

Physiological response of *Lactococcus lactis* to high-pressure

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Abbreviations

<u>Symbol</u>	<u>Mean</u>	<u>Units</u>
AcmA	autolysin muramidase	
ATP	adenosintriphosphat	
a_w	water activity	[-]
b	universal constant	[9.2 Pa ⁻¹]
cFDASE	5(and 6-)-carboxyfluorescein diacetate N-succimidyl ester	
cFSE	5(and 6-)-carboxyfluorescein succimidyl ester	
CFU	colony forming units	
Csps	cold-shock proteins	
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride	
DDBJ	Japanese Genetic Center	
DEEP-BATH	deep-sea baro/thermophiles collecting and cultivating system	
DAN	desoxyribonuclein acid	
ΔV	reaction volume	[cm ³ mol ⁻¹]
ΔV_m	molar volume change between the associated and dissociated forms of the buffering acid in solution	[cm ³ mol ⁻¹]
$\Delta V^\#$	activation volume	[cm ³ mol ⁻¹]
EB	ethidium bromide	
EDTA	ethylenediaminetetraacetic acid	
FT-IR	Fourier transform infrared spectroscopy	
GP	generalized polarization	
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]	
HorA	ABC-membrane-bound enzyme of <i>Lactobacillus plantarum</i>	
HPLC	high-pressure liquid chromatography	
Hsps	heat-shock proteins	
IFM	immunofluorescence microscopy	
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)5-phenyltetrazolium chloride	
K	equilibrium constant	[s ⁻¹]
λ	lag-phase	
Laurdan	6-dodecanoyl-2-dimethylaminonaphtalene	
LmrP	secondary drug transporter located in the membrane of <i>Lactococcus lactis</i>	
μ	specific growth rate	
MDR	multidrug resistance transporter	

MES	2-[N-morpholin]ethanesulfonic acid	
N	viable counts	[CFU/ml]
No	initial viable counts	[CFU/ml]
NCBI	National Center for Biotechnology Information	
OD	optical density	
p	pressure	[MPa]
PI	propidium iodide	
pLP712	plasmid where the ability to ferment lactose is encoded	
R	gas constant	[8,3145 J mol ⁻¹ K ⁻¹]
T	absolute temperature	[°K]
T _m	transition temperature	[°C]
t ₀	initial time	[h]
t _d	detection time	[h]
TRIS	tris (hydroxymethyl)-aminomethan	
TMW	Technische Mikrobiologie Weihenstephan	
SDS	sodium n-dodecyl sulfat.	
Sv FtsZ	antibodies from <i>Shewanella violacea</i> DSS12	

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1. Introduction

The increasing demand for products that maintain the quality of fresh foods has led to a search for new technologies of food preservation. These technologies aim to replace those that involve heat treatment, which can result in a decrease in the nutritional value and a change in the sensory characteristics of the products (Smelt, 1998). In this sense, high-pressure has been investigated in order to create products of high quality and microbiological stability. Unlike heat, high-pressure affects only a few covalent bonds and therefore has little effect on ester molecules and other compounds responsible for food flavour and taste. Pressure also has negligible effects on vitamins. However pressure does cause denaturation or unfolding of proteins by disrupting hydrophobic interactions and causes phase changes in biological membranes (Rosenthal, 2002; Ulmer *et al.*, 2002).

Food preservation by high-pressure is based on the effect of pressure on spoilage microorganisms and enzymes. High-pressure allows the destruction of most vegetative microbial cells and at higher level and multiple pressure cycles the destruction of most bacterial spores (Smelt, 1998).

High-pressure induces physiological imbalances due to the internal and external structural damages, which are responsible for inactivation of microorganisms. The effect of pressure was also associated to a permanent shrinkage of cell volume, which was related to an irreversible mass transfer that occurs between the cell and pressure medium, because of a disruption or an increase in membrane permeability. Adiabatic expansion of water was also reported to be associated with inactivation of microorganisms (Alemán, 1996).

However, many aspects of the technology still have to be clarified, mainly related to the mechanism of inactivation, variation in pressure resistance of microorganisms according to the type and composition of the product, and the adaptation response to high-pressure conditions.

1.1 General principle of high-pressure

Two principles underlie the effect of high-pressure. First, the principle of le Chatelier, according to which any phenomenon (phase transition, chemical reactivity, change in molecular configuration, chemical reaction) accompanied by a decrease in volume will be enhanced by pressure (and vice versa). An antagonistic effect of temperature is expected from the fact that a temperature increase induces a volume increase through dilation. However, an increase in temperature also increases the rate of reaction according to Arrhenius' law. Secondly, the isostatic principle (isostatic pressure), which indicates that pressure is

transmitted in a uniform and quasi-instantaneous manner throughout the whole biological sample (whether the latter is in direct contact with the pressurization medium or located in an hermetic and flexible container that transmits pressure). Independent of the size and the geometry of the food, pressure is instantaneously and uniformly transmitted (Cheftel, 1995; Smelt, 1998).

Chemical equilibria respond to pressure in a way defined by the size and sign of their reaction volumes: (Gross and Jaenicke, 1994)

$$\left[\frac{\delta \ln K}{\delta p} \right]_T = - \frac{\Delta V}{RT} \quad (1)$$

where K is the equilibrium constant, p the pressure, T the absolute temperature and R the gas constant. ΔV is the difference between the final and initial volume in the entire system at equilibrium (reaction volume), including the solute and the surrounding solvent.

In formal analogy, the dependence of the rate constant k on the pressure, which can be derived from the Eyring theory, can be written:

$$\left[\frac{\delta \ln k}{\delta p} \right]_T = - \frac{\Delta V^\ddagger}{RT} \quad (2)$$

where ΔV^\ddagger is the apparent volume change of activation (activation volume) and represents the difference in volume between the reactants and the transition state.

Thus, any equilibrium connected with a non-zero volume change will be shifted toward the more compact state by the application of hydrostatic pressure; and any reaction connected with a positive (negative) activation volume will be slowed down (accelerated) by pressure. Based on equations (1) and (2), if a reaction is accompanied by a volume decrease of 300 ml mol⁻¹, it is enhanced more than 200 000-fold by applying a pressure of 100 MPa. This means that a reaction, which takes 146 days at 0.1 MPa, can be accomplished in only 1 min at 100 MPa.

Equilibrium thermodynamics determining the structure and stability of biological macromolecules depend mainly on three kinds of interactions: ionic, hydrophobic and hydrogen bonding. Ion pairs in aqueous solution are strongly destabilized by hydrostatic

pressure. This effect is attributed to the electrostrictive effect of the separated charges: each of it arranges water molecules in its vicinity more densely than bulk water. Thus, the overall volume change favours the dissociation of ionic interactions under pressure. For the same reason the pH of water is shifted by 0.3 when pressure is raised from 0.1 to 100 MPa; in certain buffer systems the effect is even larger (for buffer systems with low pressure coefficients, cf. Kitamura *et al.*, 1987)

Similarly, the exposure of hydrophobic groups to water disturbs the “loosely packed” structure of pure water and leads to a hydrophobic solvation layer, which is assumed to be more densely packed. Hence, the exposure of hydrophobic residues occurring, for instance, during the unfolding of proteins is favoured at elevated pressure. In this context, the well established fact that van der Waals’ forces contribute significantly to hydrophobic interactions should be kept in mind. Since they tend to maximize packing density, one would predict their pressure coefficient to be positive.

Formation of hydrogen bonds in biomacromolecules is connected to a negligibly small reaction volume, which may be positive or negative, depending on the model system. **Table 1.1** gives some typical values for the volume effects connected with biochemical reactions involving the various interactions. From the previous discussion of the size and sign of ΔV and ΔV^\ddagger , it is obvious that water as the main component of the cell and as a standard solvent in biochemistry plays a major role in the understanding of high-pressure effect (Gross and Jaenicke, 1994). Compared with gases, water is nearly incompressible, adiabatic compression of water increases the temperature by about 3°C per 100 MPa (Tauscher, 1995), while the freezing point decreases to -22°C at 207.5 MPa (Palou, 1999).

The effects of pressure on metabolic processes in living organisms are thought to be very complex. Even in the case of a well-known metabolic pathway such as glycolysis, elevated hydrostatic pressure might result in enhanced, neutral or inhibitory effects as a result of the variation in sign and size of the volume changes at each step. Although the value of ΔV or ΔV^\ddagger for biochemical reactions are known, it is still difficult to predict how elevated hydrostatic pressure would affect metabolic pathways or alter the pool sizes of metabolites in living organisms (Abe *et al.*, 1999).

Non-adapted (mesophilic) microorganisms commonly show growth inhibition at about 40-50 MPa. The cessation of growth is accompanied by morphological changes such as formation of filaments in *Escherichia coli* (Sato *et al.*, 2002) and cell chains or pseudomycelia in the marine yeast *Rhodospiridium sphaerocarpum* (Lorenz, 1993).

In the case of *Escherichia coli*, three kinds of pressure- responses are observed, categorized as pressure-inducible, pressure-independent, and pressure-repressible responses (Kato *et al.* 2000). Welch *et al.* (1993) report a unique stress response of *Escherichia coli* to abrupt shifts in hydrostatic pressure that result in higher levels of heat-shock proteins (Hsps) and cold-shock proteins (Csps), as well as many proteins that are produced only in response to high-pressure. A pressure of 30 MPa activates the *lac* promoter region of *Escherichia coli* and induces gene expression controlled by this promoter on a plasmid, but represses expression initiated from *malK-lamB* and *malEFG* promoters (Sato *et al.*, 1996b).

In the yeast *Saccharomyces cerevisiae* it has been found that a short period of heat-shock treatment allows the cells to survive at lethal level of pressure. Exposition to a temperature higher than optimum enhances the synthesis of heat shock proteins (Hsps104) and trehalose preventing protein denaturation and promoting stabilisation of membrane fluidity (Iwahashi *et al.*, 1997).

The maintenance of appropriate membrane fluidity is thought to be one of the key factors for survival and growth under high-pressure conditions. High-pressures exert effects on membrane systems that are comparable to the effects of low temperature. The more-ordered, less bulky crystalline (gel) state of membrane lipids is stabilized by high hydrostatic pressure, as indicated by the increasing melting points of lipids with increasing pressure. At constant temperature, high-pressure will cause a transition in the lipid bilayer to the gel state. An organism adapted to high-pressure might be expected to adjust its membrane phospholipid composition to offset these lipid-solidifying effects, perhaps by increasing the proportion of unsaturated fatty acids. This would be analogous to homeoviscous adaptation to changes in temperature (DeLong and Yayanos, 1985).

1.2.2 Mechanisms of inactivation

To explain the response of microorganisms to different pressures, high-pressure effects on several biological molecules have been studied. High-pressure is known to cause morphological, biochemical, and genetic alterations in vegetative microorganisms (Cheftel, 1995). Protein denaturation, lipid phase change and enzyme inactivation can perturb the cell

morphology, genetic mechanisms, and biochemical reactions. However, the mechanisms that damage the cells are still not fully understood (Perrier-Cornet *et al.*, 1995).

Niven *et al.* (1999) have observed a correlation between loss of cell viability and decrease in ribosome-associated enthalpy in cells subjected to pressures of 50-250 MPa for 20 min and suggest that the ribosome may be a critical target of lethal as well as growth-inhibitory effects of high-pressure on bacteria. Yayanos and Pollar (1969) found that in *Escherichia coli* DNA synthesis stopped approximately at 50 MPa, protein synthesis around 58 MPa and RNA synthesis at 77 MPa.

However, it is commonly acknowledged that membrane damage is the main cause of cell inactivation under pressure. Physical damage to the bacterial cell membrane has been demonstrated as leakage of ATP or UV-absorbing material from bacterial cells subjected to pressure or increased uptake of fluorescent dyes as propidium iodide (PI) that do not normally penetrate the membranes of healthy cells (Isaacs *et al.*, 1995; Pagan and Mackey, 2000; Ulmer *et al.*, 2000).

The intracellular pH in *Saccharomyces cerevisiae* can be measured under high-pressure conditions using pH-sensitive fluorescent probes. Hydrostatic pressure promotes the acidification of vacuoles in a manner dependent on the magnitude of the pressure applied up to 60 MPa (Abe and Horikoshi, 1997). Pressure-induced vacuole acidification is caused by the production of carbon dioxide. Hydration and ionization of CO₂ is facilitated by elevated pressure because a negative volume change ($\Delta V < 0$) accompanies the chemical reaction. Abe *et al.* (1997) suggest that the yeast vacuole works as a proton sequestrant under high-pressure conditions to maintain a favourable cytoplasmic pH.

The similarity between pressure-induced protein denaturation and pressure-induced inactivation of microorganisms and the observations of membrane damage and decrease of intracellular pH at high-pressure suggest that membrane-bound enzymes are one of the major target for pressure-inactivation (Smelt, 1998; Ulmer *et al.*, 2000, 2002; Wouters *et al.*, 1998). In *Lactobacillus plantarum*, pressure treatment causes partial inactivation of the F₀F₁ ATPase such that the ability of cells to maintain a Δ pH is reduced, and the acid efflux mechanism is impaired (Wouters *et al.*, 1998). Ulmer *et al.* (2000, 2002) report a reduction of the membrane-bound enzyme HorA activity prior loss of cell viability of *Lactobacillus plantarum* during high-pressure treatment, indicating loss of hop resistance of pressurized cells. The effects of pressure on the activity of membrane proteins have been studied also with the Na⁺/K⁺-ATPase from teleost gills (Gibbs and Somero, 1989). The decrease of activity was found to correlate with the reduction of membrane fluidity. The physical state of the lipids

that surround membrane proteins plays a crucial role in the activity of membrane-bound enzymes, and there is considerable evidence that pressure tends to loosen the contact between attached enzymes and membrane surfaces as a consequence of the changes in the physical state of lipids that control enzyme activity (Heremans, 1995).

Loss of membrane functions such as active transport or passive permeability would be expected to impair the cell homeostasis and this might account for the increased sensitivity to environmental conditions.

High-pressure treatment also induces morphological changes in microbial cells. Separation of the cell wall and disruption in the homogeneity of the intermediate layer between the cell wall and the cytoplasmic membrane occur. Isaacs *et al.* (1995) have demonstrated with electron microscopy studies that ribosomal destruction in cells of *E. coli* and *L. monocytogenes* results in metabolic malfunctions that can cause cell death. For *Schizosaccharomyces pombe*, after a treatment at 100 MPa the nuclear membrane is damaged and fragmented (Sato *et al.*, 1996a). In the same study, a pressure treatment above 250 MPa dramatically changes the cytoplasmic substance, the cellular organelles can hardly be detected, and the fragmented nuclear membrane is barely visible (Sato *et al.*, 1996a). In *Lactobacillus viridescens* empty cavities between cytoplasmic membrane and outer cell wall and DNA denaturation are observed after pressure treatment above 400 MPa (Park *et al.*, 2001).

1.3 Effects and interactions of treatment variables

The extent of high-pressure inactivation depends on several parameters, such as the type of microorganism, the pressure level, the process temperature and time, and the composition of the dispersion medium (Cheftel, 1995).

