at the end of the storage time (Fig. 4C). An appreciable effect of ethanol on either viable cell counts or sublethal injury was not observed. This result indicates that the medium employed for determination of sublethal injury correlated well to the ability of the cells to tolerate cold storage in model beer in the absence of hop bitter acids. The presence of hop compounds during storage had a far more pronounced effect on the survival pressure treated *L. plantarum* TMW 1.460 than ethanol (Fig 4D). Hop extract in a concentration of 50 ppm resulted in sublethal injury of virtually all cells within 3 h of storage. Viable cell counts were reduced by 90% within 3 hours, indicating rapid inactivation of cells that were already sublethally injured by the pressure treatment, and viable cell counts dropped below the detection level (50 cfu ml^{-1} or 10^{-5} % surviving cells) after 27 hours of storage. In commercially available beer, cell counts on either agar were below the detection level after 2 h storage time (data not shown).

Pressure mediated killing of micro-organisms has been considered as a two stage process involving sublethal injury of cells followed by cell death (Heinz and Knorr, 1996), but these authors failed to provide physiological evidence for the metastable state of sublethally injured cells. The characterization of pressure treated cells on selective and non-selective media, and the evaluation of their ability to survive under adverse conditions presented in this work provided evidence that pressure treatment of *L. plantarum* eliminated first hop resistance and then tolerance to low pH and conditions of cold storage. The cell counts of pressure treated *L. plantarum* TMW1.460 on MRS-NaCl correctly predicted the part of the population tolerating storage in beer in the absence of hops, however, this medium failed to indicate physiological changes resulting in sensitivity towards hop compounds. MRS-NaCl was previously used to

indicate injury in *L. bulgaricus* subjected to heat stress and failure to grow on this medium was considered to reflect membrane damage (Teixeira et al. 1997). This study provides a rationale for the observation that pressure treated, viable cells fail to survive under conditions tolerated by untreated cells and our data indicate that three different physiological states of pressurized cells may be distinguished: (i) viable, uninjured cells, (ii) injured cells that lost hop resistance, and (iii) injured cells unable to survive in model beer. It can be concluded that the transition from uninjured cells to sublethally injured cells reflects rather a continuos change from uninjured to sublethally injured cells.

It is remarkable that addition of ethanol greatly enhanced bacterial inactivation during the pressure treatment, whereas it had little or no effect on cell viability during storage after the pressure treatment. Hop bitter acids, to the contrary, exerted a far less pronounced effect on cell inactivation during pressure treatment yet effectively inactivated pressure treated cells during subsequent storage. These finding may relate to the different mode of action of these inhibitory compounds. Ethanol is a membrane-active solvent, and micro-organisms respond to moderate ethanol levels by adaptation of membrane fluidity (Couto et al., 1996). It is well established that pressure treatment of lipid bilayers elicits phase transitions from the liquid crystalline phase to gel phase (Erbes et al., 1996; Tsuchido et al., 1996; Kaneshima et al., 1996). Deep sea animals and micro-organisms exposed to high pressure in their natural habitat respond to pressure by homoviscous adaptation of membrane fluidity (Cossins and Macdonalds, 1989; Yano et al., 1998). Accordingly, pressure effects on the cytoplasmic membrane as well as inactivation of membrane bound transport systems have been suggested to be a primary cause of bacterial inactivation under high pressure (ter Steeg et al., 1999; Marquis et al., 1984; Perrier-Cornet et al., 1999; Wouters et al., 1998; Macdonald, 1987). The increased efficacy of pressure treatment in the presence of ethanol may reflect synergistic effects of either agent on phase transitions in the cytoplasmic membrane.

The antimicrobial activity of hop bitter acids is based on their ability to penetrate the cytoplasmic membrane, thereby dissipating the transmembrane pH gradient. In hop-resistant lactobacilli, their activity is counteracted via the activity of a membrane bound, ATP-dependent multiple drug resistance protein, HorA (Sami et al., 1997a). Moderate pressure treatment of *L. plantarum* TMW 1.460 at 200 MPa has been shown to facilitate diffusion of inhibitors across the cytoplasmic membrane, and to inactivate the hop resistance protein HorA (Ulmer et al., 2000). This loss of specific hop resistance mechanisms by mild pressure treatment accounts for the observation that hop bitter acids rapidly inactivated pressure treated cells although they were found to have little effect on the kinetics of pressure inactivation of *L. plantarum*. The most likely explanation for the pressure induced HorA inactivation (Ulmer et al., 2000) and the concomitant loss of hop resistance observed in the work presented here is an indirect effect of membrane damage on HorA activity. Phase transitions on the cytoplasmic membrane have been shown to affect the activity of membrane bound transport systems (Marquis, 1984; Macdonald, 1987) and recent data provide evidence for structural rearrangements of the HorA analogue LmrA during its catalytic cycle in reconstituted membrane vesicles (Vigano et al., 2000). Although the use of hops as a preservative and hop resistance is rather specific for beer and beer spoiling bacteria, we anticipate that pressure-mediated injury in *E. coli* and *L. plantarum* resulting in loss of acid tolerance (Garcia Graells et al., 1998, Linton et al., 1999, Wouters et al., 1998) is governed by comparable mechanisms.

Conclusion

Our investigations on the effect of ethanol and hop extract on inactivation and killing of *L. plantarum* in a food model system during and after pressure treatment revealed that ethanol is effective mainly during pressure treatment whereas hop extract acts mainly during storage of pressure treated cells. Sublethal cell damage corresponding to the ability of *L. plantarum* to survive under conditions of cold storage at acidic conditions could be demonstrated by the use of selective media. These data are in accordance with the hypothesis that HP inactivation must be considered as a two stage process involving initial sublethal damage. The cytoplasmic membrane and membrane-associated transport systems appear to be main targets for this first inactivation step. Cell death is a secondary event as a result of loss of resistance to adverse environmental conditions. Accordingly, antagonists present in food may interact differently on primary and secondary pressure inactivation, i.e. compounds may be inert during pressurization yet exert a much stronger activity on pressurized cells compared to untreated organisms.

The observation that the ability of micro-organisms to survive in harsh environments is eliminated by high pressure suggests that different strategies must be employed for high pressure treatment of food depending on the aim of the treatment. Inhibition of growth of spoilage required for prolonged shelf life required solely a pressure treatment sufficient for sublethal injury of major spoilage bacteria. For example, in order to eliminate the beer spoiling capability of *L. plantarum* TMW1.460 used on our work, a pressure treatment of less than 5 min at 300 MPa was fully sufficient, whereas more than 3h pressurization were required for sterilization of model beer. Such prolonged pressure treatments are, however, required to ensure safe elimination of pathogenic microorganisms. Advances in the knowledge of mechanisms governing HP inactivation of micro-organisms are anticipated to

further the understanding of HP interactions with the food matrix, and to allow the establishment of mechanistic primary and secondary mathematical models for prediction of pressure death time data in food.

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Chapter III: Effect of compressed gases on the high pressure inactivation of *Lactobacillus plantarum* TMW 1.460

ABSTRACT

The efficiency of high pressure (HP)- treatment as preservation method in foods depends on environmental conditions. The presence of gases may affect the behaviour of micro organisms during HP-treatment. These additional inactivation effects were investigated in model beer (MB) at 200 MPa and 15°C. Oxygen (O_2), nitrogen (N_2) and carbon dioxide (CO_2) were dissolved by two different methods. The effect of CO_2 was additional tested at 12 MPa under conditions where a liquid CO_2 phase was present. Gases in dissolved state had no additional inactivation effect on *L. plantarum*. In contrast, the application of liquid CO_2 induced a fast inactivation depending on the ratio of the CO_2 - aqueous phase interface to the volume of the aqueous phase. A subcritical extraction of *L. plantarum* cells with CO_2 followed by GC-analysis of the extracts demonstrated that fatty acids in form of triglycerides or phospholipids were extracted off the cellular cytoplasmic membrane. The bactericidal effect of liquid and critical CO_2 thus appears to involve the extraction of membrane compounds.

INTRODUCTION

The application of high pressure (HP) in the range of 200 – 800 MPa as preservation method of foods requires insight into mechanisms of HP-mediated cell injury and death. Gases present during HP-treatment may affect microbial survival based on their general effects on Eh- and pH-values or specific effects. Oxygen dissolved during HP treatment may accelerate oxidation of membrane components and thus result in sublethal injury or cell death [1]. Supercritical and subcritical CO_2 in the range of 5 – 20.5 MPa is bactericidal [2, 3, 4]. Several mechanisms were suggested to account for this bactericidal effect of CO_2 [3, 4, 5]. (i) CO_2 may diffuse into the cell and dissipate the transmembrane pH-gradient. This pH-lowering effect should act synergistic with HP-treatment. (ii) Liquid CO_2 may act as a non-polar solvent and dissolve cell compounds, especially membrane phospholipids, and thus results in membrane perturbation and cell death. It was the aim of this study to determine whether dissolved gases affect HP-mediated inactivation of *L. plantarum*, a beer spoiling bacterium, at 200 MPa in the presence of different concentrations of N_2, O_2 and CO_2. To examine whether the bactericidal effect of CO_2 requires a liquid CO_2 phase, CO_2 was applied additionally at 12 MPa and 15°C. The effects on microbial inactivation as well as the ability to extract membrane compounds from *L. plantarum* were investigated.

MATERIALS AND METHODS

Effect of dissolved gases in the HP inactivation of L. plantarum. The HP-inactivation kinetics of *L. plantarum* were investigated in MB, a yeast fermented malt extract prepared according to previously published procedures [6]. The pH was adjusted to 4.0. Cells were grown at 30°C for 24h, harvested and resuspended in new MB. The HP-treatment was done at 15°C and 200 MPa. Compression and decompression rates were 200 MPa min^{-1}. The concentrations of N_2, O_2 and CO_2 dissolved in MB were adjusted by two methods. (i) Equilibration method: MB was equilibrated with a head space pressure of 0.4 MPa air, N_2 or CO_2 at 5°C for 3 d before pressure treatment. (ii) Cylinder-piston method: a gas filled head space of defined volume O_2, N_2 or CO_2 was pressed into MB during pressure treatment as shown in Fig. 1.

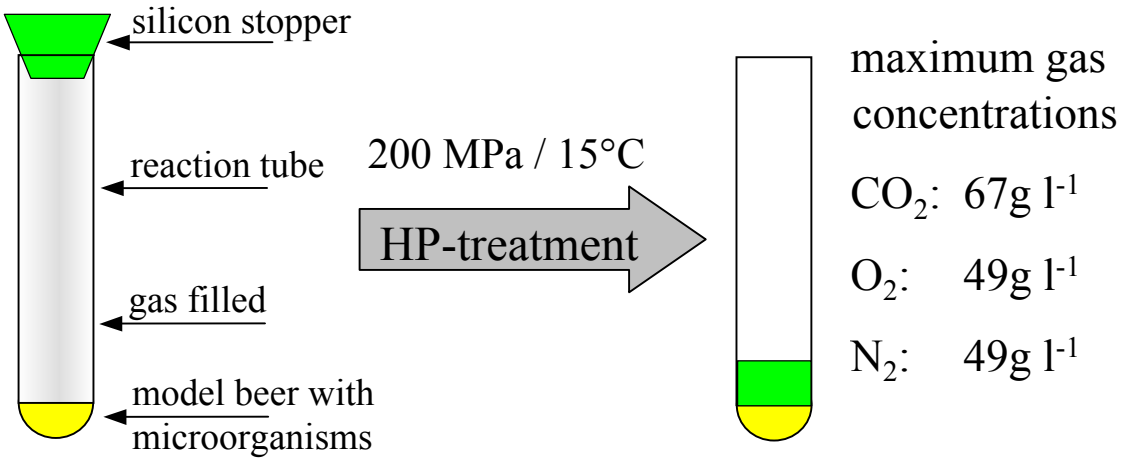

Figure 1. High pressure treatment with the cylinder-piston method

The maximum concentration of dissolved gases with these methods was (i) Equilibrium method, 0.048 g l^{-1} O_2, 0.082 g l^{-1} N_2, and 13.5 g $l^{-1}CO_2$, and (ii) cylinder-piston-method 49 g l^{-1} O_2, 49 g l^{-1} N_2, 67 g l^{-1} CO_2.

Bactericidal effect of liquid CO_2 on L. plantarum. The bactericidal effect of liquid CO_2 at 12 MPa and 15°C was determined with a system shown in Fig. 2. At these conditions, CO_2 remains fluid and a phase interphase between liquid CO_2 and MB is present. A portion of CO_2 is dissolved in MB and the CO_2 concentration is 69 g l^{-1} [7]. By opening the outlet valve of the gas cylinder CO_2 rises up in the pressure vessel until pressure is equilibrated to 12 MPa. Increasing the volume of MB in the reaction tube containing the sample leads to decreasing surface-volume ratios. The surface-volume ratio was varied in the range of 121 to 7770 m^{-1}.

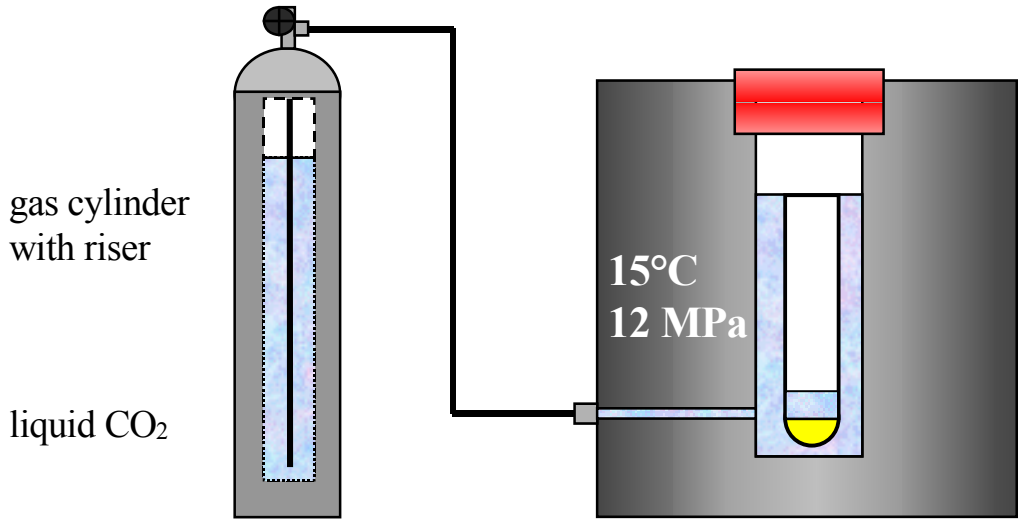

Figure 2. Apparatus used for HP-treatment of *L. plantarum* with liquid CO_2

Viable cell counts were determined by plating pressure treated samples on MRS agar, the degree of sublethal injury was estimated by plating on MRS + 4% NaCl. Membrane permeability to propidium iodide (PI) was determined by staining with the DeadLive® Kit (Molecular Probes, Eugene, USA) and reported as percentage membrane integrity as described previously [7].

Extraction of membrane fatty acids by liquid CO_2. The subcritical CO_2 extraction and GC-analysis of membrane compounds was executed by filling 633 mg of *L. plantarum* cells and 1 g of water in a thimble (extraction chamber: 79 mm x 11 I.D. mm). The thimble was kept at a

temperature of 4°C for 12 hours. After that, the extraction chamber was treated in an analytical SFE system (HP SFE Module 7680 T, HP Waldbronn) for 5 hours at 25 °C and 11.5 MPa with liquid CO_2. The extracted analytes were collected on an RP18 solid phase trap and eluted with 1 ml heptane. For the GC analysis of the extract on membrane compounds, triglycerides or phospholipids were transesterified to give the corresponding fatty acid ethyl esters (ee). This was done by refluxing 1 ml of the heptane fraction for 1 hour in 20 ml (H_2SO_4/Ethanol, 1/9, v/v) followed by three extractions with 20 ml heptane. Heptane was removed by distillation to result in a solution of 100 µl containing fatty acid ethyl esters. Analysis was performed on an DANI GC 6500 system, column: SUPELCOWAX TM 10 (30 m x 0,25 mm I.D., 0,15 µm), FID: 250 °C, injector: 250 °C, temperature program: 140 °C (1 min), 8 °C/min to 230 °C (15 min). Tricosanic acid ethyl ester (23:0) was used as internal standard. The membrane fatty acid composition of *L. plantarum* TMW 1.460 was determined by the DSMZ, Braunschweig, Germany.

RESULTS

Effect of dissolved gases in the HP inactivation of L. plantarum. The inactivation of *L. plantarum* in the presence of 0.048 g $l^{-1}O_2$, 0.082 g l^{-1} N_2, and 13.5 g l^{-1} CO_2 was determined by the equilibration method.

These gas concentrations had no effect on the inactivation kinetics at 200 MPa (data not shown). The inactivation of *L. plantarum* TMW 1.460 at 200 MPa in the presence of N_2, O_2, and CO_2 in concentrations of 49, 49, and 67 g l^{-1}, respectively, is shown in Fig. 3 (cylinder-piston method).