1.3.1 Microorganisms

The pressure sensitivity of microorganisms varies with the species and probably with the strain of the same species and with the stage of the growth cycle at which the organisms are subjected to the high-pressure treatment. Generally, yeasts and moulds are most sensitive to high-pressure, gram-positive microorganisms are most resistant, possibly because of their cell wall structure, and gram-negative microorganisms are moderately sensitive. Vegetative forms are inactivated by pressures between 400 and 600 MPa, while bacterial spores are extremely resistant and can survive pressures in excess of 1,000 MPa. Among the gram-positive bacteria *Staphylococcus* is one of the most resistant and can survive treatment at 500 MPa for more

than 60 min (Earnshaw, 1995). The stage of growth of microorganisms is also important in determining sensitivity to high-pressure, as cells in the exponential phase are more sensitive than stationary-phase cells. (Cheftel, 1995). **Table 1.2** shows the comparison of pressure and heat inactivation of some pathogens at different conditions.

Cheftel (1995) reports that when the pressure resistance of various microorganisms is compared, the survival fractions determined by different investigators vary by a factor of 1 to >8 for different species of the same genus (*Salmonella*) or by a factor 1.5-3.5 for different strains of the same microorganism (*L. monocytogenes*).

Table 1.2 Approximate heat resistance and pressure resistance for some pathogenic bacteria
Table from Smelt, 1998.

Organism	D value at 60 °C (minutes)	Inactivation (log cycles) after 15 min pressure treatment			
		Pressure (MPa)			
		300	400	500	600
<i>Aeromonas hydrophila</i>	0.1-0.2	> 6			
<i>Pseudomonas aeruginosa</i>	0.1-0.2	> 6			
<i>Campylobacter</i>	0.1-0.2	> 6			
<i>Salmonella spp.</i>	0.1-2.5	1-4.5			
<i>Yersinia enterocolitica</i>	2-3	> 6			
<i>Escherichia coli</i>	4-6	1-2			
<i>Escherichia coli O157:H7</i>	2.5			2.5	
<i>Salmonella senftenberg</i>	6-10	3			
<i>Staphylococcus aureus</i>	1-10		0.1	1.9	2.1
<i>Listeria monocytogenes</i>	3-8		1-3	> 6	

Original data from Mossel *et al.*, 1995; Patterson *et al.*, 1995; Metrick *et al.*, 1989 and Styles *et al.*, 1991.

For food preservation it is important to notice that species of foodborne pathogens contain strains that are relatively resistant to pressure (Alpas *et al.*, 1999, 2000). *Listeria monocytogenes* and *Staphylococcus aureus* required for a 6 log reduction in the initial inoculum treatments for 20 minutes at 340 and 400 MPa, respectively (Patterson *et al.*, 1995). Another pressure-tolerant pathogenic bacterium is *Escherichia coli* O157:H7. Takahashi *et al.* (1993) report that for a 6 log cycle reduction of *Escherichia coli* O157:H7, a treatment at 700 MPa for 13 min is required.

1.3.2 Pressure level and time

The magnitude of inactivation depends on pressure level and time of pressurization. In most cases, at ambient temperatures it is necessary to apply pressures above 200 MPa in order to induce inactivation of vegetative microbial cells (Cheftel, 1995). For each microorganism, there is a pressure level at which increasing treatment time causes significant reductions in the initially inoculated microbial counts (Palou *et al.*, 1999). However, there is no proportional relationship between the increase in pressure and the reduction of the bacterial population. This relation appears to follow a nonlinear pattern becoming sigmoid. The inactivation curve for microorganisms subjected to high-pressure treatments is similar to that one observed for heat treatment, with a shoulder representing a period during which the cells are able to resynthesize a vital component. Inactivation ensues only when the rate of destruction exceeds the rate of resynthesis. Heinz and Knorr (1996) postulated a two-step-model for the kinetic analysis of the survivor data after a high-pressure treatment. The transition of the bacterial cell from a stable, actively proton-releasing state **A** to a metastable intermediate state **B** with lower internal pH is assumed to take place after a certain period of time, primarily dependent on the temperature and the pressure applied. The second step in the model (**B** → **C**) describes the cell death in consequence of the internal pH decrease as a combined action of pressure, temperature and pH.

The “tailing” presented in these curves suggests that the microorganism population produced from a bacterial culture are non-homogenous (Ritz *et al.*, 2000). Pressure inactivation as function of treatment time follows the same nonlinear pattern: shoulder, exponential inactivation and tail.

1.3.3 Temperature

The temperature during pressurization can have a significant effect on the inactivation of microbial cells. Microorganisms are less sensitive to pressurization at temperatures around their optimal temperature of growth. This resistance decreases significantly at higher or lower temperatures presumably because of phase transition of membrane lipids. At sub-zero temperatures moderately pressure levels are sufficient to inactivate microorganisms such as *Saccharomyces cerevisiae*, *Aspergillus niger*, *Escherichia coli* (Hayakawa *et al.*, 1998).

The effects of temperature are of great practical interest, because combined pressure-temperature processing may cause equivalent microbial inactivation ratios while operating at lower pressure levels and/or for shorter periods of time (Cheftel, 1995).

1.3.4 pH

Mackey *et al.* (1995) indicate that a reduction in the pH of the suspending medium causes a progressive increase in cell sensitivity to pressure. Stewart *et al.* (1997) reported an additional 3 log cycle reduction in *L. monocytogenes* CA when pressurized in buffer at pH 4.0 as compared with pH 6.0 at 353 MPa and 45°C for 10 min. Pressurization in the presence of either citric or lactic acid increases the viability loss of four foodborne pathogens by an additional 1.2-3.9 log cycles at pH 4.5 for both acids at 345 MPa (Alpas *et al.*, 2000). The inactivation does not appear to depend on the type of organic acid (citric, tartaric, lactic or acetic) used for acidification (Ogawa *et al.*, 1990). An interesting hypothesis is that pressure treatment could restrict the pH range that the bacteria can tolerate, possibly because of the inhibition of ATPase-dependent transfer of protons and cations, or their direct denaturation or the dislocation of bound ATPase in the membrane (Pagan *et al.*, 2001; Wouters *et al.*, 1998).

1.3.5 Composition of medium

The composition of the medium where the microorganisms are dispersed at the moment of pressurization significantly influences the efficiency of inactivation. The addition of solutes increases the number of viable cells in a high-pressure treated sample.

A protective effect of sucrose against pressure inactivation is reported by Oxen and Knorr (1993) in experiments with the yeast *Rhodoturula rubra*. A high-pressure treatment at room temperature and 400 MPa for 15 minutes inactivates the yeast *Rhodoturula rubra* when the a_w of the suspension media is higher than 0.96, while the number of survivors is higher when the a_w is depressed.

The work from Takahashi *et al.* (1993) shows a marked protective effect of 2-4 M sodium chloride against the pressure inactivation of *Escherichia coli* or *Saccharomyces cerevisiae* dispersed in a pH 7 buffer solution. Significant baroprotective effects of NaCl or glucose are also observed by Hayakawa *et al.* (1994) with suspensions of *Zygosaccharomyces rouxii* and *Saccharomyces cerevisiae*. Ogawa *et al.* (1990) report that for *Saccharomyces cerevisiae* inoculated in concentrated fruit juices, the number of surviving microorganisms depends on the juice-soluble solids concentration and observe that the inactivation effect at pressure ≤ 200 MPa decreases as juice concentration increases. These effects are suggested to relate to the decrease in water activity. A reduced a_w may originate a cell shrinkage because of dehydration thickening the cell membrane and reducing the cell size (Knorr, 1992; Palou *et al.*, 1997). However, the influence of NaCl or of sugars on the osmotic pressure of foods or

suspension media is not sufficient to explain the marked baroprotective effects of these solutes and the mechanism of protection remains unknown.

Several mono-, and disaccharides are significantly effective in providing protection against hydrostatic pressure and high temperature damage in *Saccharomyces cerevisiae*. The extend of barotolerance and thermotolerance with different sugars shows a linear relation to the mean number of equatorial OH groups (Fujii *et al.*, 1996). Same linear relationship is seen when sugars protect protein molecules against elevating temperatures *in vitro*. These results suggest that sugars may protect cells against hydrostatic pressure and high temperature in the similar manner probably by stabilizing macromolecule(s) (Fujii *et al.*, 1996).

1.3.6 Antimicrobial compounds

The intensity of pressure required to inactivate microorganisms can be reduced in the presence of antimicrobial compounds, since moderate pressurization or short exposures can cause sublethal injury to cells, making them more susceptible to antibacterial compounds, such as bacteriocins and lysozyme (Kalchayanand *et al.* 1998a-b; Karatzas *et al.*, 2001). At ambient pressure, lysozyme is completely inactive against most gram-negative bacteria because it cannot penetrate the outer membrane to reach its target, the peptidoglycan. However, high-pressure can sensitise *Escherichia coli* and some other gram-negative bacteria to lysozyme probably through pressure-assisted self-promoted uptake (Masschalck *et al.*, 2000, 2001). The increased efficacy of the synergistic combination of high-pressure and nisin may also be explained by changes in membrane fluidity. Steeg *et al.* (1999) propose that the binding of nisin would directly increase the susceptibility of microorganisms during high-pressure treatment because of an assumed local immobilization of phospholipids. In addition, the high-pressure treatment may still cause indirect (sublethal) injury by facilitating the access of nisin to the cytoplasm membrane as a result of cell wall (and/or outer membrane for gram-negative microorganisms) permeabilization (Hauben *et al.*, 1996; Patterson *et al.*, 1995; Steeg *et al.*, 1999).

Some studies on microbial inactivation by high-pressure in combination with additives like ethanol (Tamura *et al.*, 1992), chitosan (Papineau *et al.*, 1991), nisin (Hauben *et al.*, 1996; Masschalck *et al.*, 2000, 2001; Steeg *et al.*, 1999), carvacrol (Karatzas *et al.*, 2001), CO₂ (Erkmen 2000; Haas *et al.*, 1989; Hong *et al.*, 1997; Karaman und Erkmen 2001), hop bitter compounds (Ulmer *et al.*, 2000) and EDTA (Hauben *et al.*, 1996) have been reported.

1.4 Kinetics of microbial inactivation

The patterns of high-pressure inactivation kinetics observed with different microorganisms are quite variable. Some investigators indicate first-order kinetics in the case of several bacteria and yeast (Carlez *et al.*, 1993; Cheftel, 1995; Hashizume *et al.*, 1995). Other authors observe a change in the slope and a two-phase inactivation phenomenon, the first fraction of the population being quickly inactivated, whereas the second fraction appears to be much more resistant (Cheftel, 1995; Heinz and Knorr, 1996). The pattern of inactivation kinetics is also influenced by pressure, temperature, and composition of the medium (Ludwing *et al.*, 1992).

For the use of high-pressure in food processing, it is of special interest to determine the process conditions for pressure pasteurisation in view of industrial application (Cheftel, 1995). In heat processing, inactivation is based on the assumption that death of microorganisms is log-linear with time. Although deviations from log-linearity have been found in heat inactivation, such deviations seem to be more common in pressure inactivation. Deviation from log-linearity could be explained as a two step reaction passing through an intermediate stage. This metastable intermediate state is reached after endogenous homeostatic mechanisms cannot longer balance the pressure induced displacements of equilibrium (Heinz and Knorr, 1996). A satisfactory description is possible by applying distribution models used in toxicology (Smelt, 1998).

Survival curves of pressure inactivation often show pronounced survivor tails (Patterson *et al.*, 1995). There are a number of possible theories to explain this “tail effect” in pressure-inactivation: tailing is a normal characteristic associated with the inactivation or resistance mechanisms, is the result of microbial population heterogeneity or is the result of experimental errors (Earnshaw, 1995). Another possible reason for tailing is microbial adaptation and recovery during and after pressure treatment (Palou *et al.*, 1999). Isolated, cultivated, and subjected to a second pressurization step, the pressure resistant fraction often displays again two-phase inactivation kinetics (Ulmer *et al.*, 2000).

Ritz *et al.* (2001) have shown that even if the microbial population appears totally inactivated by treatment and no culture growth is recorded, variable degrees of injury can be inflicted on the cells by high-pressure treatment. This heterogeneity of the treated cell population suggests that reversible damage may occur and cellular repair and/or growth under favourable conditions should not be ruled out. Those conditions are determining factors in further high-pressure applications in the food preservation industry. To design appropriate processing conditions for high-pressure treatments, it is essential to know the precise barotolerance levels

of different microbial species and the mechanisms by which that barotolerance can be minimized (Tewari *et al.*, 1999).

1.5 Aim of this thesis

The aim of this thesis was to study the mechanism of high-pressure induced growth inhibition and cell death distinguishing primary from secondary pressure effects. The lactic acid bacterium *Lactococcus lactis* was chosen as a model system and its responses to high-pressure were to be characterised during growth and after pressure-induced inactivation.

Therefore, using pressures between 0.1 and 100 MPa the growth under pressure conditions should be investigated. Temperature and pressure upper limits of growth were to be determined and the changes of morphology and growth rate to be reported. DAPI-staining and indirect immunofluorescence microscopy should be applied to characterise the growth of *Lactococcus lactis* under sublethal pressure.

Treatments above 200 MPa should be used to study the pressure-induced inactivation. Cells were to be characterised after high-pressure treatment by means of metabolic activity, membrane damage and viability. Modifying the composition of the medium, the effect of ionic and non-ionic solutes on the barotolerance of *Lactococcus lactis* was to be investigated. Alterations on the membrane resulting from high-pressure treatment should be investigated by online measurements of intracellular pH and membrane potential. Furthermore, the inactivation of the membrane-bound enzyme LmrP should be studied.

2. Material and methods

2.1.1 Abbreviations and solutions

cFDASE: 5(and 6-)-Carboxyfluorescein diacetate N-succimidyl ester; cFSE: 5(and 6-)-Carboxyfluorescein succimidyl ester; DAPI: 4',6-diamidino-2-phenylindole, dihydrochloride; EB: Ethidium bromide; EDTA: Ethylenediaminetetraacetic acid; HEPES: (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]); Laurdan: 6-dodecanoyl-2-dimethylaminonaphthalene; MES: (2-[N-Morpholin]ethanesulfonic acid); TRIS: Tris (hydroxymethyl)-aminomethan; SDS: Sodium n-Dodecyl sulfat.

Poly-L-lysine solution: 1 mg/ml; lysozyme solution: 25mM Tris-HCl (pH 8), 50 mM glucose, 10mM EDTA, 2 mg/ml lysozyme; PBSTE: 140mM NaCl, 2mM KCl, 8mM Na₂HPO₄, 0.05% Tween 20, 10mM EDTA.

2.1.2 Chemicals

EDTA, HEPES, MES, valinomycin and nigericin were obtained from Sigma-Aldrich (Steinheim, Germany); cFDASE from Fulka (Buchs, Switzerland); DAPI, Alexa Fluor® 488 goat anti-rabbit IgG, LIVE/DEAD® BacLight™, Laurdan from Molecular Probes (Eugene, USA); EB and TRIS from Boehringer (Mannheim, Germany). All other chemicals and M17 and MRS broths were analytical grade from Merck (Darmstadt, Germany).

2.2 Bacterial strains and media

The bacteria used in this work are shown in **Table 2.1**.

Table 2.1 Bacterial strains

Microorganisms	Origin	Reference
<i>Lactococcus lactis</i> ssp. <i>cremoris</i> TMW 1.1085	MG1363 plasmid cured strain of NCDO 712	Gasson, M.J
<i>Lactococcus lactis</i> TMW 2.26	dairy	
<i>Lactococcus lactis</i> TMW 2.27	dairy	
<i>Lactococcus lactis</i> TMW 2.28	dairy	
<i>Lactococcus lactis</i> TMW 2.29	dairy	
<i>Lactococcus lactis</i> TMW 2.118	DSM 20661	Garvie, E.I., and Farrow, J.A.E.
<i>Lactococcus lactis</i> ssp. <i>cremoris</i> TMW 1.234	NCDO 712	Davies, F.L. et al.
<i>Lactobacillus sanfranciscensis</i> TMW 1.53	DSM 20451	Kline, L., and Sugihara, T. F.
<i>Lactobacillus plantarum</i> TMW1.460	spoiled beer	

Lactococcus lactis strains were grown anaerobic at 30°C in M17 broth (Terzaghi and Sandine, 1975) supplemented with 1% glucose (GM17 broth).

Lactobacillus plantarum TMW1.460 and *Lactobacillus sanfranciscensis* TMW1.53 were grown at 30°C under anaerobic conditions in MRS and MRS-4 broth, respectively (De Man *et al.*, 1960; Stolz *et al.*, 1995).

2.3.1 Milk buffer

The milk buffer was chosen to contain the same amounts of minerals and lactose as whey from rennet casein; the buffer contained the following compounds (g·l⁻¹): KCl, 1.1; MgSO₄·7H₂O, 0.7110; Na₂HPO₄·2 H₂O, 1.874; CaSO₄·2 H₂O, 1; CaCl₂·2 H₂O, 0.99; citric acid, 2; lactose, 52. The pH was adjusted to 6.5 with KOH (2 M)

2.3.2 Milk filtrate

Milk filtrate was prepared by ultrafiltration (Filtron, Germany), the filtration surface was 0.35 m². The membrane type was minisette, omega open channel made up of polyethylensulfon (10000 dalton). The process parameters were:

volume flow: 610 l/h

pressure difference at plate-input: 3-4 bar

pressure difference at plate-output: approx. 2.3 bar

temperature: 50°C

2.4 Determination of water activity (a_w) of milk buffers

The water activity was determined by measuring the freezing point depression with a Crioscope (Type A0284, Knauer GmbH, Berlin).

2.5 Pressurization of cell suspensions

The cells of an overnight culture were harvested by centrifugation (15 min at 5500 rcf·g), washed and resuspended in milk buffer or milk filtrate to about 10⁹ cfu·ml⁻¹. The cells were suspended in 2 ml portions in sterile plastic micro test tubes, sealed with silicon stoppers and stored on ice until they were pressurized. The pressure chamber was heated/cooled to a desired level prior to pressurization with a thermostat jacket connected to a water bath. A computer program controlled the pressure level, time and temperature of pressurization. The compression/decompression rate was 200 MPa min⁻¹, the temperature was 20°C and the temperature rise due to compression was 12 °C or less. Samples were placed in the pressure

chamber 5 min prior to treatment to equilibrate the sample temperature. Cells were exposed to a pressure of 200, 300, 400 or 600 MPa for various time intervals (0-120 min). Following the release of pressure the samples were stored on ice for determination of viable counts and membrane integrity. For each HP inactivation kinetics, untreated cultures and cultures sterilized by treatment with 800 MPa for 10 min or 15 min at 80°C were used for preparation of “calibration samples” containing 100, 50, 25, 12.5, 6.25, 3.125 and 0% viable cells.

2.6 Methods for characterization of pressure-induced inactivation on cells

2.6.1 Enumeration of viable cells

The cell suspensions from each vial were serially diluted with saline immediately after the pressurization treatment. *Lactococcus lactis* strains were surface plated on GM17 agar, *Lactobacillus plantarum* TMW1.460 on MRS agar, and *Lactobacillus sanfranciscensis* TMW 1.53 on MRS-4 agar. The plates were incubated at 30° under anaerobic conditions, plates of *Lactococcus lactis* strains were counted after 24 h, plates of *Lactobacillus plantarum* and *Lactobacillus sanfranciscensis* after 48 h.

Selective agars were obtained with the addition of 3% NaCl to GM17 agar and 4% to MRS agar. Data presented are mean \pm standard deviation obtained from two to three independent experiments.

2.6.2 Membrane integrity assay

1 ml pressure-treated cell suspensions were harvested by centrifugation at 6000 rcf-g for 10 min. The supernatant was removed and the pellet resuspended in 1 ml of phosphate buffer. A stock solution of the LIVE/DEAD[®]BacLight[™] was prepared, the final concentration of each dye was 33.4 μ M SYTO[®] 9 and 200 μ M propidium iodide (PI). 100 μ l of each of the bacterial cell suspensions were mixed in 100 μ l of the stock solution, mixed thoroughly and incubated at 30°C in the dark for 5 min. The fluorescence intensities of SYTO[®] 9 and PI were measured with excitation and emission wavelengths of 485 and 520 nm, and 485 and 635 nm, respectively, using a spectraflour microtiter plate reader (TECAN, Grödig, Austria). The ratio of SYTO[®] 9 to PI fluorescence intensity was used as measure for membrane integrity. A calibration curve was established for each inactivation kinetics using the “calibration samples” described above and the results are reported as % intact membranes.

2.6.3 Metabolic activity assay

The method developed by Ulmer *et al.* (2000) was used to determine the metabolic activity. 1 ml pressure-treated cell suspensions were harvested by centrifugation. The supernatant was removed and the pellet resuspended in 1 ml of phosphate buffer. A stock solution of tetrazolium was prepared mixing 4-iodonitrotetrazolium violet (INT; 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chlorid) and glucose in phosphate buffer. The final concentration of each was 4 mM and 20 mM respectively. 100 μ l of each of the bacterial cell suspensions were mixed with 100 μ l of the stock solution of tetrazolium salt. The absorbance was measured at 590 nm during 30 min with a spectraflour microtiter plate reader (TECAN, Grödigg, Austria). A calibration curve was established for each inactivation kinetics using the “calibration samples” described above and the results are reported as % metabolic activity.

2.6.4 LmrP activity assay

The method developed by Ulmer *et al.* (2000) was used to determine the LmrP activity. 1 ml pressure-treated cell suspensions were harvested by centrifugation. The supernatant was removed and the pellet resuspended in 1 ml of phosphate buffer with 40 μ M ethidium bromide. Samples were mixed and incubated at 30°C for 2 h in the dark. After incubation, cells were harvested, resuspended in phosphate buffer and transferred to black microtiter plates. The fluorescence of cell suspensions was measured after addition of glucose using excitation and emission wavelengths of 485 and 595nm, respectively. The slope of the curves were calculated with the Software Sigma Plot, and the difference between slope of starved cells and that of untreated energized cells was considered to indicate LmrP activity.

2.6.5 Measurement of detection time of growth

Cells were pressurized as described above during 0 or 20 min at 300 MPa and 20°C. Untreated and pressurized cells were diluted in GM17 broth to obtain statistical probability of one cell per well. The total volume of each well was 150 μ l, and the media were overlaid with 100 μ l of paraffin to achieve anoxic growth conditions and to avoid evaporation losses of water. The growth of the organisms was monitored at 30°C by measuring the optical density (OD) of the growth media at 590 nm with a spectraflour microtiter plate reader (TECAN, Grödigg, Austria). Measurements were taken every 30 min for 48 h with preshaking at high intensity for 10 s prior to OD reading. The initial OD was substrated of each measurement. Results were reported as t_d (h) and defined as the time required for the spectrafluormeter to record a 0.03 increase in optical density from t_0 .

2.7 Methods for characterization of growth under high-pressure conditions

2.7.1 Determination of maximal pressure of growth

Fresh broth were inoculated with 0.1% of cells in stationary phase and placed in polypropylene tubes (Cryotubes; Nunc). After being sealed with parafilm, the samples were stored on ice until the application of hydrostatic pressure. The samples were put into titanium pressure vessels and subjected to hydrostatic pressure between 10 and 100 MPa at 30°C. The required hydrostatic pressures were reached within 2 min using a hand pump (Teramecs, Kyoto). The pressure was released in approximately 15 s. According to Abe and Horikoshi (1997) the changes in temperature because of the increase and decrease of pressure were negligible. The decrease of temperature because of decompression was about 0.5°C. Each chamber was opened after 20 and 48 h for *Lactococcus lactis* MG1363 and 25 and 48 h for *Lactobacillus sanfranciscensis*, the samples were stored on ice until they were required for further analysis. A control was kept at atmospheric pressure (0.1 MPa) at 30°C.

2.7.2 Determination of growth rate under pressure

For the determination of growth rate under pressure fresh broth was inoculated with 0.1% of cells in stationary phase and cultivated up to 48 h in the large-scale cultivation system “DEEP-BATH” (deep-sea baro/thermophiles collecting and cultivating system). This cultivation system has been designed to work within a 0-300°C range and up to 68 MPa, it is suitable for continuous sampling without decompression of the culture (Canganella *et al.*, 1997; Moriya *et al.*, 1995). Sample were taken at regular intervals, aseptically transferred to sterile vials and stored on ice until further measurements were performed. From each growth curve, the maximum growth rate μ_{\max} was obtained by fitting the OD readings to the logistic growth curve (Gänzle *et al.*, 1998). Sigma Plot 4.0 software was used for all curve fit routines.

2.7.3 Measurements of optical density

The optical density (OD) of the cells was measured using a U-2000A spectrometer (Hitachi, Japan) at 660 nm. If the OD was higher than 1.0, the samples were diluted in GM17 or MRS-4 broths.

2.7.4 Microscopic counts of cells

Samples were diluted with saline solution and the cells were counted using a Bacteria counting chamber (deep 0.02 mm, Erma, Tokyo) under a 3-phase-contrast microscope (Nikon, Japan).

2.7.5 Determination of maximal and optimal temperature of growth

Fresh broth was inoculated with 0.1% of an overnight culture. 15 ml were placed in each test tube from a bio-photorecorder (Temperature Gradient Bio-Photorecorder, Advantec, Japan) and incubated for 24 h. The first temperature gradient was between 12 and 57°C, the second between 25 and 45°C.

2.7.6 Indirect immunofluorescence microscopy

Immunofluorescence microscopy was performed as described by Sato *et al.* (2002) with the following modifications. Cells of *Lactococcus lactis* in exponential phase (OD 0.6) were fixed with 70% ethanol during 24 h at 4°C, washed and affixed to poly-L-lysine-treated slides. The slides were air dried, treated for 7.5 min with lysozyme solution and washed with PBSTE. The wells were pretreated with 2% (w/v) bovine serum albumin in phosphate buffered saline prior to incubation with the primary antibodies. The slides were incubated with α -Sv FtsZ rabbit IgG (1:3500 dilution) for 1 h, washed with PBSTE and treated with bovine serum albumin solution, before treatment for 1 h in dark with the secondary antibodies Alexa Fluor® 488 goat anti-rabbit IgG (1:1000 dilution). After washing the slides with PBSTE 5 μ l of DAPI stock solution (1mg/ml) was added to each well.

2.8 Method for measurement of internal pH

2.8.1 Labeling of cells with cFSE for pH_{in} determination

The fluorescence method developed by Breeuwer *et al.* (1996) was adapted to *Lactococcus lactis* ssp. *cremoris* MG1363 and *Lactobacillus plantarum*. Harvested cells were washed and resuspended in 50mM HEPES buffer, pH 8.0. Subsequently, the cells were incubated for 15 min at 30°C in the presence of 10.0 μ M cFDASE, washed, and resuspended in 50mM potassium phosphate buffer, pH 7.0. To eliminate nonconjugated cFSE, glucose (final concentration 10mM) was added and the cells were incubated for an additional 30 min at 30°C. The cells were then washed twice, resuspended in corresponding milk buffer and placed on ice until required.

2.8.2 Offline measurement of pH_{in} at ambient pressure

Stained cells were pipeted in a 2ml cuvette (Sarstedt, Nümbrecht, Germany) and placed in the cuvette holder of a spectrofluorometer (Perkin-Elmer Luminescence Spectrometer LS-50B, Überlingen, Germany). Fluorescence intensities were measured at excitation wavelengths of 485 and 410nm by rapidly alternating the monochromator between both wavelengths. The emission wavelength was 520, and the excitation and emission slit widths were 5 and 4nm, respectively. The 485-to-410-nm ratios were corrected for the background of the buffer. The incubation temperature was 30°C. Calibration curves were determined in buffers with pH values ranging from 4.0 to 8.0. Buffers were prepared from citric acid pH 4.0 and 5.0 (50mM), MES pH 5.5, 6.0 and 6.5 (50mM) and HEPES pH 7.0, 7.5 and 8.0 (50mM). The pH was adjusted with either NaOH or HCl. The pH_{in} and pH_{out} were equilibrated by addition of valinomycin (1 μM) and nigericin (1 μM), and the ratios were determined as described previously. Calibration curves were established for experiments performed on a single day.

2.8.3 *In-situ* measurement of pH_{in}

Fluorescence measurements under hydrostatic pressures were performed in a pressure chamber equipped with a cylindrical sapphire window (10 x 8mm). 2ml stained cells were placed in the pressure chamber and energized with 10mM glucose. The lid was closed and connected with an optical fiber to a spectrofluorometer (Perkin-Elmer Luminescence Spectrometer LS-50B, Überlingen, Germany). Fluorescence intensities were measured at excitation wavelengths of 485 and 410nm by rapidly alternating the monochromator between both wavelengths. The emission wavelength was 520, and the excitation and emission slit widths were 15nm to compensate the loss of fluorescence intensity caused by the optical fiber. The incubation temperature was 20°C, 5 min were allowed prior to high-pressure treatment to equilibrate the temperature.

Calibration curves were determined in buffers with pH values ranging from 4.0 to 8.0. Buffers were prepared from milk buffer pH 4.0, 5.0 and 6.0, and 50 mM HEPES buffer at pH 7.0 and 8.0. The pH was adjusted with either NaOH or HCl for HEPES buffers and with KOH for the milk buffers. The pH_{in} and pH_{out} were equilibrated by addition of valinomycin (1 μM) and nigericin (1 μM), and the fluorescence was determined as described above. Since the signal to noise ratio during measurements in the pressure chamber decreased compared to the measurement in cuvette, the fluorescence intensities were measured over a period of 5 min at either 0.1, 200, or 300 MPa, and the means were calculated for each buffer. A calibration curve was established for each culture stained and pressure treated on a single day.

2.8.4 Reversibility test

After the pressure treatment at pH 6.5, 1ml of the samples was placed in a cuvette and the fluorescence intensities were determined as described above. After 3 min of equilibration, glucose was added to a final concentration of 10mM and the changes in internal pH were monitored over up to 30 min.

2.8.5 Computation of the pH-values during pressure treatment

The measurements of the pressure-induced changes of pH in buffers used in this work were carried out by Volker Stippel at the Technische Universität München, Lehrstuhl für Fluidmechanik und Prozessautomation.