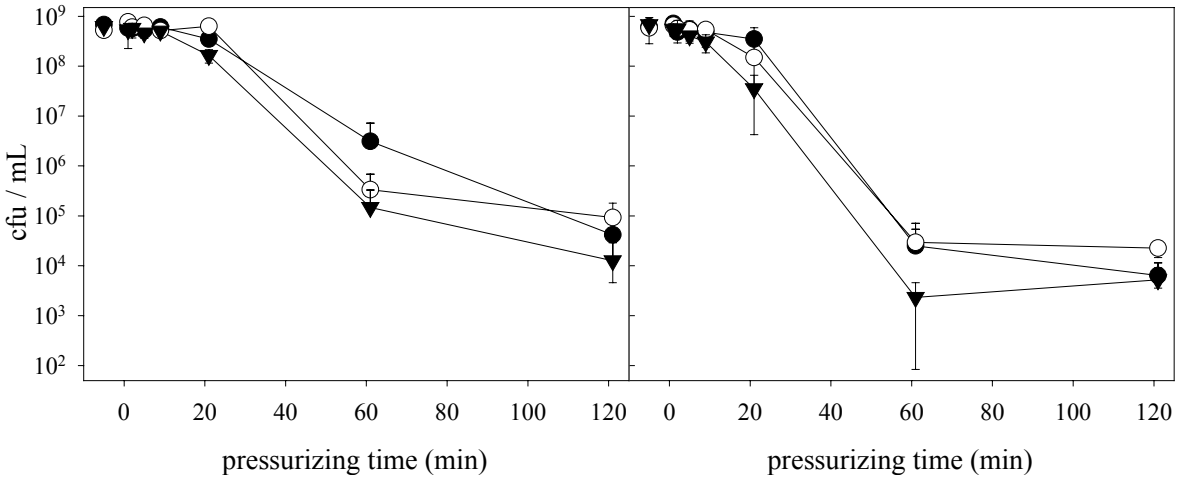

Figure 3. HP-inactivation kinetic of *L. plantarum* in presence of different gases at 200 MPa and 15°C by cylinder-piston-method. The gas concentrations were as follows: 49 gl^{-1} N_2 (❍), 49 gl^{-1} O_2 (●), and 67 gl^{-1} CO_2 (▼).

Plate counts on MRS and MRS + 4% NaCl showed the typical sigmoidal shape of microbial inactivation by HP-treatment. Curves exhibited pronounced shoulders and tailing. During the exponential inactivation phase, the cell counts on MRS + NaCl were lower by about two log cycles than the viable cell counts, indicating sublethal injury. Neither N_2, O_2 and CO_2 affected

HP-mediated cell death or sublethal injury of *L. plantarum*. The inactivation curves presented in Fig. 3 do not differ from previously published death-time data using air saturated MB but otherwise identical conditions [6].

The membrane integrity was determined with PI assay (Fig. 4). The cytoplasmic membranes were disrupted by HP-treatment; however, the loss of membrane integrity was observed only after cell death. The presence of dissolved N_2, O_2, or CO_2 had no additional detrimental effect on the membrane.

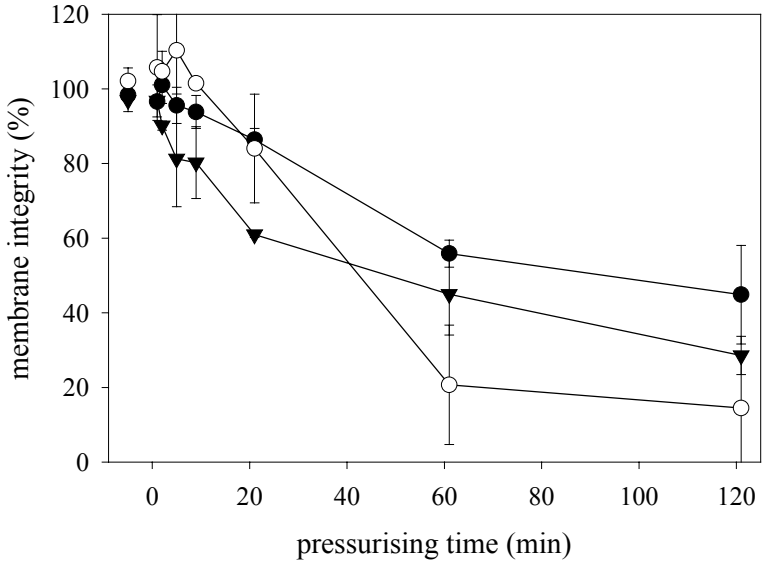

Figure 4. HP-destruction kinetic of *L. plantarum* membranes in presence of different gases at 200 MPa and 15°C by the cylinder-piston-method. The gas concentrations were as follows: 49 gl^{-1} N_2 (❍), 49 gl^{-1} O_2 (●), and 67 gl^{-1} CO_2 (▼).

Bactericidal effect of liquid CO_2 on L. plantarum. The experimental setup in these investigations ensured the presence of a liquid CO_2-phase during pressure treatment. Pressure and temperature – 12 MPa, 15°C – were chosen to obtain levels of dissolved CO_2 that are comparable to those obtained with the cylinder-piston method. In order to assess the

relevance of the CO_2 – MB phase interface, experiments were performed at different surface-volume ratios of MB (Fig. 5).

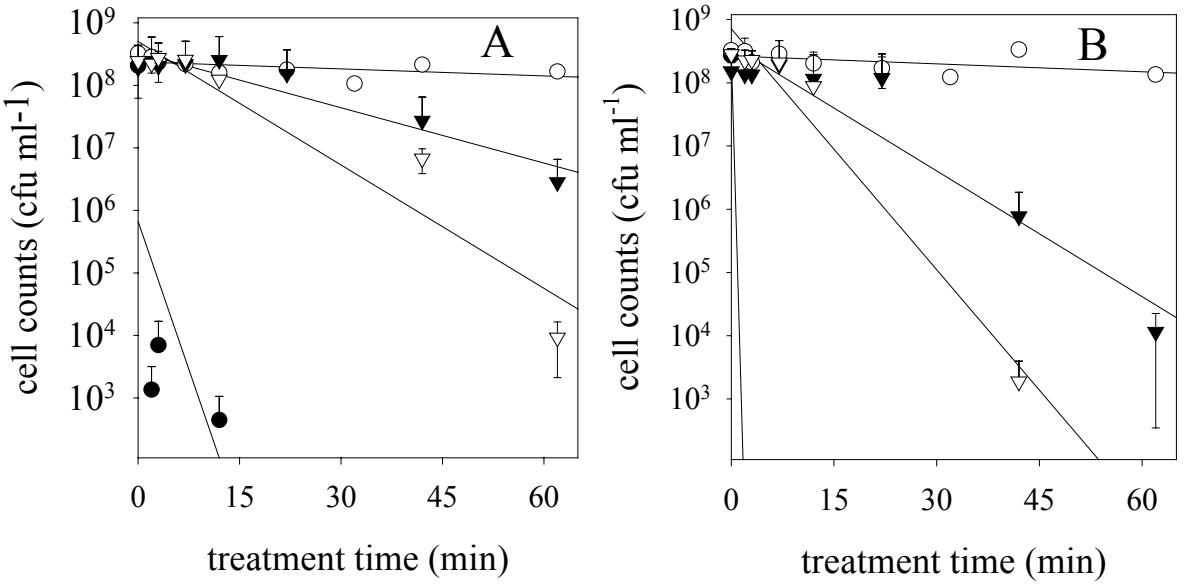

Fig. 5. Inactivation of *L. plantarum* in MB at 12 MPa, 15°C in the presence of liquid CO_2 at different surface-volume-ratios of MB. A: cell counts on MRS. B: Cell counts on MRS + NaCl. The surfaces to volume ratios were set to 121 m^{-1} (❍), 304 m^{-1} (▼), 1216 m^{-1} (▽) and 7800 m^{-1} (●). Lines represent results of log-linear regression.

In contrast to the experiments with dissolved CO_2, pressurization of *L. plantarum* with liquid CO_2 had a strong bactericidal effect. The death time curves were linear and did not exhibit a shoulder or tailing because of barotolerant cells, which argues in favour for different inactivation mechanism compared to HP-inactivation at 200 MPa in the presence of dissolved CO_2 only. The bactericidal effect strongly depended on the surface to volume ratio. To visualize this relationship of bactericidal effect vs. the surface-volume ratio, the bactericidal effect was estimated by a first order kinetics (log-linear regression) and the rate constants are plotted against the surface-volume ratio in Fig. 6 (next page). The variation of the surface-volume ratio revealed that the inactivation rate of *L. plantarum* is proportional to this ratio

(r^2 = 0.996) and thus corresponds to the amount of cells exposed to liquid CO_2 at the phase interface.

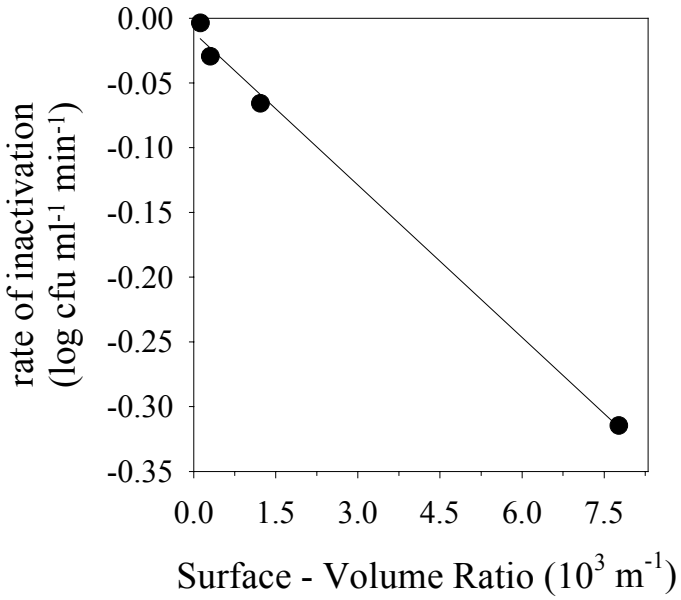

Fig. 6. Plot of the inactivation rate, estimated by log-linear regression of the death-time data on MRS against the surface – volume ratio.

Extraction of membrane fatty acids by liquid CO_2. To determine whether cellular compounds are extracted by treatment with liquid CO_2, cultures of *L. plantarum* were subjected to a continuous flow of liquid CO_2 at 25°C and 11.5 MPa. Fatty acids present in the extract as free fatty acids, phospholipids or triglycerides were quantified with GC (Fig. 7).

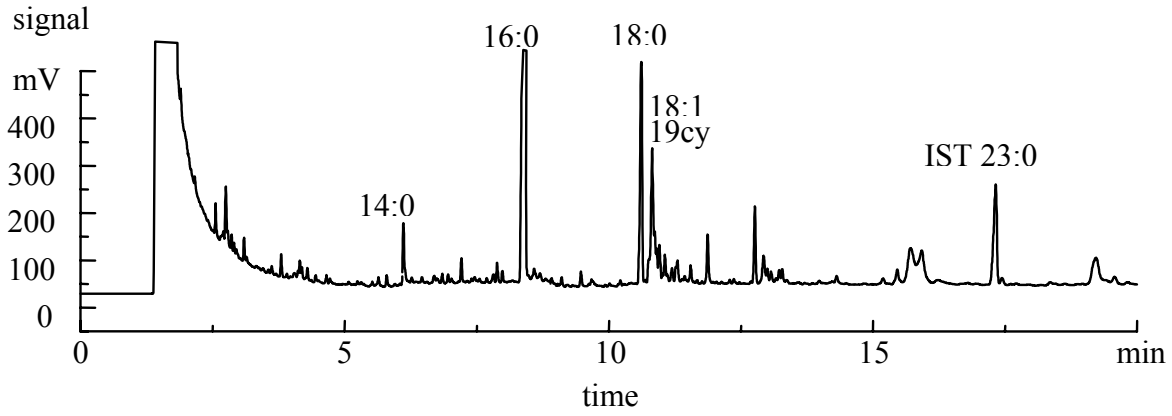

Figure 7. Chromatogram of cell extract (CO_2 extract obtained at 11.5 MPa) with 23:0 ethyl ester as internal standard.

This chromatogram was compared with membrane fatty acids obtained by a conventional extraction procedure (Fig. 8).

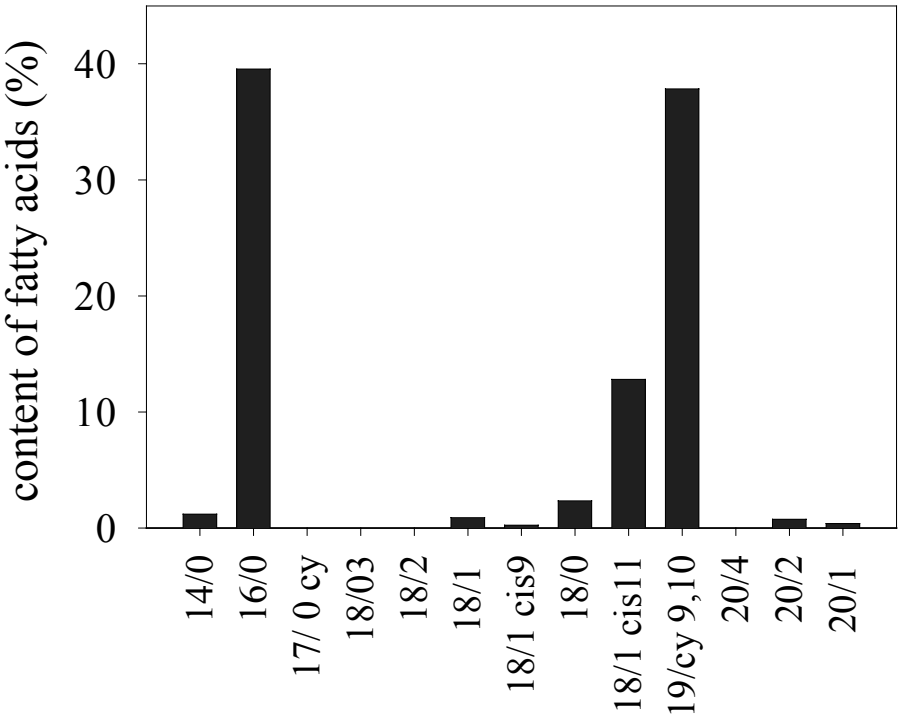

Figure 8. Percental portions of membrane fatty acid contents after conventional extraction method of *L. plantarum*.

The analytical chromatogram (Fig. 7) of the cell extract shows clearly that 14:0, 16:0, 18:0 and 18:1 fatty acids have been extracted. The fatty acid pattern obtained by subcritical CO_2 extraction of *L. plantarum* corresponds to the fatty acid pattern of the cytoplasmic membrane (Fig. 8). High amounts of 16:0 fatty acid were found in both cases, however, the peaks for the oleic acid (18/1 cis9) and lactobacillic acid (19/cy 9,10) ethyl esters were not resolved on our GC set up.

DISCUSSION

Oxygen, nitrogen and carbon dioxide dissolved in MB did not accelerate the pressure mediated inactivation and inactivation of *L. plantarum*. Even at the high concentrations reached with the cylinder-piston-method, these gases showed no additional inactivation or membrane disrupting effect.

The oxidation of membrane phospholipids was reported to contribute to cell death during drying of lactic starter cultures [1]. However, O_2 present during HP treatment did not oxidise membrane phospholipids in a way accelerating cell death.

In the presence of excess dissolved gases, gas bubbles formed during explosive decompression may mechanically disrupt cells. However, compression and decompressing rates of 200 MPa min^{-1} as used in our work maintained the system in thermo dynamical equilibrium, allowing dissolved gases to diffuse out off the cells before gas bubbles burst the cells.

Dissolved CO_2 may act bactericidal by its pH-lowering effect. At pH-values around the first pK_a of CO_2 (6.35) inside the cell, it lowers the intracellular pH by about 0.3 pH units [8]. It was furthermore suggested that carbonic acid penetrates membranes and dissipates transmembrane pH gradients [4, 9]. In our work, we did not observe a synergistic effect of dissolved CO_2 with HP inactivation of *L. plantarum*. The inactivation kinetics with HP-treatment and gases did not differ from previous published data without CO_2 [6]. It must be noticed that the pH of MB used in our work (pH 4.0) is well removed from the pK_a of carbonic acid, thus excluding an effect on the medium pH.

The results on the effect of liquid CO_2 conform to previously published data on bacterial inactivation by subcritical or supercritical CO_2 [9, 4, 5]. These authors used an experimental setup allowing for a phase interface of aqueous suspension of micro organisms and liquid or supercritical CO_2 as it was used in this work. A proportional relationship of the inactivation rate of *L. plantarum* to the surface-volume ratio was found. CO_2 thus appears to inactivate *L. plantarum* mainly at the interface of aqueous phase to liquid CO_2 phase. This suggests a cell inactivation mechanism involving membrane disturbance by liquid CO_2 rather than a bactericidal effect of dissolved CO_2. The subcritical CO_2 extraction of *L. plantarum* demonstrated that fatty acid esters can be extracted from the culture. Fatty acids extracted with CO_2 included mainly those fatty acids predominating in the membrane phospholipids of *L plantarum*. It can thus be concluded that membrane phospholipids are extracted off the membrane causing cell death. Based on the amount of free fatty acids in the extracts, it can be estimated that about 10% of the membrane fatty acids have been extracted by the liquid CO_2. This organism is readily inactivated by the application of liquid CO_2, however, the applicability of this preservation method in food technology is limited because it may result in co-extraction of components relevant for food quality.