Various approaches have been proposed to calculate changes in pK_a values of water and weak acids (Lown *et al.*, 1970; Owen and Brinkley, 1941). The pK_a values were calculated using the following relation described by Hamman and El'yanov (1975):

$$pK_a = pK_a^0 + \frac{\Delta V_m^0 p}{2,303RT(1+bp)} \quad \text{equation 1}$$

where the superscript ⁰ denotes the value at atmospheric pressure, ΔV_m is the molar volume change between the associated and dissociated forms of the buffering acid in solution, R is the universal gas constant (8,3145 J/K mol), T the absolute temperature and b an universal constant (9.2 Pa⁻¹). Hamman and El'yanov (1975) showed that this equation fits very well to experimental data obtained with various buffers and pressures of up to 120 MPa. This equation was found empirically, is in good agreement to the electrostatic theory of Born for the interactions between ions in solutions (Born, 1920).

The application of equation 1 to mixtures of buffer salts exploits the balance of H₃O⁺ ions. The pH value at ambient pressure, e.g. measured with a pH glass electrode must be known. Furthermore, the pK_w , pK_a and ΔV values at ambient pressure for all reactions must be available from literature data. It is sufficient to know the sum of the concentrations c° at ambient pressure of all components from one species e.g. $c_{\text{H}_3\text{PO}_4}^0 + c_{\text{H}_2\text{PO}_4^-}^0 + c_{\text{HPO}_4^{2-}}^0 + c_{\text{PO}_4^{3-}}^0$ to calculate the concentration of all components upon pressure shift with the law of mass action. Equation 1 can be used to calculate the pK_w and pK_a values at high-pressure. For a starting pH_i (subscript i for iteration) the concentration of all components are calculated based on the law of mass action. Based on the change of these concentrations, the number of H₃O⁺ ions

formed or consumed by a reaction is calculated. Equation 2 shows an example for calculation of ΔH_3O^+ for phosphoric acid and its salts:

$$\Delta H_3O^+_{(PO_4)} = 3 \cdot c_{H_3PO_4}^0 + 2 \cdot c_{H_2PO_4^-}^0 + c_{HPO_4^{2-}}^0 - 3 \cdot c_{H_3PO_4} - 2 \cdot c_{H_2PO_4^-} - c_{HPO_4^{2-}} \quad \text{equation 2}$$

where c^0 and c denote the concentrations at ambient and high-pressure of the subscripted component, respectively. Water is treated like an acid (equation 3).

$$\Delta H_3O^+_{(H_2O)} = c_{OH^-} - c_{OH^-}^0 \quad \text{equation 3}$$

The iteration has converged, if the predicted sum of H_3O^+ formed by individual dissociation reactions is equal to the predicted change of pH (in equation 3 and fig. 1 shorted with $\Delta H_3O^+_{pH}$)

$$\sum_i \Delta H_3O_i^+ \underbrace{-(10^{-pH} - 10^{-pH_0})}_{= \Delta H_3O^+_{pH}} = 0 \quad \text{equation 4}$$

If the difference in equation 3 (with pH_i instead of pH) is lower than 0 one has to start the calculation with a smaller value of pH_i , and vice versa.

2.9 Methods for measurement of membrane phase state

2.9.1 Determination of temperature-dependent phase state of the membrane by FT-IR spectroscopy

Lipid phase transitions were measured by Fourier transform infrared (FT-IR) spectroscopy following methods described previously (Leslie *et al.*, 1995; Ulmer *et al.*, 2002). 2 ml cell suspension were harvested by centrifugation (3 min at 10000 rcf·g), washed twice and incubated 30 min in milk buffer, milk buffer with 0.5 M sucrose or milk buffer with 4 M NaCl. After incubation, the cells were harvested and the pellets were poured into a 20 μm thick infrared cell with CaF_2 windows. The FT-IR spectra were recorded with an Equinox 55 spectrometer (Bruker, Ettlingen, Germany) equipped with a liquid-nitrogen-cooled MCT (HgCdTe) detector. Twenty scans were averaged at each temperature point. Spectra were taken every 2°C between 0 and 30°C, and every 5°C between 30 and 40°C. The infrared cell was controlled by an external water thermostat.

2.9.2 Determination of the temperature and 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 (6-dodecanoyl-2-dimethylaminonaphthalene) (Molecular Probes, Eugene, USA) is an amphiphilic fluorescence probe, which allows the determination of gel-to-fluid phase transitions in biological membranes (Parasassi *et al.*, 1990, 1991, 1995, 1998). The cells of an overnight culture were harvested by centrifugation (15 min at 5500 rcf·g), washed twice and resuspended in milk buffer or milk buffer with additives. The cell suspensions were diluted to an optical density of 1 (OD₅₉₀). Laurdan stock solution in ethanol (2 mmol liter⁻¹) was added to the cell suspension to obtain an effective staining concentration of 40 μmol liter⁻¹. The cells were stained for 30 min at 30°C in the dark. After the incubation the cells were washed twice, resuspended in corresponding milk buffer and placed on ice until required.

For the determination of the temperature-dependent phase state of the membrane, stained cells were pipeted in a 2ml cuvette (Sarstedt, Nümbrecht, Germany) and placed in the cuvette holder of a spectrofluorometer (Perkin-Elmer Luminescence Spectrometer LS-50B, Überlingen, Germany). The excitation wavelength was 360 nm, and emission spectra were collected from 380 to 550 nm with steps of 1nm. The steady-state fluorescence parameter known as generalized polarization (GP) was calculated as follows:

$$GP = (I_{440nm} - I_{490nm}) / (I_{440nm} + I_{490nm})$$

where I is the relative fluorescence intensity at the respective wavelengths (Parasassi *et al.*, 1990). The temperature was controlled by a circulation water bath. Spectra were taken every 10°C between 10 and 40°C. For the high-pressure fluorescence studies the pressure chamber described above, equipped with a cylindrical sapphire window (10 x 8mm) was used. The pressure steps were 50 MPa with a ramp of 200 MPa min⁻¹. The time left for equilibration after each pressure step was 3 min. Data presented are mean ± standard deviation obtained from two to three independent experiments.

2.10 Determination of accumulated osmolytes after hyper-osmotic shock

2.10.1 Cell extract

Cells in the stationary phase (10⁹ cfu·ml⁻¹) were harvested (3 min at 9000 rcf·g) after incubation for 30 min in milk buffer or milk buffer with additives. Cell pellets were

resuspended in SDS-Puffer (100 mM Tris/HCl pH 9.5, 1% SDS (w/v)). Cell lysis was performed with ultrasonification (Bandelin Sonoplus), 3 x 1 min, 30% cycle and 100% power. After centrifugation at 14.000 rcf·g for 30 min at 4°C, the total supernatant fraction was recovered for analysis.

2.10.2 Determination of accumulated osmolytes by HPLC analysis of cell extracts

The method developed by Clarke *et al.* (1999) was used for the detection of sugars and amino acids in cell extracts of *Lactococcus lactis*. All experiments were performed using a high-pressure liquid chromatograph from Gynkotek. The system consisted of a gradient pump model 480, a Gina 50 autosampler, a column thermostat and an ED40 electrochemical detector containing a gold working electrode and a pH reference electrode. Norleucin was used as internal standard.

3. Results

3.1 Growth under high-pressure conditions

Many surface-dwelling bacteria are able to grow, albeit slowly, at pressures of up to 60 MPa. Above that pressure, however, growth will cease as a result of the complete inhibition of protein synthesis and the occurrence of abnormal phenomena, such as the formation of filaments and the disorganization of the cytoplasm (Mozhaev *et al.*, 1994; Sato *et al.*, 2002). The potential use of high-pressure in biotechnological processes depends on the elucidation of the mechanisms of high-pressure adaptation of microorganisms. Therefore, the effect of pressure on mesophilic bacteria used in food biotechnology, i.e. *Lactococcus lactis* and *Lactobacillus sanfranciscensis* at the level of growth rate, cell morphology and cellular organization was investigated. Pressures between 0.1 and 100 MPa were used to determine the pressure upper limit of growth of *Lactococcus lactis* and *Lactobacillus sanfranciscensis* cells. **Figure 3.1** shows the results for *Lactococcus lactis* after 20 and 48 h of incubation at different pressure levels. The cell densities were reduced according to the increase of pressure. At 50 MPa the cell density was just 2.3% of the control at atmospheric pressure. 60 to 100 MPa caused a 2-3 log cycle reduction of CFU. After 48 h the cells incubated between 0.1 and 40 MPa already went in the lysis phase, an increase of the CFU at 50 MPa comparing to the CFU after 20 h was observed. 60-100 MPa reduced the CFU by 3-5 log cycles.

Lactobacillus sanfranciscensis was more piezosensitive than *Lactococcus lactis*, this strain did not grow at 50 MPa (**Fig. 3.1**). 10 MPa had no significant effect on the CFU, 30 and 40 MPa showed a strong inhibitory effect on the growth after 25 h of incubation. 50 to 100 MPa inhibited the growth, but did not inactivate the inoculation cells. Cells grown at 0.1 MPa went into the lysis phase after 48 h of incubation, whereas cells grown between 10 and 40 MPa showed an increase of the CFU. At 40 MPa, the generation time was about 24 h and the final density after 48 h was 1% of the culture at ambient pressure. 50 MPa inhibited the growth, but did not inactivate the cells, 60 to 100 MPa caused a 1 log cycle reduction of CFU.

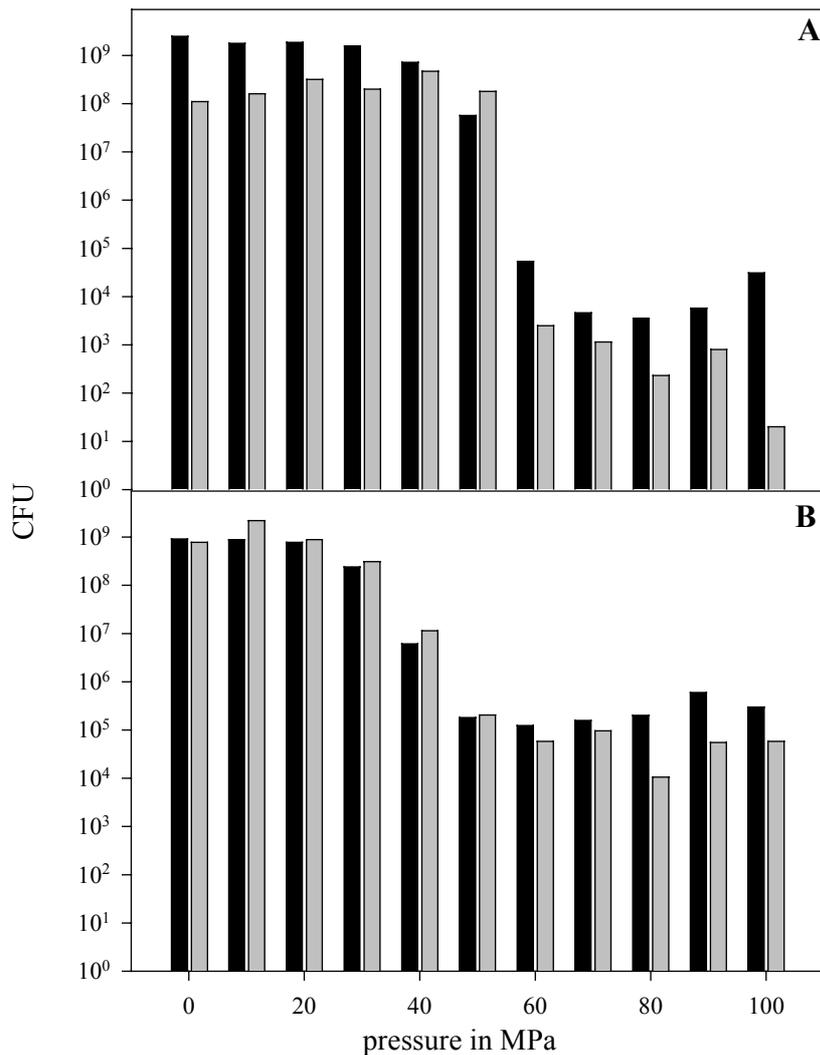


Fig. 3.1 Effect of pressure on growth and survival of *Lactococcus lactis* MG1363 (A) after 20 (■) and 48 h (▒) and *Lactobacillus sanfranciscensis* (B) after 25 (■) and 48 h (▒). Corresponding results were achieved by counts and optical density measurements.

3.1.1 Effect of temperature and pressure on growth rate

The specific growth rate profiles of deep-sea bacteria have indicated that they exhibit optimal high-pressure growth near their upper temperature limit for growth (Kato *et al.*, 1997, 2000). Therefore, the effect of increasing the temperature of growth under high-pressure conditions for *Lactococcus lactis* and *Lactobacillus sanfranciscensis* was determined.

The optimal and maximal temperature of growth for *Lactococcus lactis* was determined using a bio-photorecorder (Temperature Gradient Bio-Photorecorder, Advantec, Japan). The results are shown in Fig. 3.2, an optimal range was observed between 30 and 33°C with a growth rate of 1.7 1/h. Temperatures above the optimal range showed to have stronger inhibitory effect on the growth rate than sub-optimal temperatures.

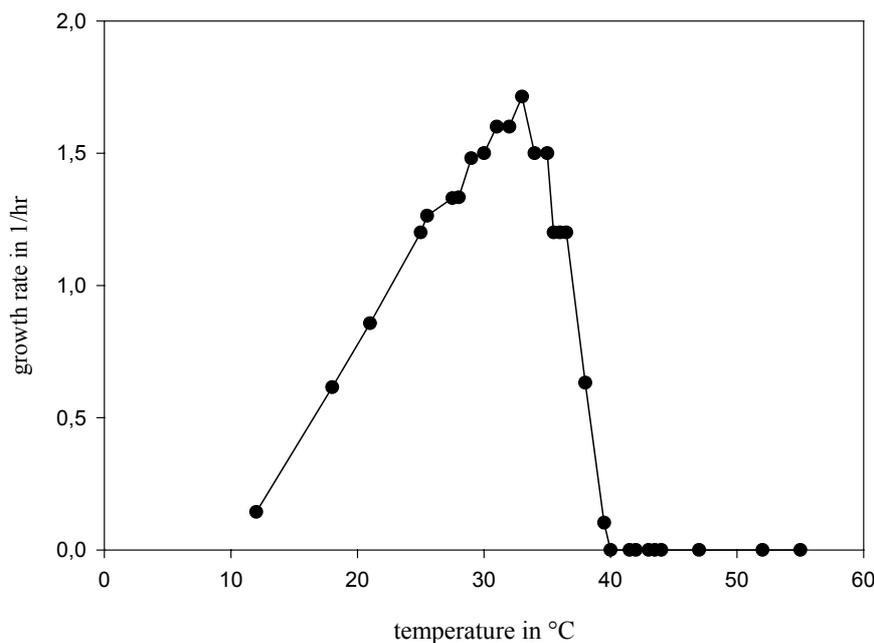


Fig. 3.2 Effect of temperature on the growth rate of *Lactococcus lactis* MG1363.