Acknowledgements

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Chapter IV: Effects of pressure induced membrane phase transitions on inactivation of HorA, an ATP-dependent multidrug resistance transporter, in *Lactobacillus plantarum*

ABSTRACT

The effects of pressure on cultures of *L. plantarum* were characterized by determination of viability and activity of HorA, an ATP-binding cassette multidrug resistance transporter. Changes were determined in the membrane composition of *L. plantarum* induced by different growth temperatures. Furthermore, the effect of the growth temperature of a culture on the pressure inactivation at 200 MPa was determined. Cells were characterised after pressure treatment by plate counts on selective and non-selective agar, and HorA activity was measured by ethidium bromide efflux. FT-IR and Laurdan fluorescence spectroscopy provided information about the thermodynamic phase state of the cytoplasmic membrane during pressure treatment. A pressure-temperature-diagram for membranes of cells was established. Cells grown at 37°C and pressure treated at 15°C lost greater than 99% of HorA activity and viable cell counts within 36 and 120 min, respectively. The membrane of these cells was in the gel phase region at ambient pressure. In contrast, cells grown at 15°C and pressure treated at 37°C lost greater 99% of HorA activity and viable cell counts within 4 and 8 min, respectively. The membrane of these cells was in the liquid-crystalline phase region at ambient pressure. The kinetic analysis of inactivation of *L. plantarum* provided further evidence that inactivation of HorA is a crucial step during pressure induced cell death. Comparison of the biological findings and the membrane state during pressure treatment led to the conclusion that the inactivation of cells and membrane enzymes strongly depends on the thermodynamic properties of the membrane. Pressure treatment of cells with a liquid-crystalline membrane at 0.1 MPa resulted in a more rapid HorA inactivation and cell death compared to cells with a gel phase membrane at 0.1 MPa.

INTRODUCTION

High hydrostatic pressure is used in food applications to attenuate and inactivate microorganisms. The survival of microorganisms during high pressure treatment is strongly dependent on the environmental conditions, such as temperature, pH, and the presence of antimicrobial compounds. Therefore, the knowledge of the mechanisms of pressure induced cell death is paramount for the deliberate design of high pressure processes in food technology (45, 20, 30, 18).

The mechanisms of pressure induced cell death are currently under investigations. Transient permeabilisation of the outer and inner membranes of *Escherichia coli* was observed by pressure treatment in the presence of nisin, propidium iodide and N-phenylnaphtylamine, respectively (44, 1, 17). Furthermore, lethal pressure treatment resulted in the loss of proteins from the outer membrane of *Salmonella typhimurium* (39). Sublethal pressure treatment of *Saccharomycopsis fibuligera* lead to loss of glycerol and ions through permeabilized membranes (37). The loss of acid resistance of *E. coli* upon sublethal pressure treatment suggested the inactivation of protective or repair functions relevant for pH homeostasis (32, 25). Factors involved in the loss of pH homeostasis of pressure treated *L. plantarum* were investigated by Wouters et al. (51). Sublethal pressure treatment of *L. plantarum* resulted in a loss of the cells to maintain the internal pH, decreased acid effflux, and inactivation of the F_0F_1ATP-ase, whereas the ability to generate ATP remained unaffected. Baroresistance of the same strain was furthermore reported to relate to the phospholipid head group composition (44). Denaturation of ribosomes *in vivo* correlated to pressure mediated cell death of *Eschericia coli*. The authors suggested, that ribosome denaturation was caused by leakage of Mg^{2+} from pressure permeabilized membranes (31). Taken together, these results suggest that damage of membranes or membrane bound transport systems are an important event in high

pressure mediated cell death.

Activity and inactivation of membrane bound enzymes depend on changes in either protein structure or membrane fluidity, or both (14, 7). Lipids in biological membranes are in the fluid (liquid-crystalline) phase allowing fast lateral movement of molecules. Pressure upshift and-/-or temperature downshift in pure one-component phospholipid bilayers induces a phase transition from the liquid-crystalline phase to a gel phase, characterized by an increased rigidity and reduced conformational degrees of freedom for the acyl chains. In natural membranes with a complex lipid composition, a more or less wide coexistence region of gel and liquid-crystalline phases is observed at the phase transition. Studies on the pressure induced denaturation of proteins in aqueous solution have led to the conclusion that pressure compresses cavities of proteins (22, 23). Furthermore, pressure induced ionization of amino side chains, and hydration of apolar amino acid side chains and concomitant loss of hydrophobic interactios destabilize the secondary structure of proteins, leading to protein denaturation (21, 29). As a general rule, oligomeric proteins are dissociated at relatively low pressure (< 200 MPa) whereas the irreversible denaturation of enzymes and proteins in aqueous solution requires pressures higher than 300 MPa (22, 29). Few data are available on pressure induced structural changes of proteins dissolved in membranes (50).

HorA is an ATP-dependent multi-drug-resistance (MDR) transporter of the ABC family conferring hop resistance to beer spoilage bacteria (41, 48). The observation that the fluorescent dye ethidium bromide is a substrate for HorA allows a straightforward determination of HorA activity. HorA of *Lactobacillus plantarum* is therefore a suitable model system to investigate the effect of chemical and physical membrane properties on the pressure-mediated inactivation of membrane bound enzymes, and cell death. High pressure inactivation of *L. plantarum* was previously found to involve HorA inactivation (46) and loss

of HorA activity was correlated to the failure of pressure treated cells to survive in the presence of hop bitter acids (16). HorA is homologous to mammalian MDR transporters and is a structural and functional homologue to the MDR transporter LmrA of *Lactococcus lactis* (40). The amino acid sequences of LmrA and HorA are 52% identical. LmrA possesses six hydrophobic transmembrane spanning α-helical segments (5, 48, 6). The enzyme binds amphiphilic substrates in the inner leaflet of the membrane and catalyses translocation to the outer leaflet of the bilayer (28, 47). The catalytic cycle of LmrA involves major rearrangements of the secondary structure induced by ATP binding and hydrolysis (49).

It was the objective of this work to determine membrane triggered mechanisms resulting in pressure mediated cell death and HorA inactivation of *L. plantarum*. Cultures were prepared by variation of the incubation temperature to achieve cells differing in the composition of the cytoplasmic membrane. Temperature and pressure induced phase transitions of cytoplasmic membrane were determined by FT-IR and Laurdan fluorescence spectroscopy, respectively. The consequences of changes in membrane properties for the resistance of cells to pressure as well as pressure induced HorA inactivation were evaluated.

MATERIALS AND METHODS

Strains and culture conditions. *Lactobacillus plantarum* TMW 1.460, an organism previously isolated from spoiled beer, was cultivated using model beer (MB). Where indicated, model beer containing 5% ethanol (MB+5%) was used. MB was prepared by fermentation of malt extract medium with *Saccharomyces cerevisiae* TMW 3.001 as previously described (46). *L. plantarum* was cultured in MB for 24 h and subcultured at 15°C, 30°C and 37°C for 64, 24 and 20 h, respectively, to late stationary growth phase. Growth time in MB+5% at 30°C was 36 hours.

High pressure treatment. An overnight culture of *L. plantarum* in MB was subcultured with 1% inoculum. Late stationary cells were harvested by centrifugation and resuspended in an equal volume of MB. This cell suspension was transferred to 2 ml Eppendorf reaction tubes, sealed with silicon stoppers, and stored on ice until pressurization. The high pressure (HP) inactivation kinetics of *L. plantarum* were investigated in HP-autoclaves precooled to 15°C or 37°C. Compression and decompression rates were 200 MPa min^{-1}. Upon pressurization, samples were stored on ice until further analysis. For each HP inactivation kinetics, untreated cultures and cultures sterilized by treatment with 800 MPa for 10 min were used for comparison. The samples' nomenclature was chosen with respect to the growth temperature (T_G) and the temperature of high pressure treatment (T_P). For example, a culture grown at 15°C and pressure treated at 37°C is labelled T_G15/T_P37.

Determination of plate counts. Cell counts were determined on MRS-agar (13) or MRS-agar containing 4% NaCl (MRS-NaCl) agar for determination of viable and sublethally injured cells. Appropriate dilutions were plated using a spiral plater (IUL, Königswinter, Germany) and plates were incubated at 30°C for 2 days under controlled atmosphere (76% N_2, 20%

CO_2, 4 % O_2). Cell counts of cultures of *L. plantarum* in MB were 4 ± 2 x 10^8 CFU ml^{-1} on either MRS or MRS-NaCl agar (mean of 20 determinations). The cell counts on MRS are referred to as viable cells and the difference between cell counts on MRS and MRS-NaCl is referred to as sublethally injured cells.

Determination of HorA activity. HorA-activity was determined by using ethidum bromide (EB) as substrate according to Ulmer et al., 2000 (46). EB stock solutions were prepared by solving 40 µmol l^{-1} EB in phosphate buffer (PB; 50 mM H_2KPO_4, 0.1 g l^{-1} MgSO4 x 7 H_2O, and 0.05 g l^{-1} $MnSO_4$ x H_2O, pH 6.5). After high pressure treatment the cells were harvested by centrifugation (10 min at 15°C, 6000 x g) and resuspended in PB. Each sample received EB stock solution to obtain a staining EB concentration of 20 µmol l^{-1}. Samples were mixed and incubated at 30°C for 90 min in the dark to load cells with EB in the absence of an energy source. After incubation cells were harvested and resuspended in PB. 200 µl of this cell suspension were transferred to black microtiterplates. To determine the EB efflux kinetics 2 µl of glucose stock solution (1 g ml^{-1} glucose) were added to the samples. Reenergized cells export EB resulting in a lower fluorescence of the EB-nucleotide complex. Fluorescence was measured in a spectrafluor microtiterplate reader (Tecan, Grödig, Austria) in 1 min intervals for 60 min at 30°C (λ_{Ex} = 485 nm, λ_{Em} = 595 nm). The initial rate of fluorescence reduction of every sample was calculated. EB efflux activity was normalized to the activity of untreated cells with each experiment. It was verified that *L. plantarum* carries a functional *horA* gene (46). EB transport in the presence of glucose was fully inhibited by 50µM reserpine, an inhibitor of the HorA homologues human MDR1 and LmrA of *Lactococcus lactis* (48). This indicates that EB efflux in *L. plantarum* is mainly attributable to HorA activity.

Determination of membrane composition. Cells were inoculated and grown as described above in 50 ml MB for different hours and at different temperatures to get samples T_G15,

T_G30, T_G37 and T_G30+5%. After lyophilisation for 24 h the samples were packed under N_2 atmosphere and sent to DSMZ (Braunschweig, Germany). Membranes fatty acids were extracted, transesterified and analysed by GC.

Determination of temperature dependent membrane phase state by FT-IR spectroscopy. In order to avoid overlapping of acyl chain vibrational bands with IR absorption of H_2O, the FT-IR measurements were performed in D_2O as solvent. 1 ml cell suspension of samples T_G15, T_G30, T_G37 and T_G30+5% were washed and incubated in 1 ml D_2O for 1 h at 15°C to exchange remaining H_2O for D_2O. After incubation, cells were harvested and resuspended in 100 µl D_2O. 35 µl of cell suspension were filled into a 25 µm thick infrared cell with CaF_2 windows. The FT-IR spectra were recorded with a MAGNA 550 (ThermoNicolet, Madison, USA) spectrometer equipped with a liquid nitrogen cooled MCT (HgCdTe)-detector. Each spectrum was obtained by co-adding 256 scans at a spectral resolution of 2 cm^{-1} and was apodized with a Happ-Genzel function. The sample chamber was purged with dry and carbon dioxide free air. The infrared cell was thermo-stated by an external water thermostat. To achieve thermal equilibrium conditions in the CaF_2 cell 20 min elapsed between two measurements. Data were collected at temperatures – 4°C to 45°C in steps of 2°C. Each sample was measured twice in two independent experiments.

The internal vibrational modes of the lipid acyl chains are assigned on the basis of the well-known studies on polymethylenes and polymethylene-chain compounds (43, 38). In the 2,800 – 3,100 cm^{-1} region there are infrared absorption bands due to symmetric and antisymmetric modes of the methylene chain, at about 2,850 and 2,920 cm^{-1}, respectively. The wavenumbers of these bands are conformation-sensitive and thus respond to temperature- and pressure induced changes of the trans/gauche ratio in acyl chains. The vibrational mode (antisymmetric stretch) of the terminal CH_3 group occurs at about 2,960 cm^{-1}.

Determination of the pressure-dependent phase state of the membrane by fluorescence spectroscopy. To study the polarity of the lipid interface and to detect phase changes of the lipid bilayer membrane, Laurdan fluorescence spectroscopy was used. Laurdan is an amphiphilic fluorescence probe, which allows the determination of gel to fluid phase transitions in biological membranes (33, 34, 36). The incubations of cells were identical to those applied for FT-IR analyses. After incubation, 1 ml cell suspension of samples T_G15, T_G30, T_G30+5% and T_G37 were washed and incubated in 1 ml D_2O for 1 h at 15°C to exchange remaining H_2O for D_2O. After incubation cells were harvested and resuspended in 980 µl D_2O. After addition of 20 µl 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) (Molecular Probes, Eugene, USA) stock solution in ethanol (2 mmol l^{-1}) an effective staining concentration of 40 µmol l^{-1} was reached. Cells were stained for 30 min at 30°C in the dark. To measure the fluorescence spectra, cells were harvested, resuspended in 1 ml D_2O and filled into a glass vial. The vial was inserted into a high pressure vessel with quartz windows. The vessel was connected to a high pressure pump. Laurdan emission spectra were obtained using the multifrequency phase and modulation fluorometer K2 (ISS Inc., Champaign, IL). The fluorometer uses a xenon arc lamp as light source. The monochromator bandpass was 8 nm. The spectra were only corrected for lamp intensity variation. The excitation wavelength was 360 nm, emission spectra were collected from 380 nm to 550 nm with steps of 1 nm. The steady state fluorescence parameter known as generalized polarization (GP) was calculated as GP = [I(440 nm)–I(490 nm)]/[I(440 nm)+I(490 nm)], where I is the relative fluorescence intensity at the respective wavelengths (35). Temperature was controlled to ± 0,1 °C by a water circulating bath. For the high pressure fluorescence studies, the ISS HP cell with quartz windows was used. Pressure was controlled with the APP Automated Pressure Control System. Pressure steps were 20 MPa with a ramp of 10 MPa min^{-1}. The time left for equilibration after each pressure step was 10 min.

RESULTS

Membrane composition of *Lactobacillus plantarum* as a function of growth temperature. According to the theory of homeoviscous adaptation, cells change the composition of their membrane to achieve a state of optimal membrane functionality in response to non ambient conditions (8, 2, 4). *Lactobacilli* change membrane fatty acid composition to higher contents of unsaturated fatty acids if grown at low temperatures (44, 15). *Lactobacillus plantarum* was grown under various growth temperatures (T_G15, T_G30, T_G37, T_G30+5%) and the fatty acid composition of membranous phospholipids was determined. Figure 1 shows the most prominent fatty acids of *L. plantarum*.

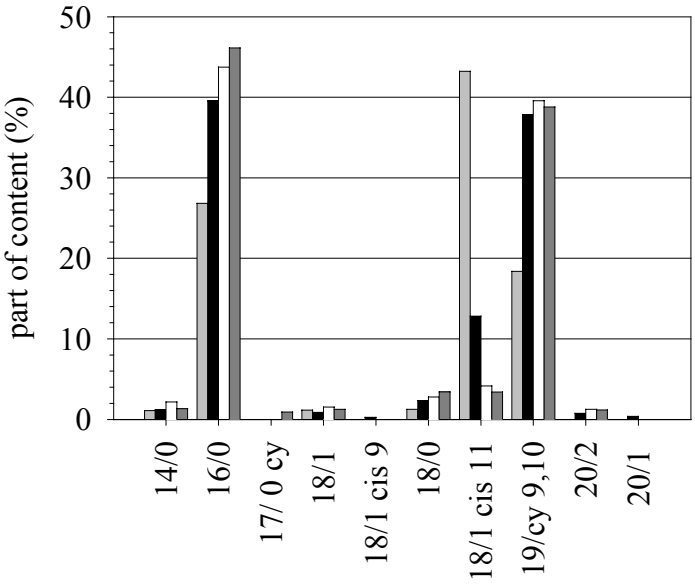

Figure 1. Fatty acid composition of *L. plantarum*'s cytoplasmic membranes in %. Cells were grown at different temperatures T_G (▒ = 15°C, ■ = 30°C, □ = 30°C + 5% ethanol, ▒ = 37°C). Fatty acids are designated by number of carbon atoms / number of double bonds.

Hexadecanoic acid (16/0) is the most important of the saturated fatty acids. *Cis*-11-octadecenoic acid (18/1 cis 11) and *cis*-11,12-methyleneoctadecanoic acid (19/cy 9,10) are the most important representatives of the unsaturated fatty acids. *cis*-11-Octadecenoic acid and *cis*-11,12-methyleneoctadecanoic acid haven an equivalent effect on membrane fluidity. The proportion of saturated fatty acids was increased with increasing growth temperature while the number of unsaturated fatty acids was decreased. The ratio of unsaturated to saturated fatty acids changed between 15°C to 37°C from 70% : 30% to 51% : 49%. Harvesting temperature adapted microorganisms for high pressure experiments thus provides microorganisms with different membrane composition.