The effects of pressure and temperature on the growth rate are shown in **Fig. 3.3**. The experiments were performed in the “DEEP-BATH”. The temperature error was $\pm 1^\circ\text{C}$. The growth rate at 0.1 MPa and 30°C was 1.7 1/h and agreed with the result obtained by bio-photorecorder. The growth rate was reduced with an increase of pressure. 50 MPa was the upper limit of pressure for growth, the growth rate was 14% of that one at optimal conditions. The growth rate at 40°C and 0.1 MPa was higher than expected according to our results with the bio-photorecorder. This can be explained because the anaerobic conditions in the “DEEP-BATH” System were more favourable for the bacteria than the continuous aeration produced by shaking in the bio-photorecorder. A rapid reduction of the growth rate was observed with the raise of pressure, at 50 MPa the cell growth was inhibited.

Further experiments were performed at 35°C to determine the inhibitory effect under high-pressure conditions caused by a temperature between the optimal range and the upper limit. The growth rate at 0.1 MPa was 1.39 1/h approaching the value obtained by the bio-photorecorder. At 50 MPa the cells were still able to growth, but the growth rate was lower than the rate at 30°C . A raise of the temperature did not increase the piezotolerance of *Lactococcus lactis*.

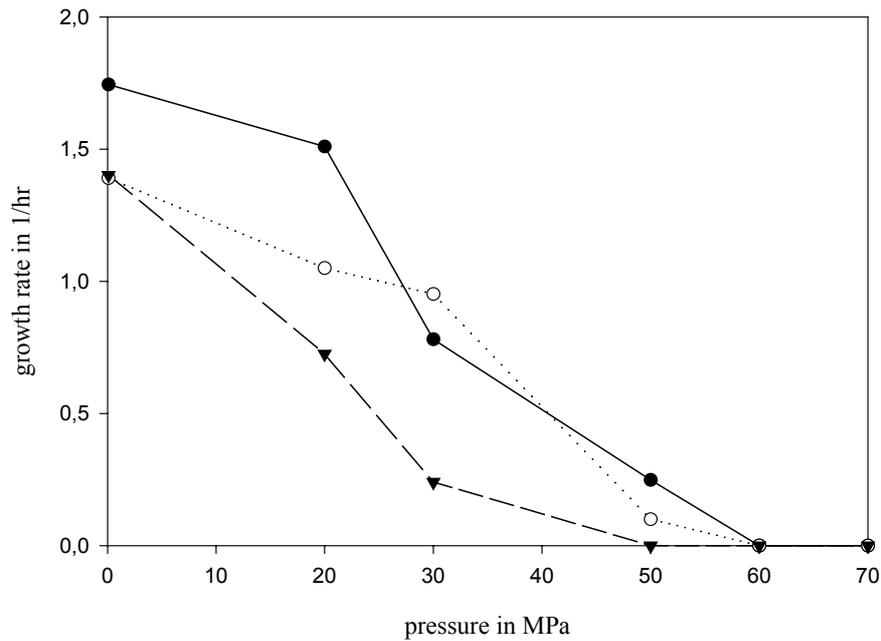


Fig. 3.3 Effect of pressure and temperature on the growth rate of *Lactococcus lactis* MG1363. (●) Growth rate at 30°C, (○) at 35°C and (▼) at 40°C.

The growth rate of *Lactobacillus sanfranciscensis* at 30°C is shown in **Fig. 3.4**. The growth rate at 30°C and 0.1 MPa was 0.66 1/h and was reduced proportional to the increase of the pressure. Since the strain *Lactobacillus sanfranciscensis* grows in anaerobic conditions, it was not possible to determine the upper limit growth temperature with the bio-photorecorder, which works under continuous shaking. According to Gänzle *et al.* (1998) we chose 38°C as upper limit temperature.

The results of the “DEEP-BATH” System did not show any apparent growth or inactivation at 0.1 MPa and 38°C after 48 h. 30 MPa caused a reduction of 1 log cycle after 24 h of incubation. The effect on the growth rate at atmospheric pressure of a temperature between the upper limit and the optimal was determined. The growth rates at 35 and 37°C were 85 and 58% of the growth at 30°C, respectively. As in the case of *Lactococcus lactis* increasing the temperature of growth of *Lactobacillus sanfranciscensis* did not increase its piezotolerance.

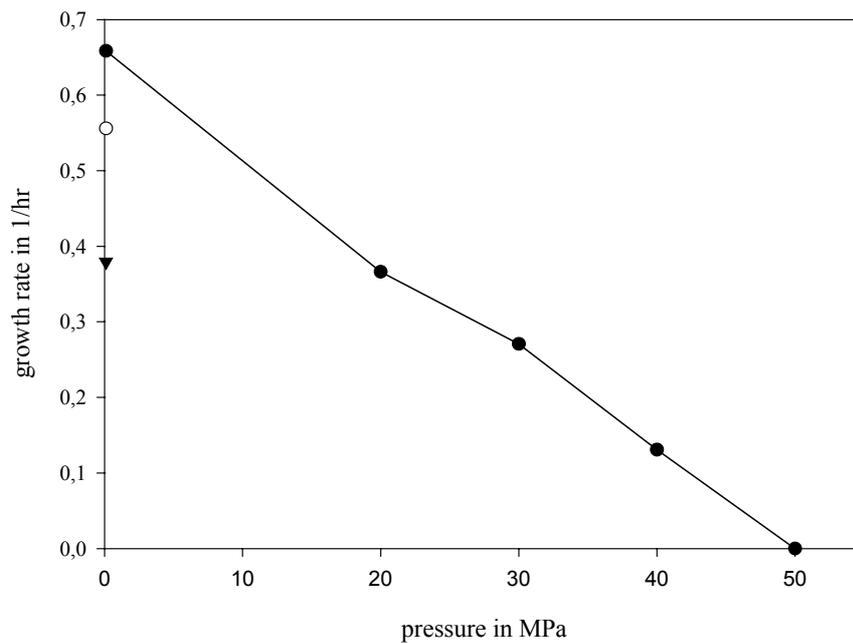


Fig. 3.4 Effect of pressure and temperature on the growth rate of *Lactobacillus sanfranciscensis* TMW1.53. (●) Growth rate at 30°C, (○) at 35°C and (▼) at 37°C.

3.1.2 Morphologic changes and immunofluorescence microscopy

Pressure induced changes in morphology of *Escherichia coli* cells have been observed by Sato *et al.* (2002). Therefore, cells of *Lactococcus lactis* and *Lactobacillus sanfranciscensis* grown between 0.1 and 100 MPa were observed with a 3-phase-contrast microscope. Additional observations were made after staining cells of *Lactococcus lactis* with FtsZ antibodies and DAPI by fluorescence microscopy.

Fig. 3.5A-C show cells of *Lactococcus lactis* grown at 0.1, 30 and 50 MPa. Under normal growth conditions *Lactococcus lactis* cells are present in form of diplococci and form chains in response to stress conditions such as insufficient energy sources. Our experiments showed that an increase of growth pressure also produced a chain formation. The size and incidence of the chains depended on the level of pressure. At 10 and 20 MPa 3-4 cells chains were observed, but the diplococcal appearance was predominant. At 30 MPa approximately 50% of the cells were in chains, these chains were formed with 4-6 cells. In addition a deformation of cell morphology was observed, the cells adopted a slight rod shape form (**Fig. 3.5B**).

Cells grown at 40-50 MPa were mainly in chains, the length varied from 3 to 14 cells, the exact count of the cells was difficult to determine because there was no clear septum between cells. A change in the cell morphology was noticed, they lost their spherical shape adopting a rod shape in the direction of the chain length. The presence of oversized cells was apparent

(Fig. 3.5C). At 60 MPa and higher pressures the cells did not grow, therefore they did not present noticeable morphological changes.

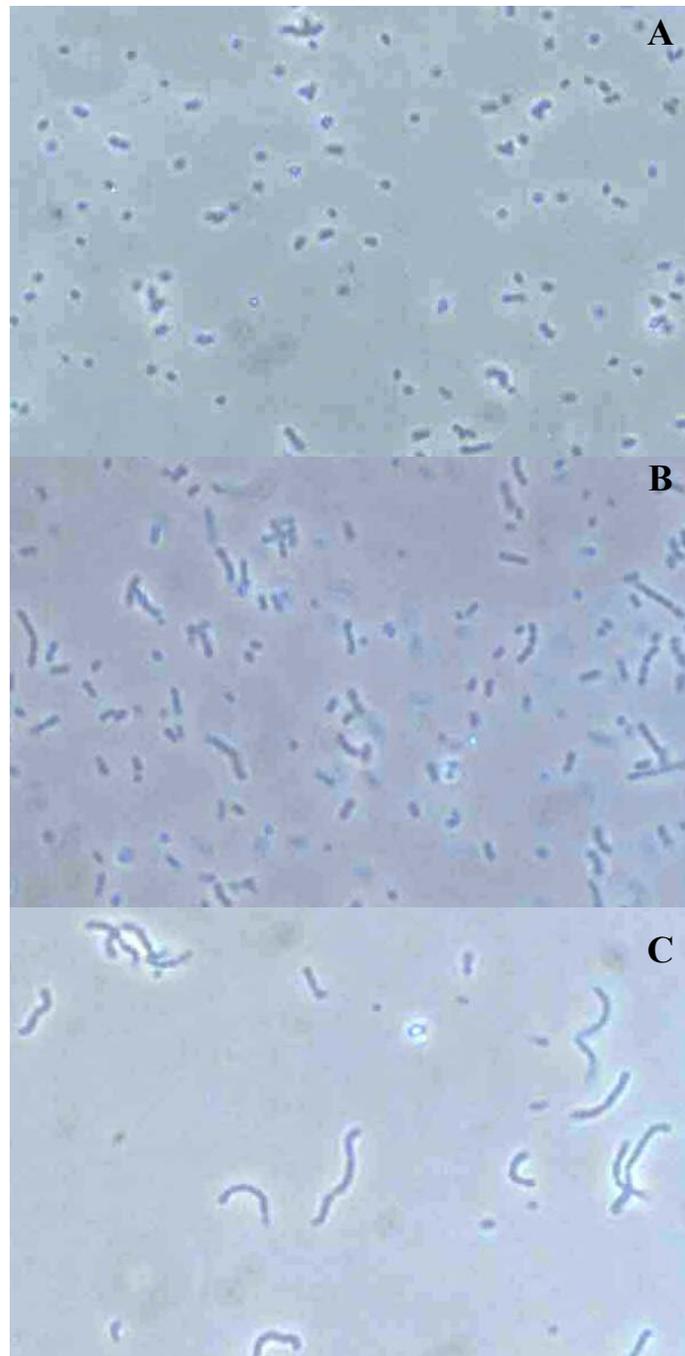


Fig. 3.5 3-phase-contrast microscopic images of *Lactococcus lactis* MG1363 grown at 0.1 MPa (A), 30 MPa (B) and 50 MPa (C).

Chain formation and changes in the cell morphology were also observed at temperatures of growth above the optimal range. The combination of higher pressure and temperature enhanced the changes of cell morphology.

Lactobacillus sanfranciscensis cells under normal conditions of growth have a short rod-shaped form. Cells grown at 10 MPa did not show any change of morphology. At 20 MPa the morphology of the cells did not change after 25 h of incubation, but after 48 h the cells were elongated. At 30 and 40 MPa elongated cells and nodal formation were apparent. Comparable morphological changes were observed during growth at temperatures above the optimum (data not shown).

Fig. 3.6 to 3.8 show microscopic immunofluorescence and DAPI-staining images of *Lactococcus lactis* cells grown at 0.1, 20 and 50 MPa in the exponential phase. The method used for the immunofluorescence microscopy (IFM) was developed for the gram-negative bacterium *Escherichia coli*, thus the following modifications were realised.

According to the National Center for Biotechnology Information (NCBI) and the Japanese Genetic Center (DDBJ), *Lactococcus lactis* FtsZ (Y15422) shares approximately 40% DNA-identity and 30% protein-identity with *Escherichia coli* FtsZ (X55034), and 57.5% DNA-identity and 48% protein-identity with *Shewanella violacea* FtsZ (AB052554). A Western blotting analysis was performed to verify the immunoreaction and to select the antibodies (data not shown). *Lactococcus lactis* FtsZ reacted positive to FtsZ antibodies of *Escherichia coli* and stronger to the antibodies of the piezophilic bacterium *Shewanella violacea* DSS12. Therefore, the antibodies of *Shewanella violacea* DSS12 were used for the IFM.

Cells in exponential phase grown at 0.1 MPa were harvested, washed and resuspended either in saline solution (0.85% NaCl) or in PBSTE and fixed 24 h at 4°C with 80% methanol or 70% ethanol. The fixation with methanol damaged the cells impairing the formation of pellets after fixation. Both treatments with ethanol were effective for cell fixation, but superior images were obtained with ethanol/PBSTE. Fixation of cells grown at 20 and 50 MPa were made with 70% ethanol and PBSTE during 24 h at 4°C.

The target of the lysozyme treatment is the permeabilization of the cell membrane to the antibodies. To determine the optimal duration of treatment, cells were exposed to the lysozyme solution during 0, 1.5, 3, 5 and 7.5 min. The best permeabilization was obtained after 7.5 min of treatment, and a slight cell shape deformation was also observed. This elongation did not interfere with the observation of high-pressure induced changes.

The morphological changes observed with a 3-phase-contrast microscope were confirmed. Diplococci were the prevalent form in cells grown at 0.1 and 20 MPa, filament were present

at 50 MPa. Chains of 4 cells were also observed at 20 MPa. Oversized cells were present at both high-pressure conditions.

DAPI staining did not show differences between growth conditions. Cells grown at high-pressure were stained and showed separated nucleoids, even though they formed chains and cell division was not apparent by light microscopy. The blue fluorescence of DAPI upon binding to AT regions of DNA indicated that the chromosomal DNA segregation and condensation was independent of the changes in morphology.

IFM exhibited relevant differences between *Lactococcus lactis* FtsZ at atmospheric and high-pressure conditions. Whenever all samples were in the exponential phase (OD_{600} 0.6), the presence of FtsZ rings was noticeable just in cells grown at 0.1 MPa. FtsZ rings are shown in **Fig. 3.6C**. At 20 MPa FtsZ rings were not observed. A reduced number of FtsZ rings was observed in slide areas where the cell density was high (**Fig. 3.9**).

To emphasise differences between cells grown at 0.1 and 50 MPa, **Fig. 3.10** shows selected cells observed with light microscopy, DAPI staining and IFM. **Fig. 3.10B** and **C** show cells before and during cell division at 0.1 MPa. During division the segregation and condensation of DNA indicated by separated nucleoids is accompanied with the presence of FtsZ ring between cells. Whereas in cells prior division the intensity of DAPI fluorescence increases because of DNA replication, the nucleoids are not clearly divided and no FtsZ ring was observed. **Fig. 3.10D-F** show cells grown at 50 MPa, the division between cells is difficult to determine with light microscopy, but DAPI staining indicates the presence of several nucleoids in one chain corresponding to duplication, segregation and condensation of DNA. However, FtsZ rings were not observed between cells.

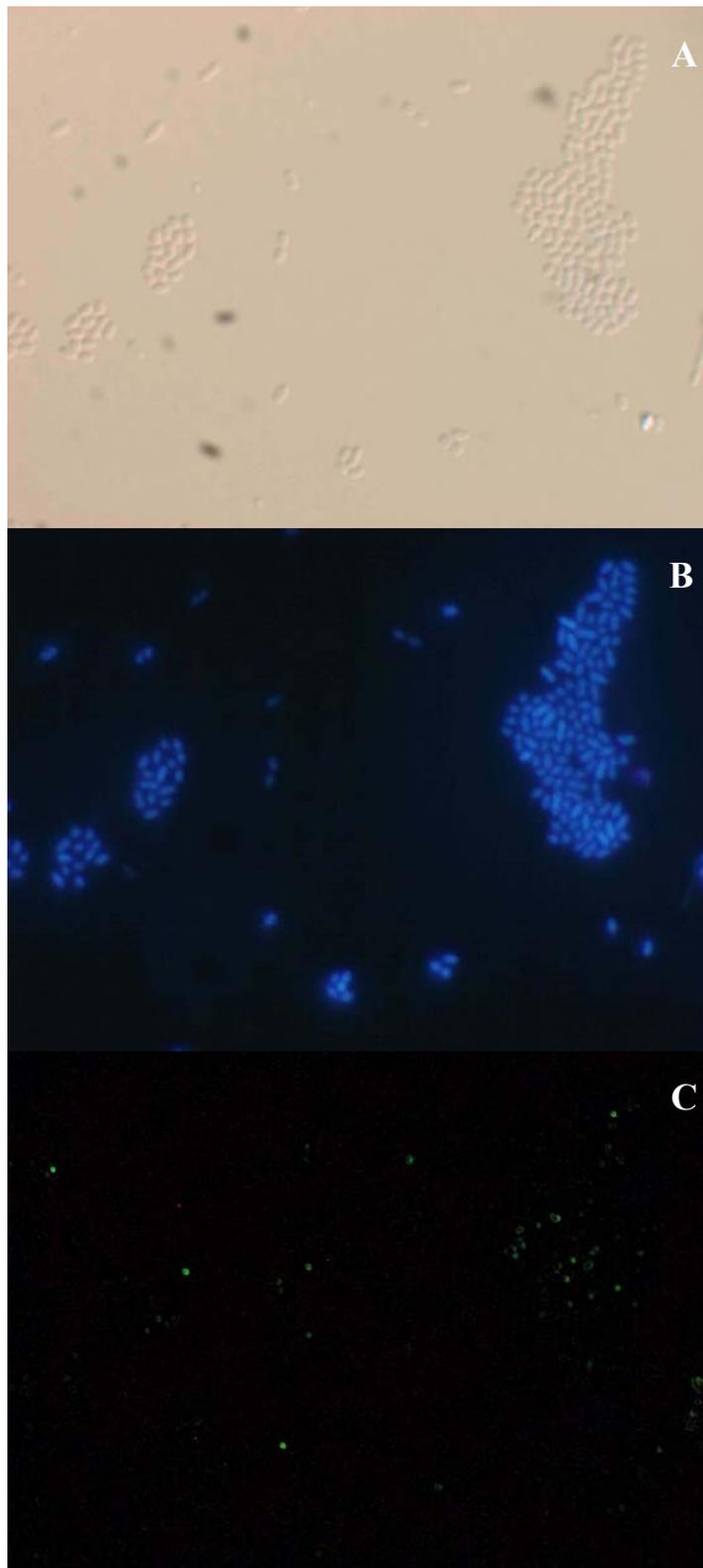


Fig. 3.6 Light contrast microscopic image of *Lactococcus lactis* MG1363 grown at 0.1 MPa (A), cells stained with DAPI (B) and immunofluorescence microscopy with Sv FtsZ antibodies (C).

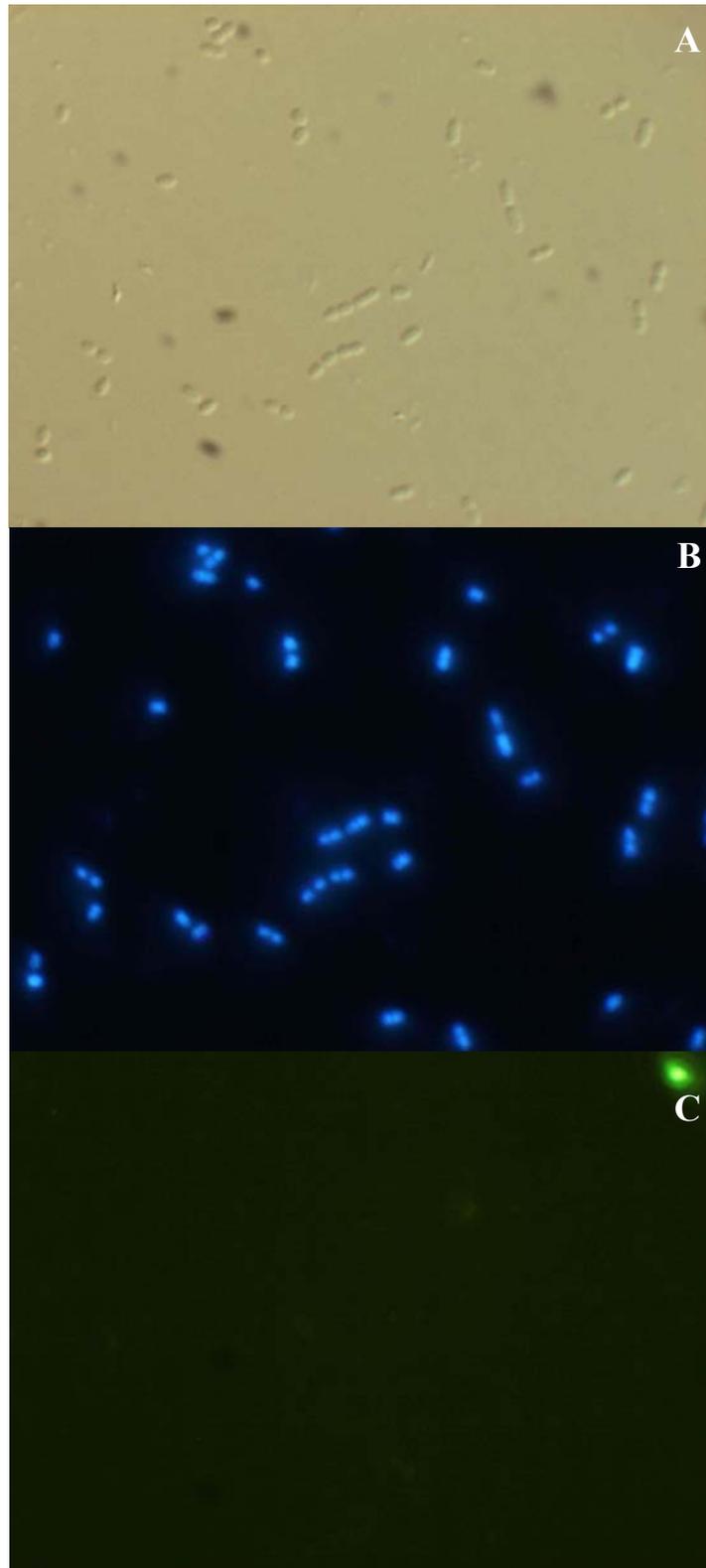


Fig. 3.7 Light contrast microscopic image of *Lactococcus lactis* MG1363 grown at 20 MPa (A), cells stained with DAPI (B) and immunofluorescence microscopy with Sv FtsZ antibodies (C).

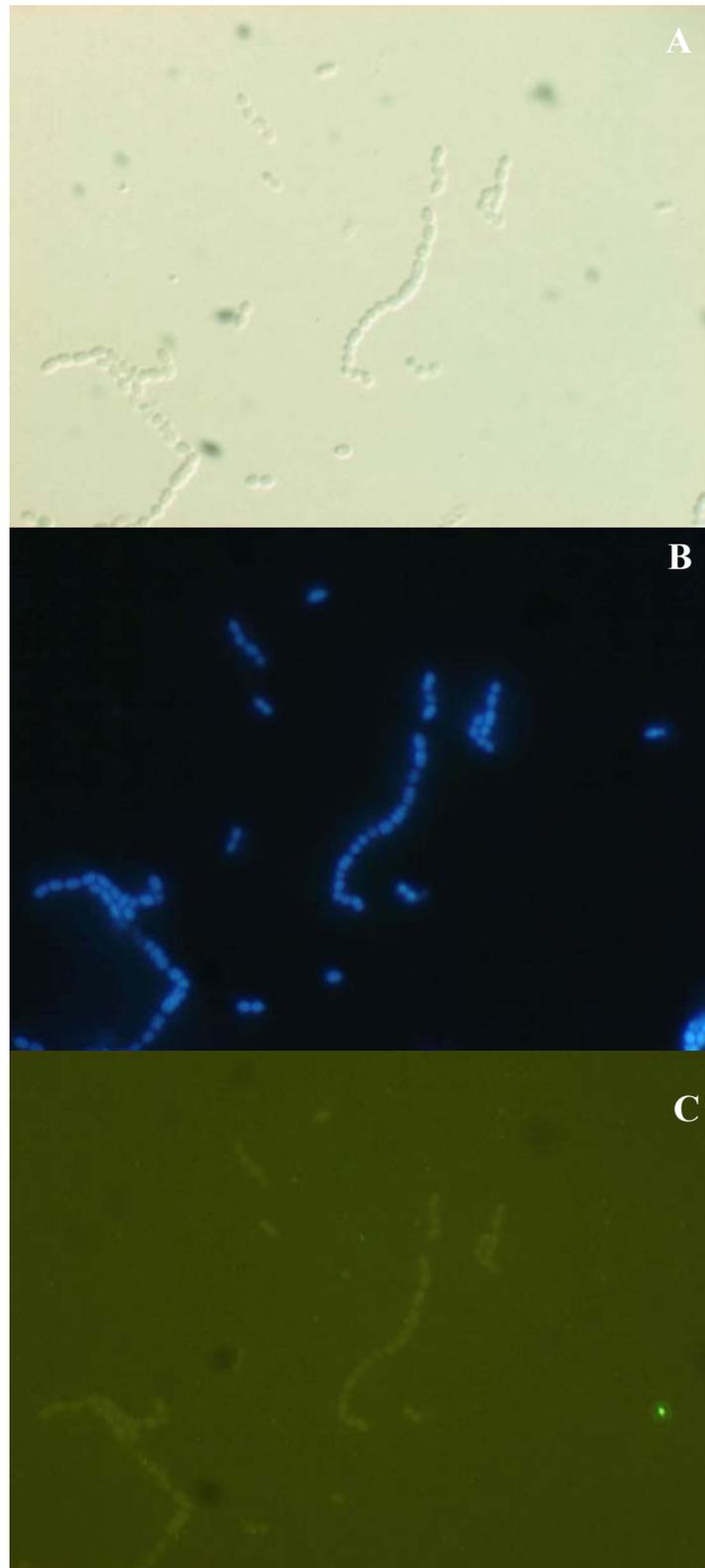


Fig. 3.8 Light contrast microscopic image of *Lactococcus lactis* MG1363 grown at 50 MPa (A), cells stained with DAPI (B) and immunofluorescence microscopy with Sv FtsZ antibodies (C).

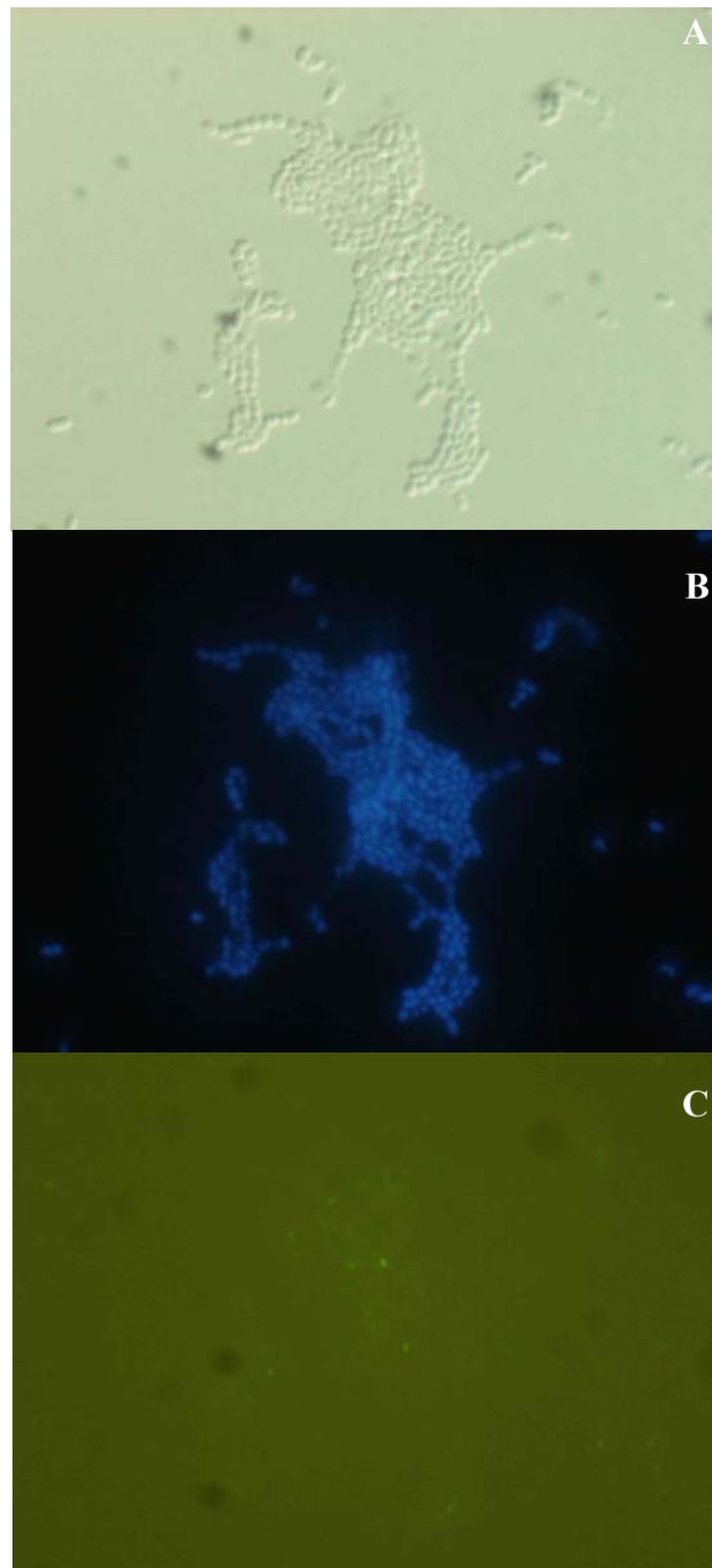


Fig. 3.9 Presence of FtsZ rings in cells grown at 50 MPa. **A:** light contrast microscopic image. **B:** DAPI staining. **C:** immunofluorescence microscopy with Sv FtsZ antibodies.