Membrane phase state at different temperatures measured by FT-IR spectroscopy. Using FT-IR spectroscopy the effect of altered membrane composition on conformational and thermodynamic membrane properties was determined. In Figure 2 (next page), the peak-wavenumber of the CH_2 stretching vibrations of the membrane lipids is shown. Wavenumbers around 2,850.5 cm^{-1} represent a relatively rigid molecular packing of the fatty acyl chains, and the peak is shifted to higher wavenumbers if the chains are "melting" and a high conformational disorder is reached at high temperatures in the liquid-crystalline phase. The FT-IR melting curves were fitted to logistic function $y = y_0 + \frac{A}{1 + e^{\frac{-(T - T_m)}{b}}}$ where y_0 and A denote the lower and upper asymptotes, respectively, and b is a shape coefficient. The melting temperature of the membranes was defined as the turning point of the melting curve. The all liquid-crystalline or gel phase regions are characterised by a nearly horizontal slope. Figure 2 shows that the methylen stretching vibrations of samples T_G15, T_G30, T_G37 occur at about 2,850.5 cm^{-1} at –4°C. The corresponding value for sample T_G30+5% was 2,849.5 cm^{-1}, pointing to a higher membrane rigidity at that temperature.

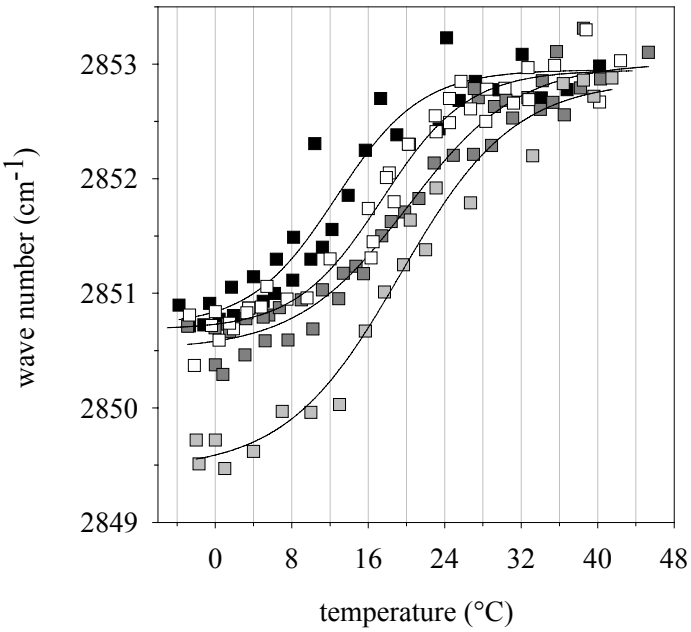

Figure 2. Peak maxima of FT-IR spectroscopy measurements of *L. plantarum* cells grown at different temperatures T_G (■ = 15°C, □ = 30°C, ■ = 30°C + 5% ethanol, ■ = 37°C). Shown are the wavenumbers of two independent experiments. Every experiment was done by heating the cells from –4°C to 45°C and cooling from 45°C to –4°C. The curves represent the best fit of the logistic function ($r^2 = 0.98$)

The samples T_G15, T_G30, T_G37 and $T_G30+5\%$ exhibit mean chain melting temperatures of 12.6, 17.4, 20.1 and 19.3°C, respectively. A decreased incubation temperature thus induced a change in chain melting behaviour which can be detected by FT-IR spectroscopy. Increasing amounts of unsaturated fatty acids shift the phase transition towards lower temperatures. However, the lowering of the melting points of the membranes is not proportional to the growth temperature changes. For example, a downshift of growth temperature by 15°C from 30°C to 15°C induced a reduction of the membrane melting point by 5°C only.

Membrane phase state at different temperatures and pressures as measured by Laurdan fluorescence spectroscopy. The FT-IR spectroscopy data provided information about the temperature-induced membrane phase transitions at ambient pressure. The pressure-dependent membrane phase behaviour was determined by Laurdan fluorescence spectroscopy.

The Laurdan fluorescence spectra depend on changes in environmental polarity induced by membrane phase transitions (34, 3). The determination of the pressure dependence of the GP values of cells grown (T_G) and high pressure treated (T_P) at different temperatures are shown in Figure 3.

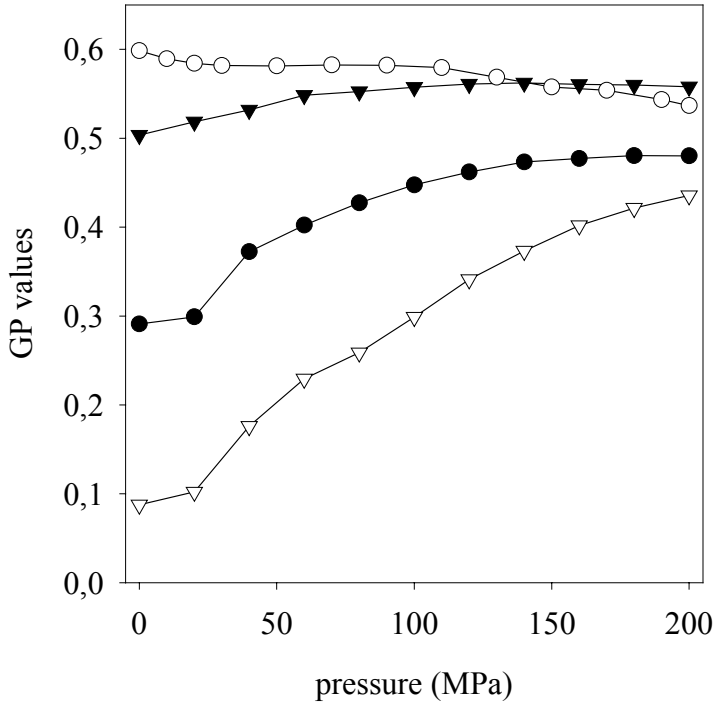

Figure 3. GP values of *L. plantarum* cells stained with Laurdan under pressure conditions. The cells were grown at temperature T_G and were high pressure treated at temperature T_P. (● T_G37/T_P37, ○ T_G37/T_P15, ▼ T_G30/T_P15, ▽ T_G15/T_P37,). Data from samples T_G15/T_P15 and T_G30+5% ethanol /T_P15 were equivalent to those obtained with samples T_G30/T_P15 (data not shown).

Data from samples T_G15/T_P15 and T_G30+5% ethanol /T_P15 were equivalent to those obtained with samples T_G30/T_P15 (data not shown). With increasing pressure, the polarity around the fluorophor in fluid membranes decreases resulting in higher GP values. The change can be interpreted as pressure induced phase transition from liquid-crystalline to coexistence and gel phase regions. T_G15/T_P37 showed the greatest increase of GP with pressure. These cells possess a high content of unsaturated fatty acids. An increase of pressure at 37°C induces a

marked phase transition indicated by a shifting in GP values from 0.09 to 0.42. T_G37/T_P37 showed a less pronounced phase transition with increasing pressure. On the contrary, the samples T_G30/T_P15, $T_G30+5\%/T_P15$ and T_G15/T_P15 exhibited no significant changes of phase state with pressure. Their membranes stay essentially in a rigid, gel-like type of state. The influence of ethanol during growth on membrane phase behaviour with high pressure is negligible. The membrane in T_G37/T_P15 experiments was fully converted to a gel phase by cooling from 37°C to 15°C and an additional change by pressure was not detectable.

Pressure inactivation of *L. plantarum*. To evaluate the influence of altered chemical and physical properties on cytoplasmic membranes, cells were high pressure treated (200 MPa) and the kinetics of inactivation were determined by plate counts on selective and non-selective agar. Figure 4 shows the cell counts on MRS-agar which were high pressure treated at 15°C and 37°C.

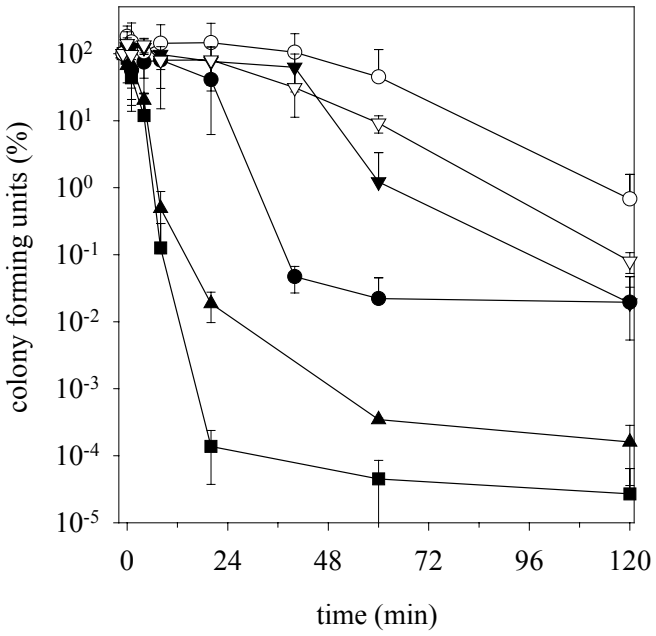

Figure 4. High pressure inactivation kinetics of *L. plantarum* at 200 MPa. The cells were grown at temperatures T_G and were pressurized at temperatures T_P. Shown are the means and standard deviations of viable cell counts of two experiments plated on MRS relative to initial cell counts in percent (● T_G15/T_P15, ○ $T_G30+5\%$ethanol$/T_P15$, ▼ T_G30/T_P15, ▽ T_G37/T_P15, ▲ T_G37/T_P37, ■ T_G15/T_P37).

The sample T_G15/T_P37 showed the fastest inactivation with more than 6 log cycles after 30 min. This speed of inactivation is equivalent to previous investigations on T_G30/T_P15 cells at 400 MPa (46). The inactivation velocity decreased in the order $T_G15/T_P37 > T_G37/T_P37 > T_G15/T_P15 > T_G30/T_P15$, T_G37/T_P15 and $T_G30+5\%/T_P15$. The latter cells showed no inactivation after 45 min. The incubation with 5% ethanol during growth apparently provided a baroprotection. The results on selective plates (MRS-NaCl) showed the same sequence of inactivation. In general, the rate of inactivation is three fold faster (Figure 5).

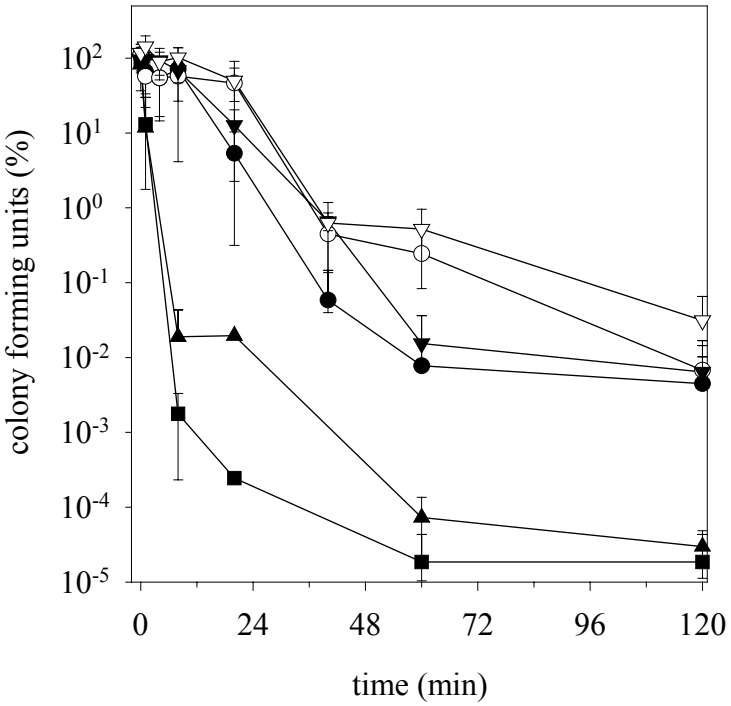

Figure 5. High pressure inactivation kinetics of *L. plantarum* at 200 MPa. The cells were grown at temperatures T_G and were pressurized at temperatures T_P. Shown are the means and standard deviations of viable cell counts of two experiments plated on MRS + 4% NaCl relative to initial cell counts in percent (● T_G15/T_P15, ○ T_G30+5%ethanol/T_P15, ▼ T_G30/T_P15, ▽ T_G37/T_P15, ▲ T_G37/T_P37, ■ T_G15/T_P37).

Pressure inactivation of HorA. In addition to plate counts high pressure treated cultures were also characterized with respect to HorA activity. The results of the fluorimetric assay for HorA activity are shown in Figure 6. The EB transport activity of the samples T_G15/T_P15,

$T_G30+5\%/T_P15$, T_G30/T_P15 and T_G37/T_P15 was more pressure resistant than in T_G15/T_P37 and T_G37/T_P37.

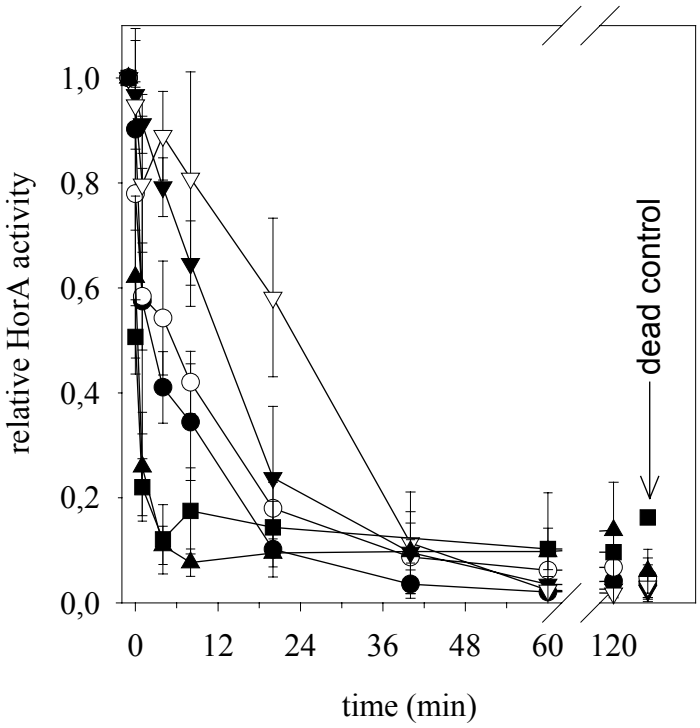

Figure 6. High pressure inactivation kinetics of HorA at 200 MPa. The cells were grown at temperatures T_G and were pressurized at temperatures T_P. HorA activity was expressed relative to untreated cells. Shown are the means and standard deviations of three independent experiments (● T_G15/T_P15, ○ $T_G30+5\%$ethanol$/T_P15$, ▼ T_G30/T_P15, ▽ T_G37/T_P15, ▲ T_G37/T_P37, ■ T_G15/T_P37). Control dead cells were prepared by pressure treatment at 800 MPa for 10 min.

After the first pressure application, the relative activity of the samples T_G15/T_P37 and T_G37/T_P37 decreases from 1.0 to 0.5 or 0.6, respectively. The HorA activity then decreased rapidly and after 7 min of pressure treatment reached the level of control dead cells (treated at 800 MPa for 10 min). The inactivation of HorA in samples T_G15/T_P15, $T_G30+5\%/T_P15$, T_G30/T_P15 and T_G37/T_P15 is slower and reaches the level of control cells between 36 and 60 min. No significant differences in HorA inactivation between samples $T_G30+5\%/T_P15$, T_G15/T_P15 and T_G30/T_P15 are detected. Although the $T_G30+5\%/T_P15$ membrane fatty acids composition is similar to that of cells grown at 37°C, their HorA pressure resistance is not

increased. The most pressure resistant sample was T_G37/T_P15, characterized by high level of saturated fatty acids in its membrane. The inactivation of HorA is in general about three times faster than plate counts on MRS. A linear plot of HorA activity versus plate counts on MRS-NaCl shows that these data sets are highly correlated (r^2 of 0.91). For example, after 40 min treatment of the sample T_G30/T_P15, HorA activity, MRS-NaCl and MRS cell counts were reduced by 80, 87, and 20%, respectively. Arranging the different samples in the order of decreasing speed of HorA inactivation shows the same pressure resistance of cells as it was observed with viable cell counts and sublethal injury. However, $T_G30+5\%/T_P15$ cells were about as resistant as T_G37/T_P15 cells considering viable cell counts and sublethal injury, but were not different from T_G15/T_P15 cells with respect to HorA inactivation.

DISCUSSION

Cells of *L. plantarum* differing in physical and chemical membrane properties, and membrane phase state were high pressure treated. It could be demonstrated that membrane properties affect the rate of inactivation of viable cells as well as the inactivation of the membrane bound ethidium transport activity. The kinetic analysis of cell physiology during high pressure treatment provided further evidence that inactivation of MDR transport and possibly other membrane bound transport enzymes is a crucial step during pressure mediated elimination of microorganisms. Furthermore, HorA inactivation was shown to correlate to sublethal injury in *L. plantarum*.

In this study, energy dependent ethidium efflux in whole cells of *L. plantarum* was determined and the results were expressed as HorA activity based on the observation that a funcitonal HorA is required for hop and ethidium resistance in beer spoiling lactobacilli (41, 42), and the finding that reserpine, an inhibitor of human MDR1 transporter and homologues, fully inhibited ethidium transport in *L. plantarum* TMW1.460. In *Lactococcus lactis*, 4 different drug efflux systems have been characterized (52), and two of these transporters, ATP dependend LmrA and proton motive force dependend LmrP, mediate ethidium efflux. Therefore, it is possible that a minor contribution to ethidium efflux in energized *L. plantarum* stems from transport systems other than HorA.

An intact cytoplasmic membrane as well as a source of ATP are a prerequisite for ATP-dependend, MDR-transporter mediated ethidium efflux. Generally, pressure treatment of *L. plantarum* could either affect membrane integrity, metabolic activity and hence energy generation, and HorA activity. It was previously shown that the glycolytic activity as well as the membrane integrity of *L. plantarum* TMW 1.460 were not irreversibly affected by

pressure treatment as long as cells were viable (46). This observation conforms with results of Wouters et al. (51) that ATP generation in pressure treated *L. plantarum* is not impaired as long as cells remain viable. Therefore, a decreased ethidium efflux activity in in pressure treated *L. plantarum* is not caused by lack of ATP, or a compromised membrane, but is attributable to inactivation of HorA transporter.

In this study, pressure mediated sublethal injury of *L. plantarum* was determined by plating on MRS-NaCl agar. HorA inactivation correlated to plate counts on MRS-NaCl rather than viable cell counts. This finding provides a rationale for the observation that pressure treated, sublethally injured cells of *L. plantarum* TMW1.460 specifically lost the ability to survive in hopped beer (16). Resistance of *L. plantarum* to 4% NaCl requires transport enzymes to balance osmotic pressure (19). Apparently, protective and repair functions required for growth under osmotic stress follow a comparable pressure inactivation kinetics as HorA.

The membrane of *L. plantarum* was altered in chemical composition by incubation at different temperatures without changing ethidium efflux activity. The physiological reaction to low temperature during growth is the incorporation of unsaturated acyl chains to maintain the fluidity of the membrane approximately constant and to ensure the functionality of membranes and membranes bound enzymes. Measurements using FT-IR spectroscopy provided information about the lipid bilayer phase transitions induced by temperature in *Lactobacillus plantarum*. At optimal growth conditions (30°C) the membrane of microorganisms is in the lamellar liquid-crystalline or L_α phase, where the phospholipid molecules are conformationally disordered. In natural membranes, there is a large compositional heterogeneity of constituent lipids. Hence, between a pure liquid-crystalline and gel state, a more or less broad coexistence region, where liquid-crystalline and gel lipid domains coexist, are present. For comparison, in one-component lipid bilayers, such as

dipalmitoylphosphatidylcholine (DPPC), several different gel states occur in the temperature-pressure phase space (12). By lowering the temperature, the lipid bilayer is changing from the L_α to the $P_{\beta'}$ gel phase, whose chains are stretched and of all-trans conformation, and the surface of the membrane is undulated. A further decrease of temperature leads to the formation of the to $L_{\beta'}$ gel phase, where the acyl chains are tilted with respect to the bilayer normal. Further pressure-induced gel phases, such as an interdigitated gel phase, are observed at high pressure (50). In *L. plantarum* membranes, no sharp liquid-crystalline to gel or gel-gel phase transition was detected. The Laurdan fluorescence data allowed to study also the pressure-induced phase behaviour of the different samples. The membrane fluidity determined by Laurdan at ambient pressure was in agreement with FT-IR data.

From these temperature and pressure dependent data, a tentative temperature-pressure phase diagram (p-T-diagram) can be constructed for the *L. plantarum* membranes (Figure 7).

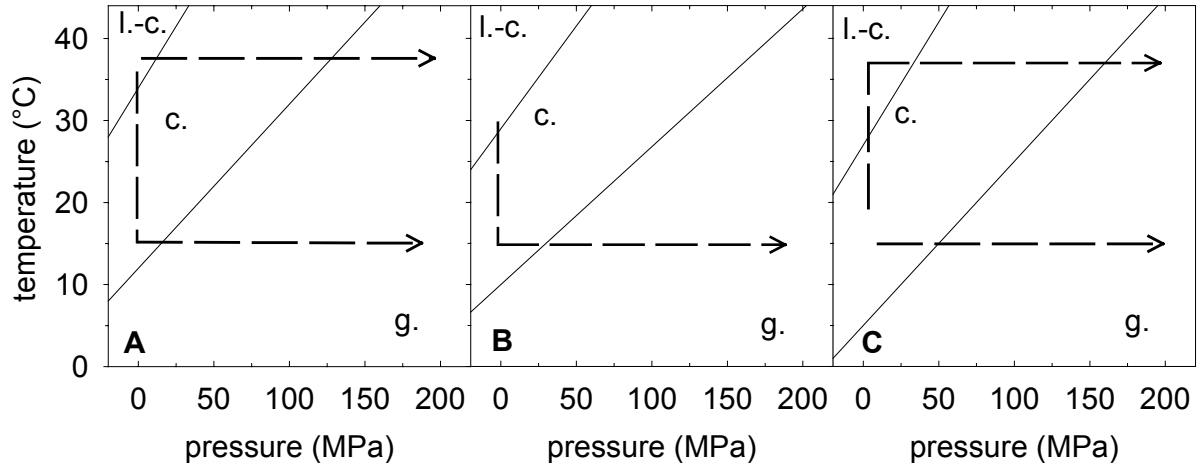

Figure 7. p-T-diagram of the cytoplasmic membrane of *L. plantarum* grown at 37°C (A), 30°C (B) and 15°C (C). The membrane phase state in the p-T-diagram is based on the data presented in Figures 2 and 3 (FT-IR measurements from -4 - 44°C at 0.1 MPa and determination of Laurdan fluorescence spectra from 0.1 to 200 MPa at 15 and 37°C). Abbreviations: l.-c.: liquid-crystalline phase; c.: coexistence phase; g.: gel phase. Lines represent phase transitions interpolated from the raw data. Dotted lines indicate temperature and pressure shifts during pressure treatment of cells with various growth temperature T_G and

pressureization temperature T_P. Cooling signifies an isobar (vertical) transit to gel region and pressurizing an isotherm (horizontal) transit to gel region in the p-T-diagram.

The temperature range of the coexistence phase are concluded from FT-IR data. The pressure induced phase transisions are concluded from the Laurdan spectra.. This p-T-diagram of cytoplasmic membranes of *L. plantarum* provides information about the membrane phase state before and during the high pressure treatment. Changes of temperature and pressure during processing of the samples can be interpreted as horizontal and vertical movements in the p-T-diagram, respectively. Cells pressurized at their growth temperature were at 0.1 MPa in the liquid-crystalline or coexistence phase region moving horizontally (isothermally) into gel phase region at 200 MPa. Cells, which are pressurized at temperatures differing from the growth temperature, experience by cooling or heating a vertical movement (isobar at 0.1 MPa) in the p-T-diagram before pressure application. All cell membranes are in the gel phase at 200 MPa.

The p-T-diagram of the various cultures can be correlated with the respective kinetics of high pressure inactivation. The phase transitions induced by variation of temperature were reversible and did not affect HorA activity or cell viability. Thus, the lipid environment does not seem to irreversibly influence HorA activity at ambient pressure over the whole temperature range covered. However, irreversible pressure denaturation of HorA varied in cells with different membrane properties. Those cells that were subjected to pressure at a temperature where their membrane is in the liquid-crystalline phase state and a gel phase of the membrane was induced only at pressures greater 100 MPa were most sensitive both with respect to HorA inactivation and cell death. Those cells that were subjected to pressure at a temperature where the membrane is in the gel phase region were most resistant with respect to HorA activity and cell viability. These results are consistent with the hypothesis that

irreversible pressure denaturation of HorA is faster if pressure is applied to protein embedded in a fluid-crystalline or coexistence membrane compared to denaturation in a gel phase membrane. Furthermore, it is conceivable that pressure induced membrane phases different from those observed at ambient pressure exert irreversible effects on HorA. Although few data are available on the effect of membrane properties on irreversible pressure denaturation of integral membrane proteins, it is well established that structure and function of these proteins strongly depend on the interactions with the lipid bilayers. The membrane phase is known to determine the activity of Na-K-ATPase activity at pressures ranging from 30 to 100 MPa (7, 26, 27). The refolding of bacteriorhodopsin in lipid bilayers in controlled by the physical pressure exerted by the membrane on the protein (11). The conformation of gramicidin, a low molecular weight peptide antibiotic, is modulated by phase transitions of the lipid matrix. Incorporation of the peptide lead to significant changes of the structure and pressure / temperature phase behaviour of the lipid bilayer system (53).

The observation that HorA inactivation of ethanol adapted cells was faster than that of 37°C adapted cells with comparable membrane properties highlights that factors other than membrane fatty acid composition and membrane fluidity affect pressure denaturation of HorA, for example the phospholipid headgroups, or chaperone proteins. The shock response of lactobacilli to lethal ethanol challenge involves the synthesis of ethanol shock proteins (24). However, the adaptation of lactobacilli to ethanol concentrations permitting growth did not involve protein synthesis but required adjustments of membrane composition (9, 10). The phospholipid headgroup composition of the membrane, which was not taken into account in this study, correlated to barosensitivity of *L plantarum* (44). Differential expression of chaperones depending on the temperature of incubation could contribute to the variation of barosensitivity of cells that were pressure treated at different temperatures. However, cells grown at 15°C and pressure treated at 37°C exhibited comparable barosensitivity as cells

grown at 37°C and pressure treated at 37°C. These cells were found to have a comparable membrane fluidity, but can not be expected to have comparable expression levels of either heat or cold shock proteins.

In conclusion, evidence is provided that the composition and the phase behaviour of cytoplasmic membranes affect pressure denaturation of HorA. The inactivation of MDR-transport activity by high pressure is measured before the loss of viability. These transport enzymes are important for survival of microorganisms in adverse conditions encountered in food (16). Our results provide insight into mechanisms governing the loss of resistance of microorganisms by high pressure processing and facilitate the application of high pressure in the context of a hurdle system for preservation of minimally processed food.

Acknowledgements

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Chapter V: Effects of pulsed electric fields on inactivation and metabolic activity of *Lactobacillus plantarum* in model beer

ABSTRACT

The adequate application of pulsed electric fields (PEF) for food preservation requires insight into mechanisms of PEF mediated cell injury and death. *Lactobacillus plantarum* TMW 1.460, a beer spoiling organism, was PEF treated at different field strengths and energy inputs in model beer (MB). The determined threshold values for field strengths and energy inputs were 13 kV cm^{-1} and 64 kJ kg^{-1}, respectively. Above these critical values irreversible cell damages occurred indicated by loss of cell counts on MRS. Below critical values metabolic activity and membrane integrity were reduced by 85% without loss of viability. Sublethal injury, defined as difference between cell counts on selective and non-selective media, was not detected with PEF. Addition of nisin and hop extracts, during PEF treatment, exerted different effects. Nisin and PEF reduced cell viability by 1.5 orders of magnitude. Hop extract and PEF reduced cell viability by 1.5 orders of magnitude and induced 99% sublethal injury. The reversibility of membrane damage below critical field strength was measured by propidium iodide (PI) staining. Identical PEF experiments were performed in presence and absence of PI. From the comparison of these results it was concluded, that 30% of membrane damage were resealed after PEF treatment. PEF experiments above critical field strength in hopped MB revealed, that cells were reduced by 5 orders of magnitude. The surviving cells were killed after 14 h of storage in hopped MB.

INTRODUCTION

The application of non-thermal sterilisation methods such as pulsed electric fields (PEF) in foods is favourably because of the possibility to preserve valuable but thermo-sensitive ingredients (1). The survival of microorganisms after PEF depends mainly on total energy input and field strength of PEF. Additionally the environmental parameters in foods influence the efficacy of PEF microorganism inactivation. Treatment setup has to be adapted to food matrix, temperature, pH, presence of antimicrobial compounds, and conductivity of media. Therefore, basic knowledge of PEF mediated sublethal injury and death of cells is of paramount importance to optimise PEF sterilisation in different foods.

Cytoplasmic membranes are considered to be the main target of PEF leading to pore formation, leakage of substances and finally cell death. Pore formation can be reversible or irreversible depending on treatment conditions: (i) reversible pore formation occurs at low field strengths and energy inputs and results in uptake of macromolecules, e.g. DNA fragments, without loss of viability. (ii) Higher field strengths and energy inputs are thought to involve irreversible pore formation leading to cell death (2, 3, 4). Based on literature data a threshold value of field strength must be exceeded to induce a critical transmembrane potential $\Delta\varphi$ = -1V. To achieve the proposed transmembrane potential of -1V a field strength above of 30 kV cm^{-1} is required for most bacteria in liquids. The threshold value of field strength is a function of geometry of microorganism, type of PEF equipment, and conductivity of medium. PEF treatment above the threshold field strength results in reduced cell membrane thickness, pore formation and finally in high loss of cell viability (5).

In various experiments with field strength above the threshold value irreversible effects of PEF treatment were measured. The loss of cells viability, detection of ruptured membranes'

integrity and determination of collapsed internal cell pH with 5 (and 6-)-carboxyfluorescein diactetate succinimidyl ester indicated PEF conditions severely affecting cell membranes (6, 7). Russel et al. (8) measured an increment of UV-absorbent substances after PEF treatment and determined consequently the sum of reversible and irreversible pore formation. In contrary to irreversible effects of PEF on cells only little information about reversible PEF effects on membranes is available. Especially, cell reactions below the postulated threshold value of field strength and energy input are of mayor interest to elucidate principal PEF mechanisms. Treatment of cells below this threshold value results in sublethal injury of cells. This sublethal injury combined with survival conditions in foods may act synergistic on loss of cell viability and gives the opportunity to use PEF at lower energy levels.

In addition to permeabilisation of cytoplasmic membranes PEF inactivates enzymes. The effect on pectin methyl esterase was reported in orange juice by Yeom et al. (9). As an explanation for pectin methyl esterase inactivation by PEF conformational changes in structure are favoured (10, 11). Ryan et al. (6) measured inactivation of membrane bound enzymes of microorganisms such as H^+-ATPase in *Salmonella typhimurium* leading to loss of proton transport. A local heating next to formed pores leading to enzymes' denaturation was suggested as reason for enzyme inactivation (12). Also, the loss of the transmembrane pH gradient of *Listeria monocytogenes* with PEF treatment demonstrated the inactivation of transport systems relevant for pH homeostasis (8). The loss of transport functions impairs the possibility of cells to survive in adverse conditions. Low pH or preservatives in foods, e.g. nisin in cheese or iso-alpha-acids of hops in beer, may contribute synergistic effects on food pasteurisation during storage.

It was the objective of this work to determine the consequences resulting from PEF mediated sublethal injury and cell inactivation. The membrane bound enzyme inactivation at discrete

energy input levels and electrical field strengths was measured. Especially, PEF effects on cell functionality below the critical field strength threshold value were of interest. In the experimental setup, cultures of *Lactobacillus plantarum* TMW 1.460, a beer spoiling microorganism, were used for PEF experiments in model beer (MB). The effects of PEF were determined by measuring metabolic activity, membrane integrity, and sublethal injury. Hop bitter acids, dissipating transmembrane pH gradient, and nisin, a pore forming bacteriocin, were added to the medium to investigate putative synergistic effects between food preservatives and PEF. Subsequent to PEF treatment storage experiments in MB were performed to determine microbial survival under food conditions.

MATERIALS AND METHODS

Strains and culture conditions. *Lactobacillus plantarum* TMW 1.460, an organism previously isolated from spoiled beer, was cultivated using MB, MRS-agar, or MRS agar containing 4% NaCl (MRS-NaCl). MB was prepared like previously described by inoculating malt extract medium with *S. uvarum var. carlsbergensis* TMW 3.001 (13). The over night cultivation of *L. plantarum* in MB was at 30°C.

PEF treatment with antimicrobial additives. An overnight culture of *L. plantarum* TMW 1.460 in MB was subcultured (24 h) with 1% inoculum at 30°C to late stationary growth phase. The optical density (OD) at 578 nm was adjusted to 0.8 ± 0.1 with MB and the pH was measured. Resulting pH values before PEF treatment were 3.5 ± 0.3. When indicated, late stationary cells were harvested by centrifugation (4000 x g, 5 min, 25°C) and resuspended in an equal volume of MB with pH = 4.0. PEF treatment was done in a continuous flow system with 2.2 l h^{-1} and a gap wideness of $2*10^{-3}$ m. The conductivity of MB for all experiments was 1.20 – 1.78 mS cm^{-1}. The experimental design allowed a variation of field strength at constant total energy input and energy input at constant field strength. This provided comparability between the PEF treatment effects in all experiments. Temperature was monitored and the suspension never exceeded 31°C during the PEF treatment. After adjustment of field strength and energy input to desired values, the system was purged for 2 min to obtain equilibration conditions. Thereafter, 20 ml of treated MB were collected to get a representative sample. Untreated cells and control cells after heat treatment with 80°C for 10 min were used for comparison.

PEF treatments in MB with additives were performed with (i) 100 ppm hop-extract (isomerized hop extract containing 22% iso-α-acids, HHV, Mainburg, Germany) at pH = 3.6 or 4.0 and (ii) 1 mg l^{-1} nisin (Sigma, Taufkirchen, Germany) at pH = 3.5.