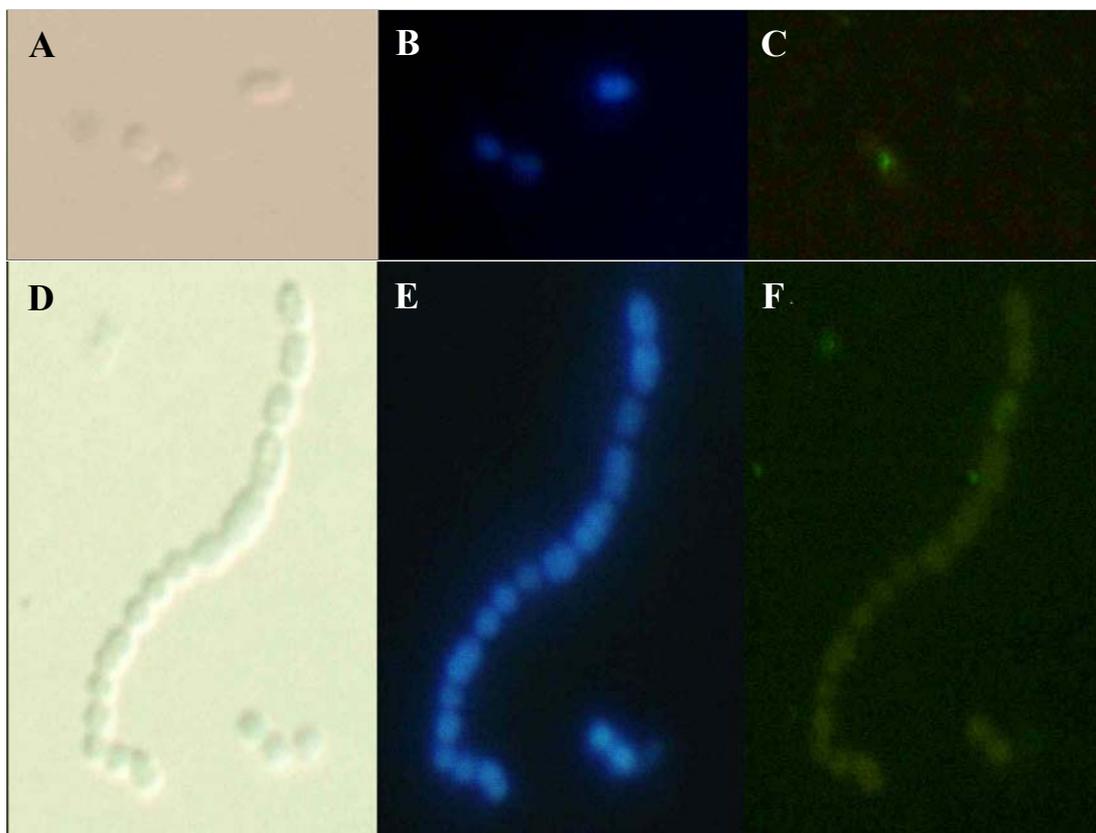


Fig. 3.10 Comparison of cells grown at 0.1 and 50 MPa. Light contrast microscopic image of *Lactococcus lactis* MG1363 grown at 0.1 MPa (A), cells stained with DAPI (B) and immunofluorescence microscopy with Sv FtsZ antibodies (C). Light contrast microscopic image of cells grown at 50 MPa (D), cells stained with DAPI (E) and immunofluorescence microscopy with Sv FtsZ antibodies (F).

3.2 Pressure inactivation. Development of model system and strain selection

3.2.1 Validation of milk buffer as food system

High-pressure treatment is considered as a non-thermal attractive process for preserving food (Knorr, 1993; Smelt *et al.*, 2002). Ideally pressure should result in microbiologically safe and stable foods over a wide range of products formulations. To be able to predict safety and stability, reliable food-like models are necessary to investigate high-pressure inactivation.

For this study a milk buffer was prepared containing the same amounts of minerals and lactose as whey from rennet casein. To verify the suitability of the milk buffer as food system, cells suspensions of the *Lactococcus lactis* ssp. *cremoris* MG1363 and *Lactococcus lactis*

subsp. *lactis* TMW 2.118 were exposed to a treatment at 300 MPa for different times intervals at 20°C in milk buffer and in milk filtrate, the results are shown in **Fig. 3.11A-B**. The inactivation curves of the bacteria in both media showed sigmoid asymmetric shapes when plotted in logarithmic scale. Within the inactivation curves two parts can be distinguished: an initial shoulder and an inactivation phase followed by tailing (Heinz and Knorr, 1996) (**Fig. 3.11A-B**). The inactivation curves of both microorganisms in milk buffer and in milk permeate did not show significant deviation, the differences were within the standard deviation of experiments. Further experiments were realized in the chemically defined milk buffer.

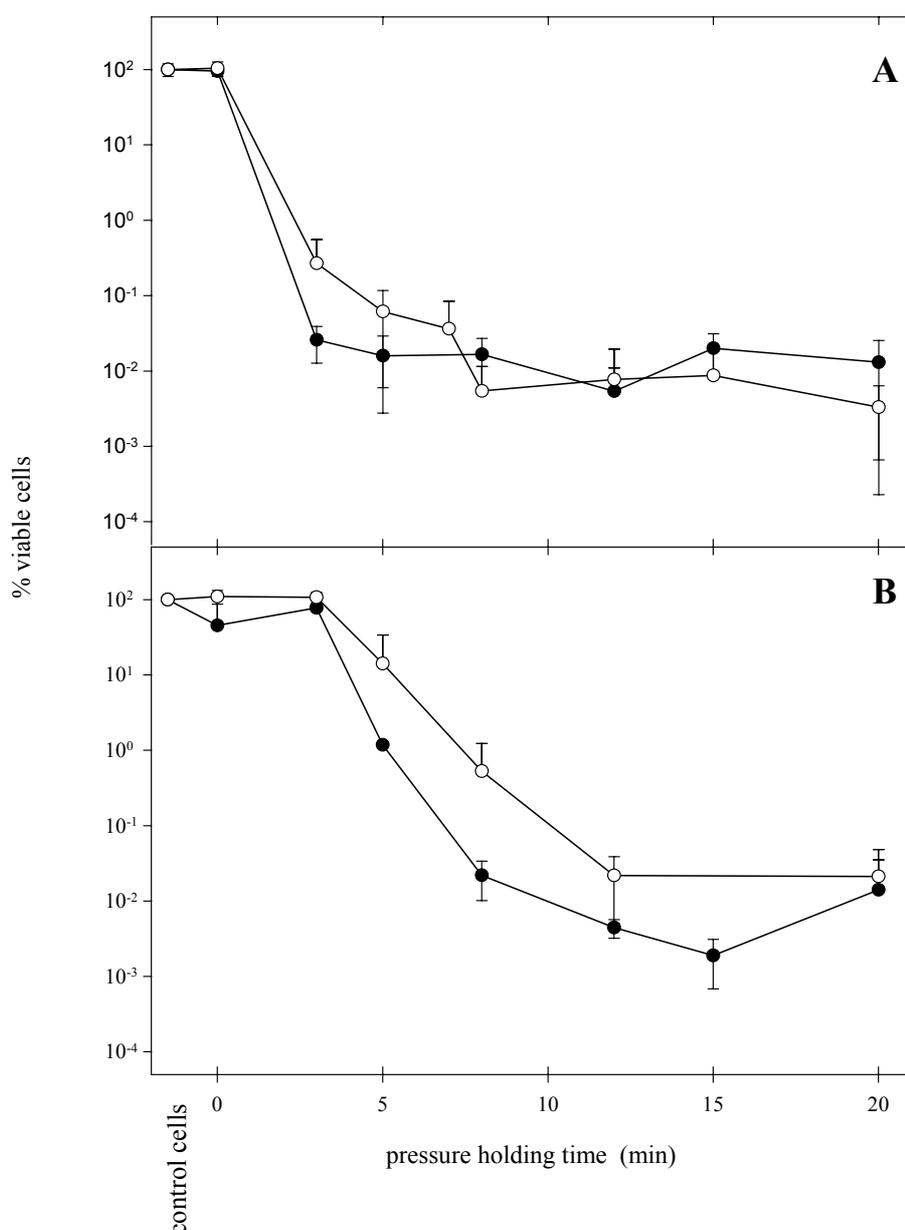


Fig. 3.11 Kinetics of inactivation of *Lactococcus lactis* cells in milk buffer (○) and milk filtrate (●) after pressure treatments at 300 MPa and 20°C. **A:** Treatments of *Lactococcus lactis* ssp. *cremoris* MG1363. **B:** Treatments of *Lactococcus lactis* subsp. *lactis* TMW 2.118.

3.2.2 Selection of strain

The choice of test strain is of paramount importance to facilitate the comparison of studies and to estimate the microbial behaviour outside the measured data points. In **Fig. 3.12A-B** the inactivation kinetics of six strains of *Lactococcus lactis* are shown. The cell suspensions were subjected to hydrostatic pressure at 300 MPa and 20°C for different time intervals (0-20 min). The inactivation curves of all strains showed sigmoid asymmetric shapes (**Fig. 3.12A-B**). However, three of the strains showed to be more resistant, *Lactococcus lactis* ssp. *lactis* TMW 2.118, *Lactococcus lactis* TMW 2.29, *Lactococcus lactis* TMW 2.27 had a shoulder phase of 5 min (**Fig. 3.12A**). Meanwhile, the shoulder phase of *Lactococcus lactis* ssp. *cremoris* MG1363, *Lactococcus lactis* TMW 2.26 and *Lactococcus lactis* TMW 2.28 was approximately 3 min (**Fig. 3.12B**). Within 12 min all strains had a microbial reduction of 4 log cycles.

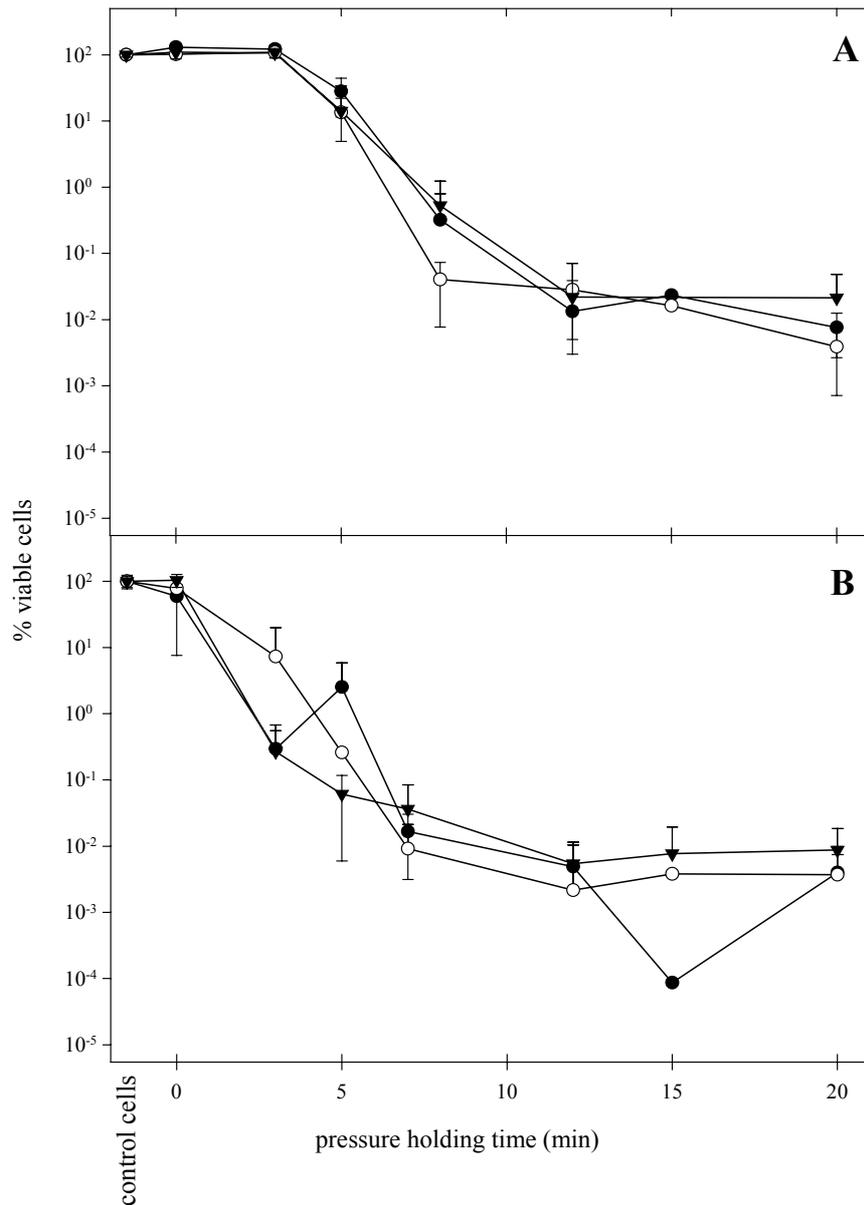


Fig. 3.12 Comparison of inactivation kinetics of *Lactococcus lactis* species at 300 MPa and 20°C. **A:** *Lactococcus lactis* TMW 2.27 (●), *Lactococcus lactis* TMW 2.29 (○), *Lactococcus lactis* ssp. *lactis* TMW 2.118 (▼). **B:** *Lactococcus lactis* TMW 2.26 (●), *Lactococcus lactis* TMW 2.28 (○), *Lactococcus lactis* ssp. *cremoris* MG1363 (▼). The results show the mean \pm standard deviation of 2 or more independent experiments.

The pressure resistance of the strains showed to correlate to their ability to utilise lactose, the more resistant strains are lactose positive. The ability to ferment lactose is encoded in the plasmid pLP712 (Gasson, 1983). The effect of this plasmid on the barotolerance of *Lactococcus lactis* was studied through inactivation kinetics of *Lactococcus lactis* NCDO712 and *Lactococcus lactis* MG1363, plasmid-cured strain of NCDO 712. As shown in **Fig. 3.13** no significant difference was observed between inactivation kinetics.

This strain of *Lactococcus lactis* ssp. *cremoris* MG1363 has been subject of several genetical and physiological studies (Bolhuis *et al.*, 1994, 1995, 1996; Buist *et al.*, 1995; Le Bourgeois *et al.*, 1995; Kilstrup *et al.*, 1997), thus further experiments were performed with this strain as model microorganism.