Determination of plate counts. MRS agar and MRS-NaCl agar plates were used to determine viable and sublethally injured cells. Appropriate dilutions were plated after PEF treatment using a spiral plater (IUL, Königswinter, Germany) and plates were incubated at 30°C for 2 days under controlled atmosphere (76% N_2, 20% CO_2, 4 % O_2). Cell counts of cultures of *L. plantarum* in MB were (2.27±1.65) x 10^8 CFU ml^{-1} on either MRS or MRS-NaCl agar (mean and standard deviation of 18 determinations). The cell counts on MRS are referred to as viable cells and the difference between cell counts on MRS and MRS-NaCl is referred to as sublethally injured cells.

Determination of metabolic activity. Cells from 20 ml sample were harvested by centrifugation at 25°C, 5.000 x g for 5 min, resuspended in 20 ml phosphate buffer with glucose (PBG, 50 mM H_2KPO_4, 0.1 g l^{-1} $MgSO_4$ x 7 H_2O, 0.05 g l^{-1} $MnSO_4$ x H_2O, and 4 g l^{-1} glucose, pH 6.5), and 200 µl were transferred to microtiterplates. The optical density measurement after addition of 2-(-iodophenyl)-3-(*p*-nitrophenyl) 5-phenyltetrazolium chloride (INT, Molecular Probes, Eugene, USA) to a final concentration of 2 mM was done as previously described (13). The results of metabolic activity are reported as % respect to untreated (100%) and heat inactivated (80°C, 10 min) cells (0%).

Determination of membrane integrity. The integrity of the cytoplasmic membranes of PEF treated cells were determined using propidium iodide (PI) in effective staining concentration of 100 µM. Membrane integrity was measured by fluorescence (λ_{EX} = 485 nm; λ_{EM} = 635 nm) and reported as % of untreated cells as previously described (13). A calibration curve

was established for each PEF inactivation using cells treated with 80°C for 10 min and mixed with untreated cells in steps of 10% from 0% to 100% intact cells.

To measure the reversibility of membrane damage mediated by PEF treatment samples of a divided cell culture were stained with 15 µM PI. One part was treated by PEF in presence of PI and second part was stained after PEF treatment. For both samples the effective staining time in MB was 15 min. Afterwards samples were washed in phosphate buffer (PB, 50 mM H_2KPO_4, 0.1 g l^{-1} $MgSO_4$ x 7 H_2O, 0.05 g l^{-1} $MnSO_4$ x H_2O, pH 6.5) before measuring PI fluorescence. PEF treatment was done at field strengths of 9.5 and 13.1 kV cm^{-1} with total energy input of 18.2 and 17.0 kJ kg^{-1}, respectively. Samples were plated on MRS and MRS-NaCl to determine sterilization efficacy of PEF treatment.

Survival of *L. plantarum* during storage in hopped MB. Stationary growth cells of *L. plantarum* were PEF treated in MB (pH = 3.6) without hop extract at field strengths of 27.6, 31.2, 35.2 kV cm^{-1} with near constant energy input of 55.5, 55.0, 48.7 kJ kg^{-1}, respectively. After these treatments samples were stored in MB with 100 ppm hop extract at pH = 3.6 and 10°C. Viable cells inactivation kinetics during storage were determined by plating on MRS and MRS-NaCl.

RESULTS

Effects of PEF treatment on *Lactobacillus plantarum* below critical field strengths. Based on calculations of Heinz et al. (5) for critical electroporative field strength *L. plantarum* was treated in MB with field strengths ranging from 10 to 19 kV cm^{-1} at corresponding energy inputs of 13 to 42 kJ kg^{-1}. The results are presented in Figure 1.

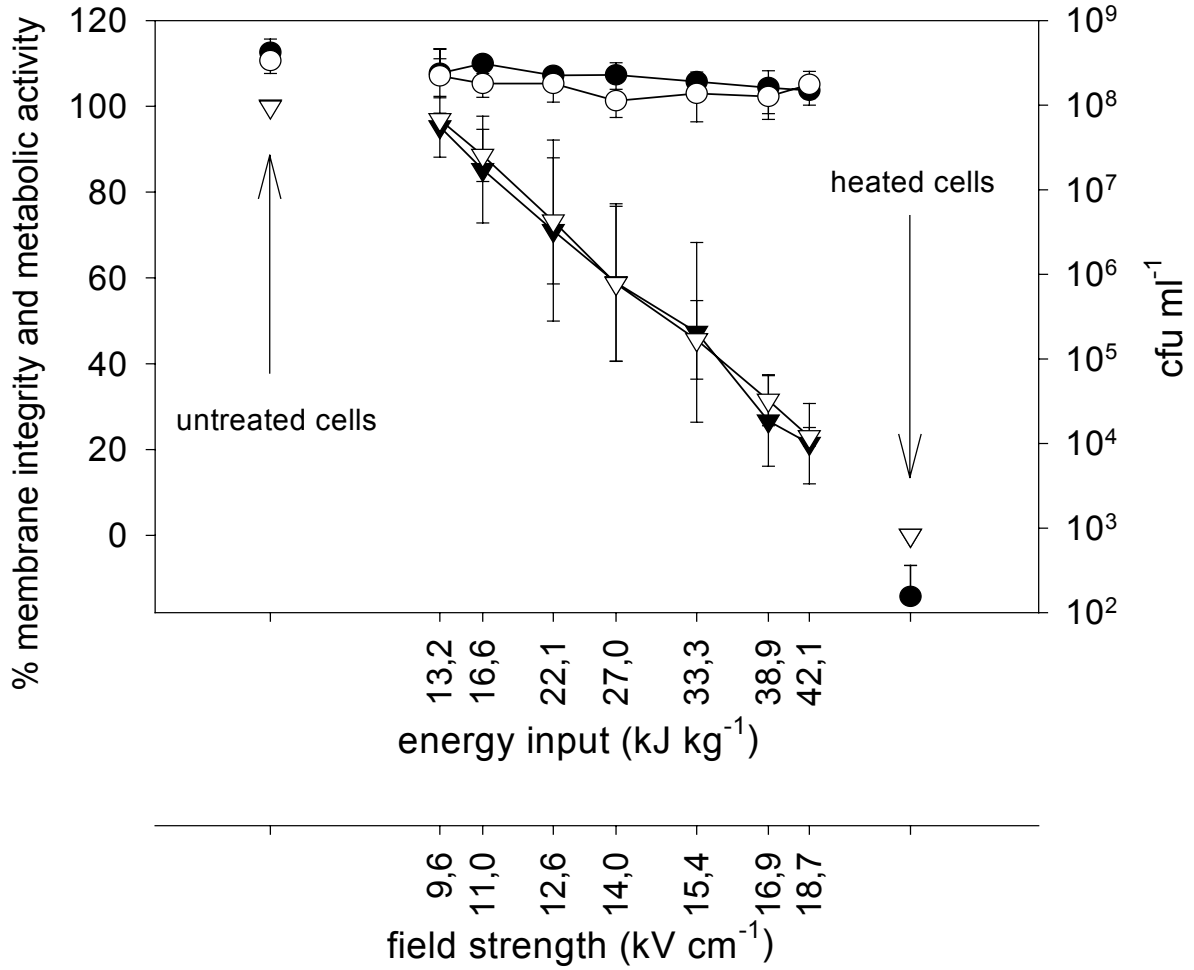

Figure 1. Effects of PEF treatment on *L. plantarum* in MB (pH = 4.0, 30°C) depending on field strength and corresponding energy input. Shown are membrane integrity (▽) and metabolic activity (▼) referred to calibration. Viability in cfu ml^{-1} plated on MRS (●) and MRS-NaCl (○). Controls of untreated and heat-treated (80°C, 10 min) cells are inserted. The results are based on means and error bars indicate standard deviations of two independent experiments.

As seen in Figure 1 the viability of cells remains unaffected by PEF treatment with 10 to 19 kV cm^{-1} indicating treatment conditions below the critical field strength. A difference in viability between samples plated on MRS and MRS-NaCl resulting in specific injury of the cells could not be detected. The metabolic activity and membrane integrity decreased linearly with applied field strength or energy input, respectively. These effects of PEF treatment were detected before loss of viability occurred.

A second experiment below critical field strength was done at 13.8 kV cm^{-1} with varied energy input. The field strength of 13.8 kV cm^{-1} and energy input of 27 kJ kg^{-1} corresponds to 60% metabolic activity and membrane integrity as depicted in Figure 1. Total energy input was increased with incremented pulse numbers. The results are shown in Figure 2.

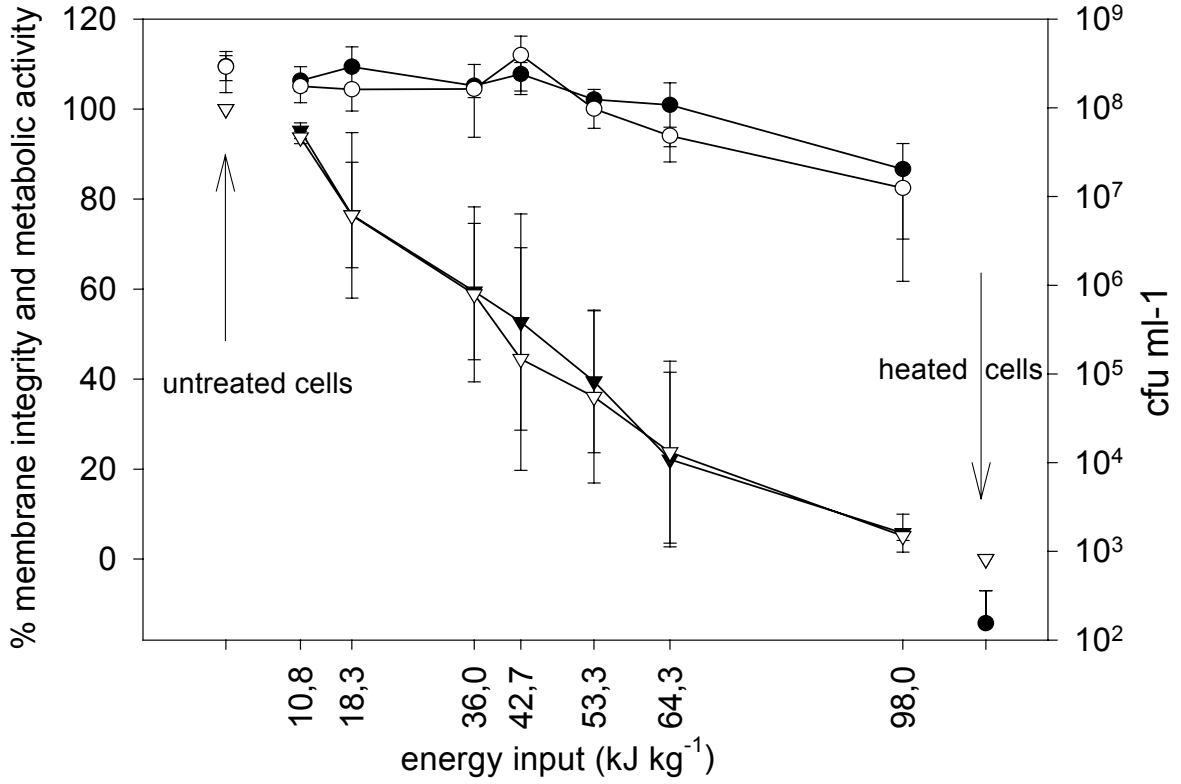

Figure 2. Effects of PEF treatment on *L. plantarum* in MB (pH = 4.0, 30°C) at constant field strength (13.8 kV cm^{-1}) and increasing energy input. Shown are membrane integrity (▽) and metabolic activity (▼) calculated in percent referred to calibration (data not shown). Viability in cfu ml^{-1} plated on MRS (●) and MRS-NaCl (○). Controls of untreated and heat-treated

(80°C, 10 min) cells are inserted. The results are based on means and error bars indicate standard deviations of two independent experiments.

In Figure 2 a slight effect of PEF treatment on the viability is seen at higher energy inputs. At field strength of 13.8 kV cm^{-1} and 98 kJ kg^{-1} a reduction of viable cell counts by one order of magnitude was achieved. The effects on metabolic activity and membrane integrity showed a linear relationship to increased energy input. With 98 kJ kg^{-1} the percental metabolic activity and membrane integrity are 95% reduced and reached nearly the level of heat-treated cells. Inactivating effects on viability can be detected at energy inputs higher than 60 kJ kg^{-1}.

Hop extract, a preserving ingredient in beer, contains iso-α-humulones and lupulones which act as ionophores in bacterial membranes destroying transmembrane pH-gradient. To detect a synergistic effect, treatments in presence of hop extract (100 ppm, pH = 4.0) were performed. PEF did not affect inactivation of metabolic activity, membrane integrity or viability (data not shown).

Effects of PEF treatment above critical field strength and energy input on *Lactobacillus plantarum*. Experiments at higher field strengths (28.8, 31.6, and 34.8 kV cm^{-1}) were done to determine the efficacy of PEF treatment on viable cells of *L. plantarum* in MB and hopped MB (pH = 3.6, 100 ppm hop extract). In Figure 3 the viability of *L. plantarum* after PEF treatment is depicted.

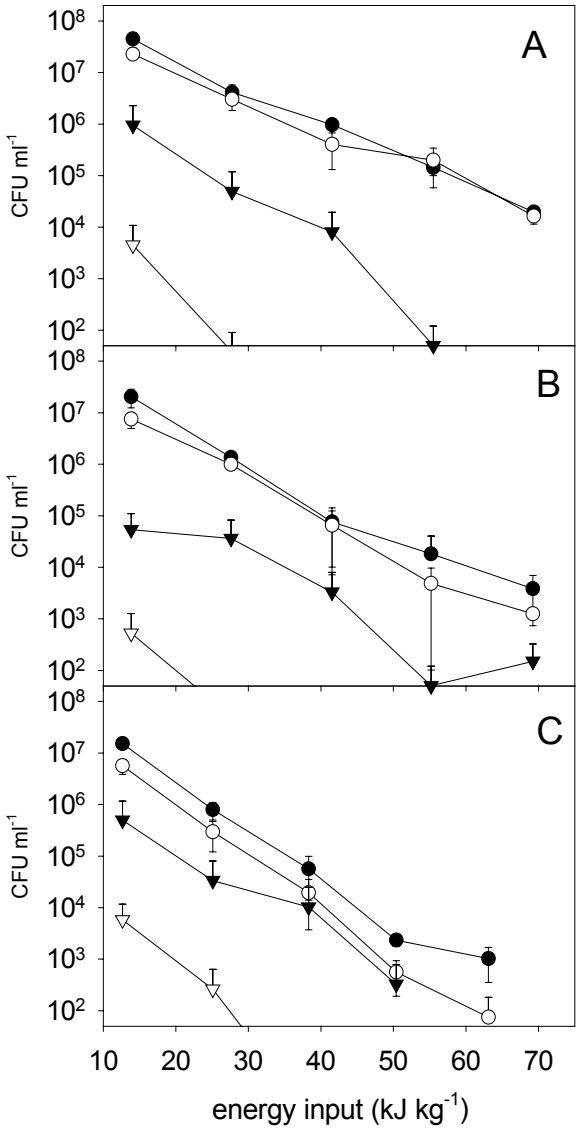

Figure 3. Effects of PEF treatment on *L. plantarum* in MB (pH = 3.6, 30°C) at different field strengths (A: 28.8 kV cm^{-1}, B: 31.6 kV cm^{-1}, C: 34.8 kV cm^{-1}) and increasing energy input. Treatment: MB and plated on MRS (●), MB and plated on MRS-NaCl (○), MB with 100 ppm hop extract and plated on MRS (▼), MB with 100 ppm hop extract and plated on MRS-NaCl (▽). The results are based on means and error bars indicate standard deviations of two independent experiments.

The reduction of viability is a log-linear relationship depending on energy input at constant field strength. Cells PEF treated in MB and plated on MRS and MRS-NaCl did not differ with respect to viability loss or sublethal injury. Increased field strengths (Figure 3. B and C) induced higher inactivation at the same energy inputs, e.g. in Figure 3. A at 28.8 kV cm^{-1} and 50 kJ kg^{-1} a viability reduction up to 2 orders of magnitude was reached. In Figure 3. C with 34.8 kV cm^{-1} the reduction was 4 orders of magnitude. The addition of hop extract at this PEF treatment conditions strongly affected the cells. In contrast to PEF conditions below threshold field strength the presence of hop extract reduced cell viability by 1.5 orders of magnitude as

compared to treatment in MB without hops. Furthermore, 99% of surviving cells were sublethally injured. The slopes of inactivation efficacy at each field strength were similar.

The metabolic activity and membrane integrity after PEF treatment at 28.8 kV cm^{-1} are show in Figure 4. Metabolic activity and membrane integrity is reduced to less than 15% at field strengths above 28.8 kV cm^{-1}.

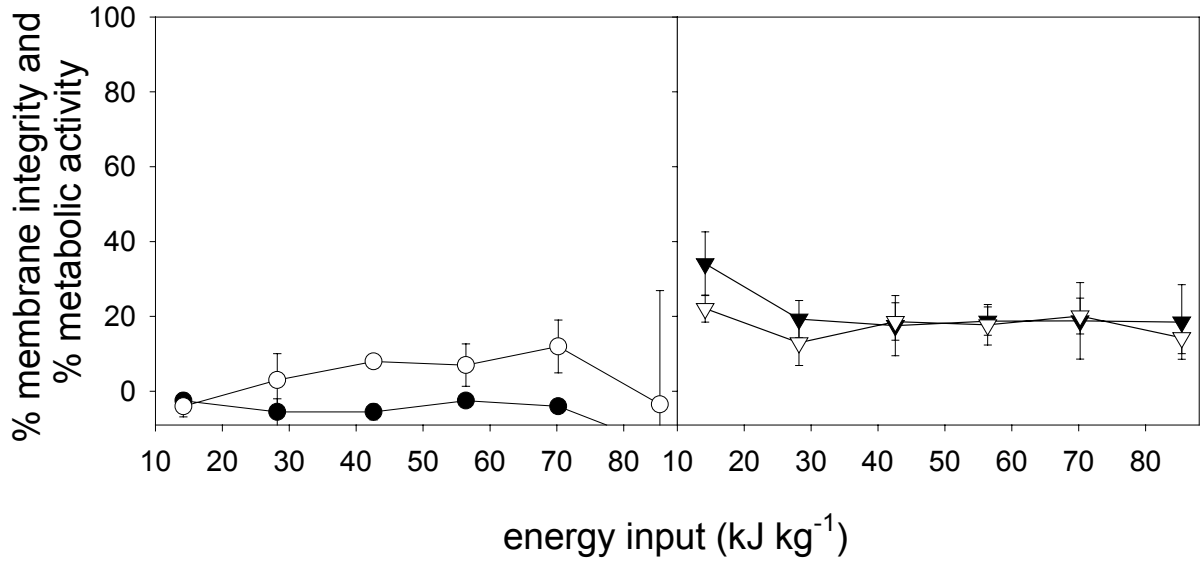

Figure 4. Effects of PEF treatment on *L. plantarum* in MB (pH = 3.6, 30°C) at field strengths 28.8 kV cm^{-1} and increasing energy input. Left: membrane integrity (● = MB, ○ = MB with 100 ppm hop extract). Right: metabolic activity (▼ = MB, ▽ = MB with 100 ppm hop extract) calculated in percent referred to calibration (data not shown). The results are based on means and error bars indicate standard deviations of two independent experiments. Experiments at field strengths of 31.6 and 34.8 kV cm^{-1} looked not different.

The hop acids did not have an additional effect on inactivation of these two physiological parameters. The detection limit of colorimetric and fluorescence assay is ± 10%. At field strength of 28.8 kV cm^{-1} only metabolic activity is reduced from 35% to 17%. An additional metabolic activity reduction is not detected with higher energy inputs or higher field strengths and Tetrazolium. PI staining indicated complete membrane integrity loss after first treatment.

PEF treatment in presence of nisin. The effect of nisin present during PEF treatment on *L. plantarum* in MB was determined. The MIC of nisin in MB for *L. plantarum* was 1 mg l^{-1} (data not shown). The field strength was set to 31.6 kV cm^{-1} with different energy inputs. The results are shown in Figure 5.

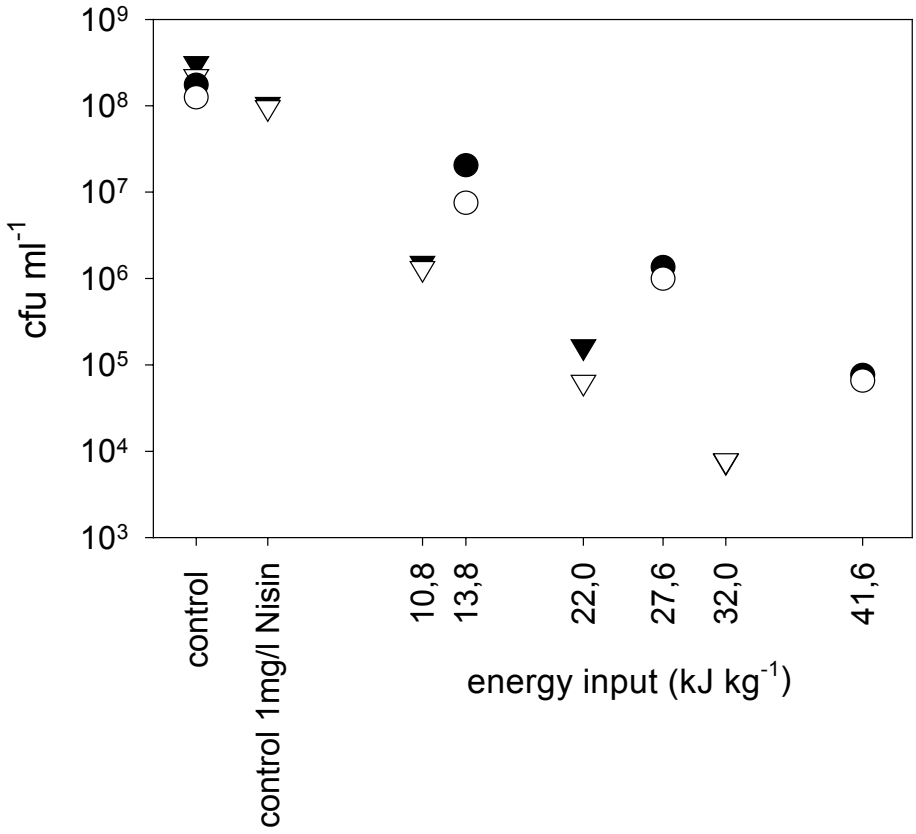

Figure 5. Effects of PEF treatment on *L. plantarum* in MB (pH = 3.5, 30°C) at constant field strengths (31.6 kV cm^{-1}) and increasing energy input. Treatment: MB and plated on MRS (●), MB and plated on MRS-NaCl (○), MB with 1 mg l^{-1} nisin and plated on MRS (▼), MB with 1mg l^{-1} nisin and plated on MRS-NaCl (▽).

In the presence of nisin upon PEF treatment the inactivation of *L. plantarum* is increased by 1.5 orders of magnitude. The control sample stored with nisin is not affected by viability. In contrast to the ability of hop extract to injure cells sublethally, no difference in viability was detected between cells plated on MRS and MRS-NaCl.

Determination of reversibility of membrane damage during PEF treatment with PI. The ability of PI to enter cells with ruptured cytoplasmic membranes was used to estimate the reversibility of membrane damages. After mild PEF treatment cells' membrane damage was measured with PI to determine the membrane integrity. To distinguish between reversible and irreversible membrane damage these results were compared with samples treated at equal field strengths and energy inputs but with PI present during PEF treatment. The results are shown in Figure 6.

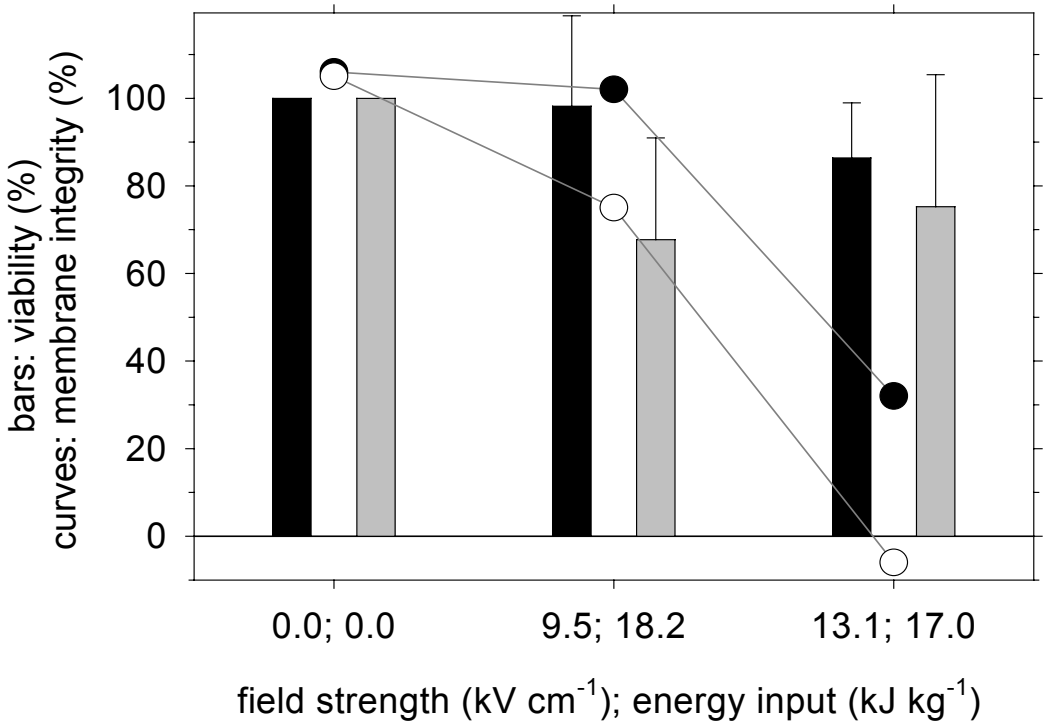

Figure 6. Reversibility of mild PEF treatment mediated membrane damage in MB (pH = 3.5, 30°C) at different field strengths and energy inputs. Bars: calculated % viability plated on MRS (■) and MRS-NaCl (■) referred to initial cfu ml^{-1}. Curves: calculated % membrane integrity after PEF treatment in MB (●) and MB with PI (○) based on calibration. Staining with PI did not affect viability of *L. plantarum* (data not shown).

Cells stained with PI during PEF treatment show 30% less membrane integrity than cells stained after PEF treatment. This increased membrane damage during PEF treatment indicates

pores, which are resealed when cells are stained with PI after treatment. In this experiment PEF conditions had no effect on viability or sublethal injury of cells.

Storage of *Lactobacillus plantarum* after PEF treatment in MB with hops. As a consequence of partial reversibility of PEF treatment mediated injuries the effect on survival after different PEF treatments was investigated. *L. plantarum* was PEF treated in MB with hop compounds and afterwards viability and sublethal injury was monitored during storage in hopped MB. Results are shown in Figure 7.

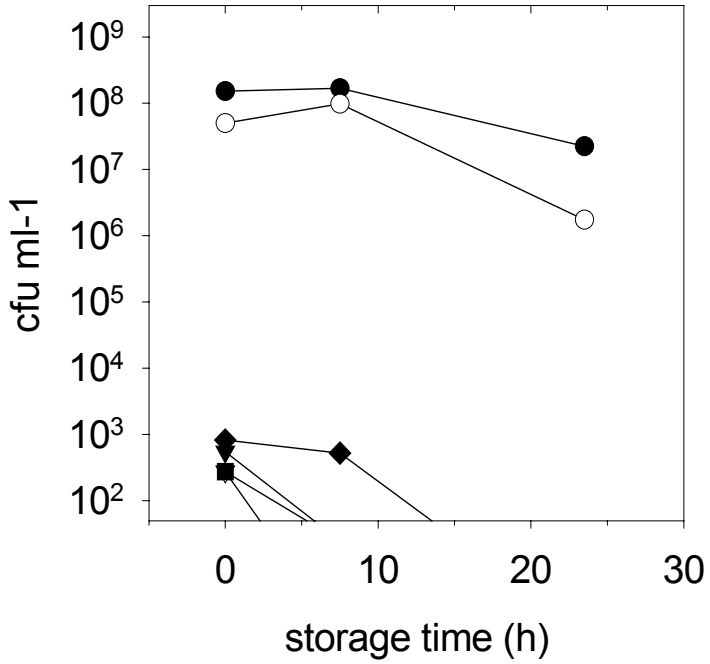

Figure 7. Effects of different PEF treatments in hopped MB (pH = 3.6; 100 ppm hop extract, 30°C) on storage viability (pH = 3.6; 100 ppm hop extract, 10°C) of *L. plantarum*. Control cells plated on MRS (●) and MRS-NaCl (○), 27.8 kV cm^{-1} and 55.7 kJ kg^{-1} plated on MRS (▼) and MRS-NaCl (▽), 31.2 kV cm^{-1} and 55.4 kJ kg^{-1} plated on MRS (■) and MRS-NaCl (□), 35.0 kV cm^{-1} and 50.2 kJ kg^{-1} plated on MRS (◆) and MRS-NaCl (◇). (Abscissa indicates detection limit).

The reduction of viability in MB with hops was minimum 5 orders of magnitude at described PEF treatment conditions. The most ineffective treatment was at 31.2 kV cm^{-1} and 55.4 kJ kg^{-1} where cells survived 13 h in MB at 10°C after treatment. During storage (100 ppm hop

extract, pH = 3.6, 10°C) untreated cells counts on MRS decreased by 50% in 25 hours. Surviving cells were not able to recuperate. Cells surviving PEF were sublethally injured and inactivated within 8 hours of storage in beer.

DISCUSSION

In this work, effects of PEF on cells have been detected below the threshold level for microbial killing. The involved inactivation mechanisms of PEF on *Lactobacillus plantarum* in MB were elucidated. Heinz et al. described the dependence of inactivation on the parameters field strength and total specific energy input for different food spoiling bacteria (4, 5). To confirm the proposed critical field strength for *L. plantarum* in beer experiments under different conditions of field strength and energy input were performed.

During PEF treatment a transmembrane potential difference leads to electrical breakdown and local conformational changes of bilayer resulting in destruction of transmembrane gradient and loss of viability (14). To verify the existence of a threshold value of field strength and energy input for inactivation of *L. plantarum* in MB. Reported by Heinz et al. (5), the effects of mild PEF treatment on metabolic activity and membrane integrity were determined by monitoring the effects at conditions where no loss of viability was detected (15). These results showed a linear relation of field strength and/or energy input with metabolic activity and membrane integrity. The threshold field strength for viability of *L. plantarum* in MB was determined at 13.8 kV cm^{-1} and energy input 60 kJ kg^{-1}. This value conforms to calculations of Heinz *et al.* where *L. plantarum* required a critical field strength in the range of 10 to 60 kV cm^{-1}(5). Noticeable loss of cell viability was first detected at levels of metabolic activity and membrane integrity below 15%. Wouters et al. measured *L. plantarum* with flow cytometry and found this linear dependence of energy input and PI uptake correlated with ruptured membranes. The experiments showed a log-linear relationship between reduction of viability and field strength of 2.5 kV cm^{-1} and energy inputs higher than 30 kJ kg^{-1} (6). This log-linear dependency of viability and energy input was verified at different field strengths

with *L. plantarum* in MB. Increasing field strengths induced increased viability loss at equal energy input levels. Metabolic activity and membrane integrity were not longer detected at these PEF treatment conditions.

The part of sublethally injured cells on selective agar was determined. In contrast to other sterilization methods, e.g. high pressure and temperature, sublethally injured cells were not found with PEF treatment (13, 16). This result supports the hypothesis that PEF treatment exerts an 'all-or-nothing' effect on cells and sublethal injury does not occur (8, 17).

PEF treatment leads to membrane damage and pore formation. Cells can reseal these damages as seen in electroporation techniques if the electrical impact was not severe enough (18). Irreversible membrane damages mediated by PEF treatment were detected by PI uptake (6). The reversibility of mediated membrane damages was visualized by the difference of PI uptake during and after PEF treatment. PEF below critical fields strength did not reduce viability on MRS or MRS-NaCl. Cells treated in presence of PI took up 30% more PI than cells, which were stained after treatment. Thus, their pore formation was reversible. PEF treatment induced membrane damages, which partly are resealed immediately after treatment. Although 0% of membrane integrity were determined during PEF treatment at 13.1 kV cm^{-1} and 17.0 kJ kg^{-1}, no reduced viability on MRS or MRS-NaCl were detected. This indicates reversibility of membrane damages and resealing, if cells are incubated in optimal conditions after PEF treatment.

The effect of antimicrobial additives on PEF treatment and *L. plantarum* in MB was studied with nisin and hop extract. Both substances interact with bacterial membranes. Nisin acts as pore forming molecule, which integrates into membranes and dissipates transmembrane gradients (19). Pol et al. (20) studied the combination of PEF treatment and nisin with

Bacillus cereus. An inactivating effect of 3.8 log cycles with nisin instead of single PEF treatment with 2 log cycles of inactivation was found in HEPES buffer (pH = 7). It was of interest, if nisin also exhibits sublethal injury on cells of *L. plantarum* in MB. The nisin concentration was augmented to 1 mg l^{-1} because of *L. planatrum´s* minimum inhibitory concentration (MIC) (data not shown). An additional lethal effect of 1.5 orders of magnitude was found at different energy inputs. Nisin did not induce sublethal injury in surviving cells.

Hop bitter acids act as a carrier of protons in the cytoplasmic membrane. It traps protons at the outer membrane surface and carries them to the inside, thus, reducing intracellular pH. This effect leads to loss of transmembrane pH gradient and starvation of cell (21). hop extract induces sensitivity of viability against sterilizing methods, which first target is the cytoplasmic membrane, e.g. high pressure (17). Gänzle et al. (16) found additional 2 orders of magnitude inactivation effect with high pressure in presence of 100 ppm hop extract with *L. plantarum*. The addition of hop extract to cells during mild PEF treatment induced neither additional viability reduction on MRS or MRS-NaCl nor metabolic activity and membrane integrity loss. PEF treatments at higher field strengths in presence of hop extract provoke additional viability loss of averaged 1.6 orders of magnitude on MRS compared to MB without hop extract. In contrast to nisin, hop extract caused 2 orders of magnitude of sublethally injured cells on MRS-NaCl during PEF treatment. Total inactivation was performed at field strengths of 28.0 – 34.8 kV cm^{-1} and energy input higher than 30 kJ kg^{-1}. Sublethally injured cells may be unable to repair PEF treatment mediated pores in the cytoplasmic membrane. A preliminary experiment showed that *L. plantarum* cells injured by PEF treatment exhibited decreased storage survival in MB with hops. The inactivation was done at field strengths and high energy inputs above critical value with hop extract in presence leading to immediate loss of 5 orders of magnitude of viability. Complete

inactivation after 15 hours can be explained by lost membrane integrity and metabolic activity as basic requirements for the functionality of energy dependent carrier systems, e.g. the hop resistance mechanism HorA (22). The result indicates that reversible membrane damage may suffice to prevent spoilers in specific food applications. The combination of PEF treatment in beer, with certain content of hop extract, seems to be a promising possibility of pasteurisation avoiding the disadvantages of thermal pasteurisation, e.g. darkness and cooking flavour augmentation by heating. Bacterial membranes are the working point of hop extract acids and with PEF treatment they are additionally weakened. This inhibits recuperation of spoiling bacteria and recontamination of foods.

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Additional insights and conclusions

High pressure opens new possibilities in food industry processes for preservation, modification and design of foods. The possibility to change texture, colour and turbidity generating ingredients, and process influential characteristics is still investigated. Fischer *et al.* and Castellari *et al.* examined the applicability of high pressure in the brewing process. 500 MPa improved the specific beer filterability from 3.6 to 5.1 arbitrary units by cracking β-glucan gels. Apart from that, the treatment reduced potential for arising of turbidity from 32.5 to 22.6 EBC units (1, 2, 3). High pressure is also known as antimicrobial method. It affects microorganisms and prevents them from spoiling foods, especially, if foods possess a preserving hurdle system (4). These promising results lead to further research to investigate the applicability of high pressure as biological preserving method.

For this reason the mechanisms of high pressure affecting the beer spoiling microorganism *Lactobacillus plantarum* TMW 1.460 were studied. The beer habitat contains several restrictive conditions, which prevent other microorganisms from growth: (i) the low content of nutritions (lack of fermentable sugars and nitrogen containing substances) is disposable only for metabolic specialists. These can be found mainly in the genus *Lactobacillus* (5, 6). (ii) Carbon dioxide and shortage of oxygen in beer excludes growth of aerobic microorganisms. (iii) Ethanol and hop acids, which dissipate transmembrane pH gradient, select specifically adapted Lactobacilli. Hop acids are substrates for membrane bound multi-drug-resistance transport enzymes. These enzymes mainly mediate resistance of beer spoiler against the hopped and acidic environment in beer. Sami *et al.* and Sakamoto *et al.* described a hop transporting enzyme (HorA), which affects efflux of hop acids out of the membrane by changing conformational structure and ATP hydrolysis (11, 7). *L. plantarum* possesses the genetic information and the ability to express the multi drug resistance enzyme HorA. The

functionality of the enzyme is determined by the position in the membrane and the supplementation of the ATP binding cassette with nucleotides. To refresh the nucleotide pool a glycolytic pathway must be on duty, and intact membranes must separate the cytoplasm from environment.

To study the working point of high pressure on cells and to elucidate the sequence of importance for viability it has to be cleared, which precondition for cell viability is irreversibly inactivated first. Colorimetric assays were established to examine activity and integrity of cell processes and compartments. The examination of the metabolic activity after mild pascalisation was implemented through staining with Tetrazolium. Active enzyme cascades reduce Tetrazolium to Formazan. This changes optical density of cells indicating intact metabolic activity. Membrane integrity was measured with propidium iodide and fluorescence of DNA-propidium-iodide-complex. The results of colorimetric assays and cell viability on MRS agar confirmed, that loss of membrane integrity and metabolic activity took place after cell inactivation. Sublethal injury of the osmostatic system indicated, that cell compartments were affected before loss off cell viability occurs. These results lead to the conclusion, that high pressure mainly did not exert its effects on the basis of membrane rupture or cytoplasmic enzyme denaturation. The destruction of transport systems plays a major role in cell inactivation (8). Experiments measuring the collapse of internal pH of *Lactococcus lactis* after high pressure treatments lead to the conclusion, that essential processes for internal pH homeostasis were seriously damaged (9). The loss to endure osmotic-stress and, as further storage experiments after pascalisation demonstrated, the loss of hop resistance, pointed out, that the cytoplasmic membrane in combination with membrane bound enzymes were the first target of high pressure (10). Membrane bound enzyme HorA, mainly responsible for hop resistance and growing in beer, is an unspecific substrate transporter. Among hop acids also ethidium bromide, a fluorescent dye is catalysed (11). This

enabled to measure HorA activity after pascalisation by fluorescence kinetic experiment (8). HorA inactivation kinetics displayed, that membrane bound enzyme activity is the threshold value for cell inactivation. If HorA activity is reduced by high pressure to approximately 10% cells' viability began exponentially to decrease. A close relationship between membrane bound enzyme HorA and the thermodynamical behaviour of the membrane during high pressure inactivation was estimated. To verify this hypothesis, the effects of varied membrane acyl chain compositions on HorA inactivation kinetics were investigated. The different compositions of acyl chains in the membrane were stimulated through growth of *L. plantarum* at different temperatures. 15°C entailed the incorporation of high level of unsaturated fatty acids. 37°C provoked high content of saturated fatty acids. According to the ordering principle of high pressure, similar to the ordering principle of low temperature, membranes with unsaturated fatty acids crystallize at higher pressures compared with membranes mainly consisting of saturated fatty acids. Crystallisation is phase transition from liquid-crystalline to gel phase of the membrane and can be initiated by lowering temperature and/or increasing pressure. The arrangement of the molecules in the gel phase is rigid and is reversibly lost, if temperature rises or pressure decreases (Introduction; Figure 3.). The point of crystallization depends on the fatty acid composition and is shifted to higher pressures in cells grown at 15°C. The HorA inactivation velocity increased in cells grown at 15°C. In other words, the membrane bound enzyme was affected more strongly by pressure in liquid-crystalline phase than in gel phase. For this reason cells grown at 15°C are killed faster because of the retarded membrane crystallisation. The pressure induced crystallization irreversibly affects the HorA enzyme activity. This is in contrast to temperature induced crystallization, which inactivated HorA activity reversibly (12). A schematic diagram showing the relationship between growth and pressurisation temperature and the baro-sensitivity, expressed in 99% inactivation at 200 MPa, of *L. plantarum* was set up in Figure 1.

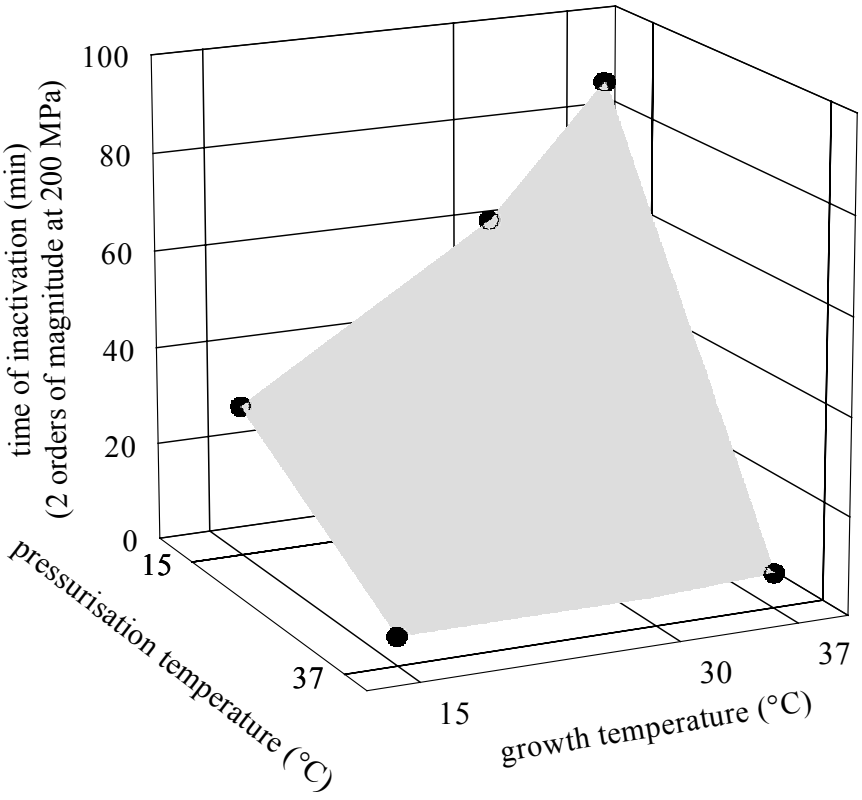

Figure 1. Schematic diagram of baro-sensitivity (time to inactivate 2 orders of magnitude of viability) at 200 MPa of *L. plantarum* depending on growth and pressurisation temperature. Depicted are means of two independent experiments. Inactivation times deviated ±10%.

According to Figure 1. baro-sensitivity at 37°C pressurisation temperature is nearly independent of growth temperature. At pressurisation temperature of 15°C the role of growth temperature was markedly intensified. The baro-sensitivity was nearly tripled from 31 min to 89 min at growth temperatures of 15°C to 37°C, respectively. If pressurisation began in the liquid-crystalline phase cells lost 99% of their viability after 7 min as seen at 15°C and 37°C growth and 37°C pressurisation temperature. A possible explanation is, that cells grown at 37°C were cooled to 15°C before pressure treatment. This provokes a temperature induced membrane phase transition to the gel phase prior to pressurisation. The temperature induced gel phase protects membrane bound enzymes and viability against high pressure (Chapter IV). The exact mechanism of protection by temperature induced gel phase before pressure application is not yet understood. Experiments are necessary to investigate to damaging effect of

pressure induced phase transition from liquid-crystalline to gel phase on cell viability and membrane enzyme activity.

General conclusions. In this work it could be demonstrated that sublethal injury occurs above a low threshold value of pressure before loss of viability takes place. The thermodynamical behaviour of membrane phospholipids was a major determinant of this sublethal injury. The high pressure induced membrane effects occur immediately with increasing pressure and generally, reversible effects are observed prior to irreversible membrane damage. The lethal effects result from the combination of damaged membrane transport systems and detrimental environmental parameters. High pressure treated cells loose the structural and functional integrity of the cytoplasmic membrane and are unable to maintain vital concentration gradients of ions and metabolites. Therefore, high pressure inactivation kinetics measured *ex-situ* during pressure holding times do not only describe high pressure effects, but the effect of adverse environmental conditions.

An explanation for these conclusions is given in Figure 2. The results of high pressure treated cells plated on MRS (viability) and sublethal injury (HorA activity) are depicted, exemplarily. Shown is the pressure ramp profile of 120 minutes pressure treated sample at 200 MPa. Sublethal injury expressed in relative HorA activity took place with raising pressure, immediately. Until 40 min pressure treatment time sublethal injury was reversible and cells could recuperate, if cultivated in optimal environment. Cell viability is first affected after 40 min pressure treatment time, when HorA activity is completely inactivated. In this state cells could not endure high pressure and pressure transmitting media conditions and were killed exponentially.

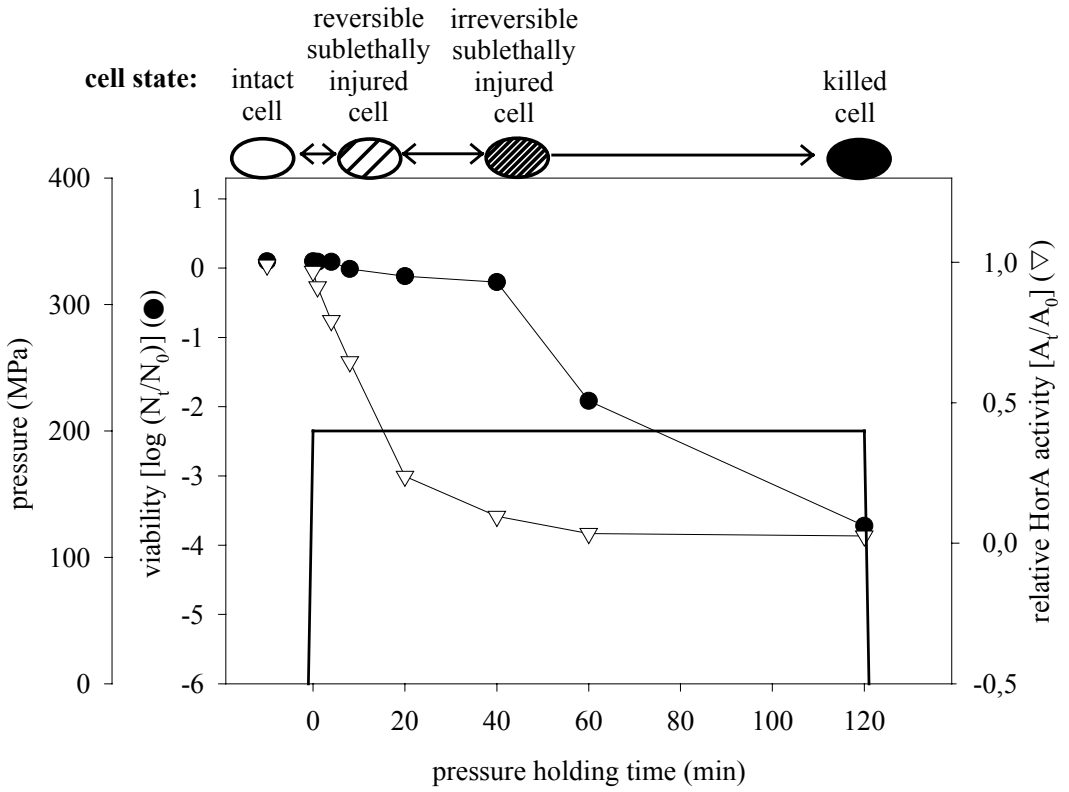

Figure 2. Results of high pressure treated cells plated on MRS (viability, ●) and sublethal injury (HorA activity, ▽). Pressure ramp profile is shown for 120 minutes at 200 MPa (▬). At 40 min pressure holding time cell state changed from reversible sublethally injured to irreversible sublethally injured. Exponential inactivation of cells began after 40 min pressure holding time.

Taking in consideration, that the main target on cells are the membrane bound enzymes, the potential of high pressure in food technology resides in synergisms at low pressures rather than in replacement of processes. The pressure level for *L. plantarum* inactivation was decreased from 600 MPa to 200 MPa by respecting the baro-sensitivity of hop resistance, its growth temperatures and the preserving hurdle system of beer (8, 10, 12). Gänzle *et al.* investigated the effect of high pressure on *Escherichia coli* outer membrane and discovered destruction of the protecting sugar calix (13). Further investigations should be done to prove, if this effect also relieves the penetration of hop acids in gram-negative beer spoilers, as *Megasphaera* and *Pectinatus*. Beer spoiling yeasts are immediately killed at pressures of 200 MPa (data not shown). Experiments at lower pressure levels may elucidate, if an optimum of

killing beer spoilers without affecting yeasts physical condition is achievable. Thus, pressure treatment in combination with storage in hopped medium could reduce contaminations during yeast management. These investigations gave opportunity to diminish the quantity of materials to be pressurized and consequently the costs for high pressure equipments enormously. Equipments with spiral shape and continuous flow, even with high throughput, for 350 MPa are known from plastics industry and first pilot equipments for fluidal foods working with a semi-continuous 4-stroke mechanism were developed (14, 15).

High pressure treatment opened new fields for research. Among the potential for preservation and technological advancements it has given the possibility to realize fundamental interrelationships between physical and microbial processes. Further experiments using *in-situ* measurement techniques will contribute to elucidate high pressure effects on living systems.

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CURRICULUM VITAE

HELGE MARTIN ULMER

PERSÖNLICHE ANGABEN

- Geburtsdatum: 27.12.1969
- Geburtsort: Bogotá / Kolumbien
- Familienstand: verheiratet

AUSBILDUNG

1976 - 1978	Deutsche Schule Bogotá
1978 - 1989	Grundschule I und Georg-Wilhelm-Steller-Gymnasium in Bad Windsheim mit Abschluss Abitur
1989 - 1990	Wehrdienst
1990 - 1992	Studium der Fachrichtung Wirtschaftsingenieurwesen an der Technischen Hochschule Karlsruhe
1992 - 1998	Studium der Fachrichtung Getränketechnologie und Brauwesen an der Technischen Universität München - Weihenstephan mit Abschluss Diplom Ingenieur
ab 1998	Promotion am Lehrstuhl für Technische Mikrobiologie der Technischen Universität München
1999 - 2001	Aufbaustudium der Fachrichtung Biotechnologie der Technischen Universität München

BERUFSERFAHRUNG UND TÄTIGKEITEN

01.06. – 30.09.1990	Praktikum Bürgerbräu Bad Windsheim
01.07. – 31.10.1992	Praktikum Landwehrbräu Reichelshofen

Diplomarbeit:	Projektierung und Anwendungsanalyse eines Meßsystems zur Untersuchung der Viskosität flüssiger Lebensmittelinhaltsstoffe unter Ultra-Hochdruckbedingungen bis 7000 bar
Promotion:	Molecular mechanisms of the high pressure inactivation of beer spoiling *Lactobacillus plantarum*

SONSTIGE KENNTNISSE

Spanisch:	zweite Muttersprache
Englisch:	sehr gut

Freising, den 6. Mai 2002