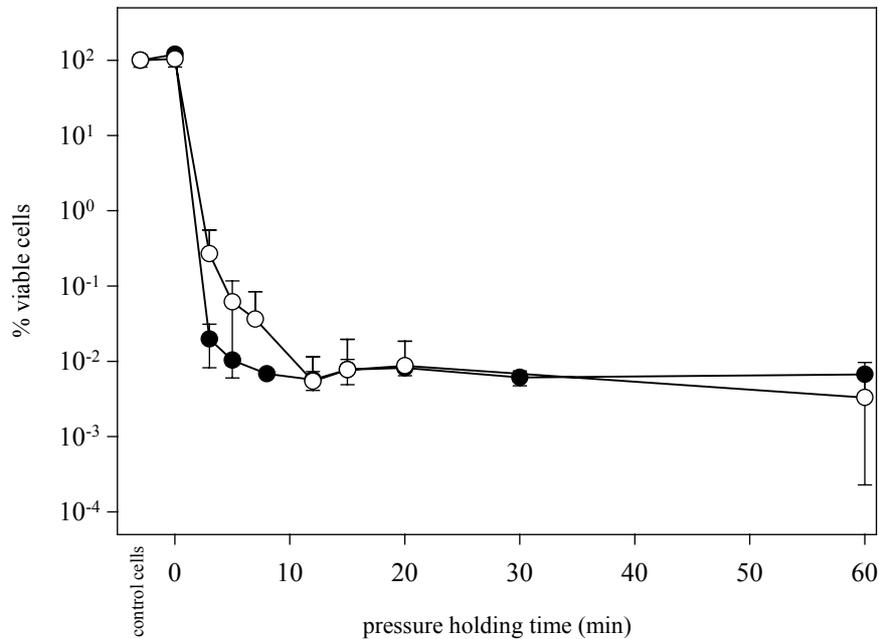


Fig. 3.13 Inactivation kinetic of *Lactococcus lactis* ssp. *cremoris* MG1363 (○) and *Lactococcus lactis* ssp. *cremoris* NCDO 712 (●). The results show the mean \pm standard deviation of 2 or more independent experiments.

3.3 Detection time

In order to characterise the physiological heterogeneity of cultures of *Lactococcus lactis* prior and after high-pressure treatment, the physiological state of individual cells was studied by means of detection times. Untreated cells and cells after a treatment at 300 MPa during 0 and 20 min were diluted in GM17 broth and incubated 48h at 30°C in a microtiter plate. The inoculum level approached the statistical probability of one cell per well. **Fig. 3.14A-C** show the distribution of detection times of untreated cells and cells treated for 0 and 20 min; **D-F** the distribution of detection times of cells cultivated after 20 min of pressure treatment (300 MPa, 20°C), and subjected to a second pressurization for 0 and 20 min. The results show a broad distribution of detection times without normal distribution. The pairwise multiple comparison procedure (Dunn's Method) indicates statistically significant difference between the distribution of untreated cells and 20 min pressurized cells, and 0 min and 20 min pressurized cells, but there is not significant difference between untreated cells and 0 min

pressurized cells. The median of detection time is extended by almost 2.5 h in cells treated during 20 min.

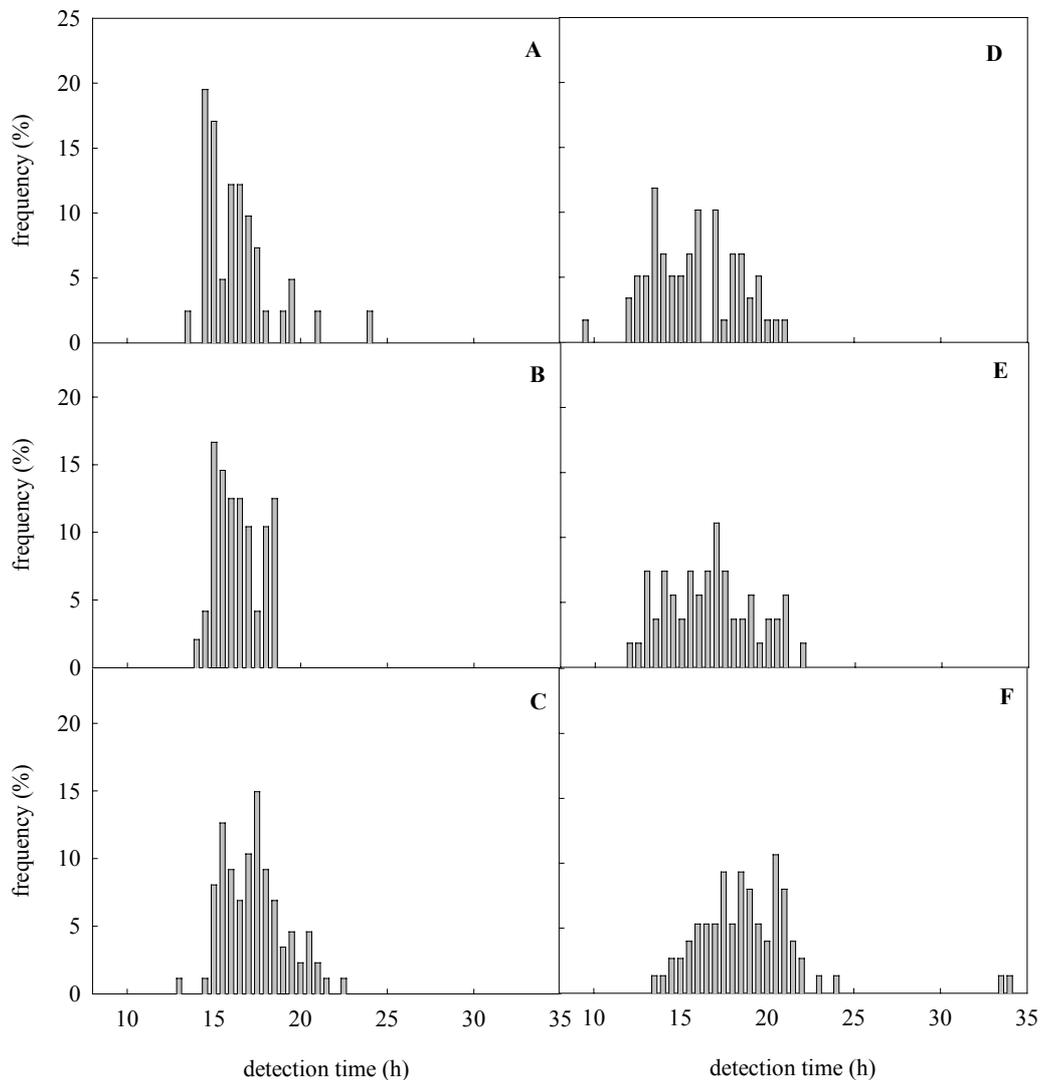


Fig. 3.14 Distribution of detection times of control and pressurized cells of *Lactococcus lactis* grown at 30°C in GM17 broth. **A:** untreated cells; **B:** 0 min treated cells at 300 MPa and 20°C; **C:** 20 min treated cells at 300 MPa at 20°C. **D-F** experiments were performed with an outgrowth of surviving *L. lactis* populations after a treatment for 20 min at 300 MPa and 20°C; **D:** untreated cells; **E:** 0 min treated cells at 300 MPa and 20°C; **F:** 20 min treated cells at 300 MPa at 20°C.

High-pressure inactivation kinetics are in many cases characterised by tailing, representing a more barotolerant fraction of the initial population. To determine the reversibility of this phenotypical baroresistance an incubation between pressure cycles was allowed. **Fig. 3.15** shows inactivation kinetics of two pressure profiles at 300 MPa and 20°C. In profile C the pressure was hold without interruption up to 120 min. The results indicated that the extension of holding time from 5 to 120 min inactivated just 1 cycle log indicating a prolonged tailing

effect. Between 10^4 and 10^5 cells were pressure-resistant. In profile B the pressure-resistant fraction obtained after 30 min of pressure treatment at 300 MPa and 20°C was allowed to recover during 30 min in GM17 broth at 30°C and 0.1 MPa. After this incubation time the cells were pressurized during 0, 45 and 90 min at 300 MPa and 20°C. The results showed an extensive inactivation by the second pressure treatment pointing to a loss of the pressure resistance of the cells.

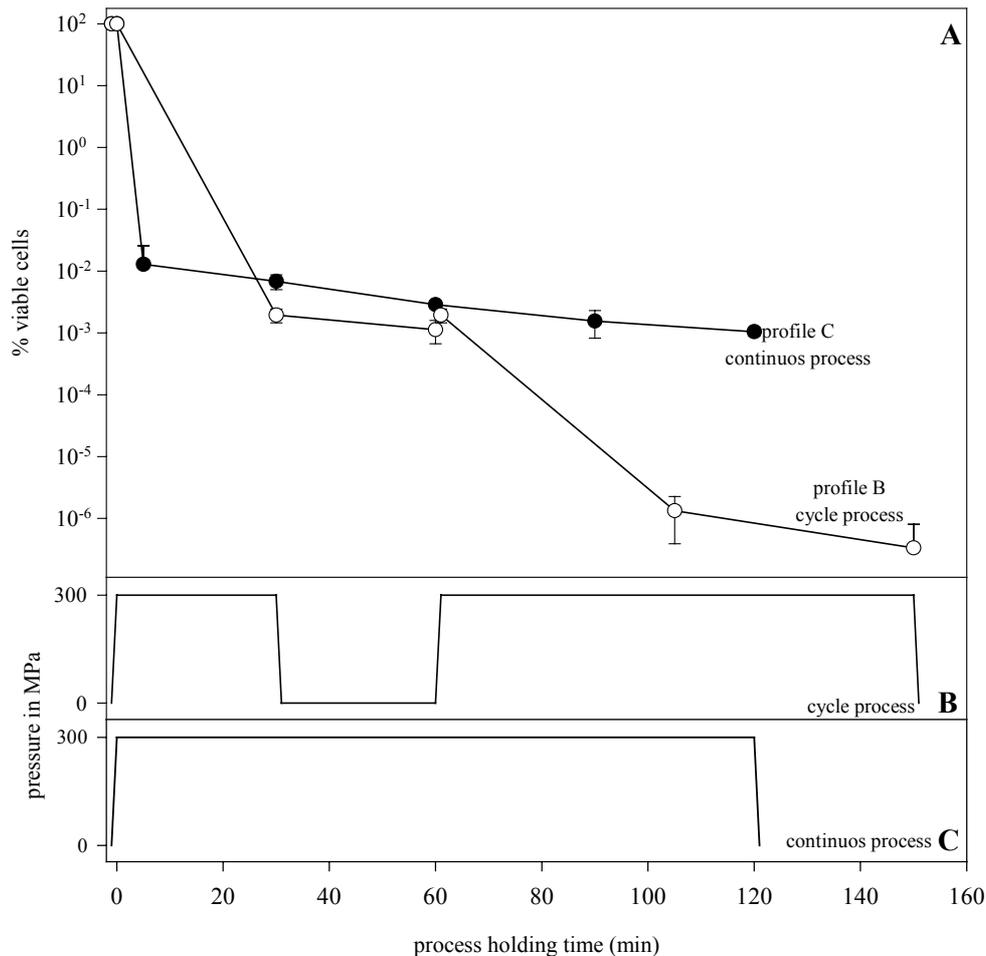


Fig. 3.15 Comparison of two pressure profiles to inactivate *Lactococcus lactis* at 300 MPa and 20°C. **A:** inactivation kinetics; **B:** two-cycle treatment with 30 min incubation at 0.1 MPa in GM17 broth to recover; **C:** continuous treatment with holding time from 0 to 120 min. The results show the mean \pm standard deviation of 2 or more independent experiments.

3.4 Effect of sucrose and sodium chloride on the survival and metabolic activity of *Lactococcus lactis* under high-pressure conditions

3.4.1 Inactivation of *Lactococcus lactis* cells in milk buffer at different pressure levels

Besides the type of microorganism the extent of pressure inactivation depends on several factors, such as pressure level, process temperature and time, and composition of the

dispersion medium (Cheftel, 1995). To verify the effect of pressure level and time suspensions of *Lactococcus lactis* ssp. *cremoris* MG1363 were exposed to 200, 300, 400 and 600 MPa at 20°C for different time intervals (**Fig. 3.16**). Pressure treatment at 200 MPa upon 60 min did not affect the viability of *Lactococcus lactis*. The inactivation curves of the bacteria at 300, 400 and 600 MPa showed the typical sigmoid asymmetric shape. The shoulder of the inactivation curves was reduced with the increase of the pressure but the level of survivors after 60 min was the same for all pressure treatments.

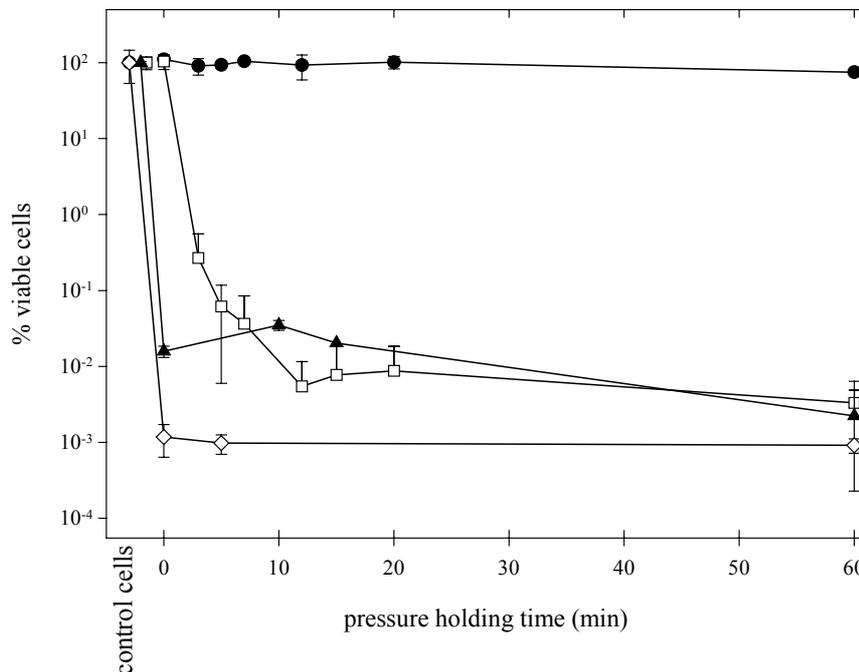


Fig. 3.16 Effect of pressure on inactivation of *Lactococcus lactis* ssp. *cremoris* MG1363 in milk buffer at 20 °C. 200 MPa (●), 300 MPa (□), 400 MPa (▲), (◇) 600 MPa. The results show the mean \pm standard deviation of 2 or more independent experiments.

3.4.2 Metabolic activity and membrane integrity after high-pressure treatments

The cells from the treatments at 200 and 300 MPa were analysed to determine their metabolic activity and membrane integrity (**Fig. 3.17A-B**). After a treatment for 5 min at 300 MPa the metabolic activity is 10-12% of the activity of untreated microorganisms, and after 12 min of treatment the cells did not show any metabolic activity. Treatments at 200 MPa up to 60 min did not inactivate the microorganisms, but reduced their metabolic activity to 50%. This reduction occurred in the first 20 min of treatment (**Fig. 3.17A**). During treatment at 300 MPa, cell death was closely followed by the loss of metabolic activity, however cultures retained about 25% of the metabolic activity, even after a 3 log reduction in cell counts (**Fig. 3.17B**).

The cytoplasmic membrane of *Lactococcus lactis* remained impermeable to PI even after cell death. Treatment at 200 MPa did not result in a reduction of membrane integrity after 20 min and cultures retained 30% of membrane integrity after 20 min at 300 MPa, corresponding to 4 log reduction of viable cell counts. These results show that the treatment of *Lactococcus lactis* ssp. *cremoris* MG1363 with high-pressure initially affects metabolic activity and subsequently damages membrane integrity.

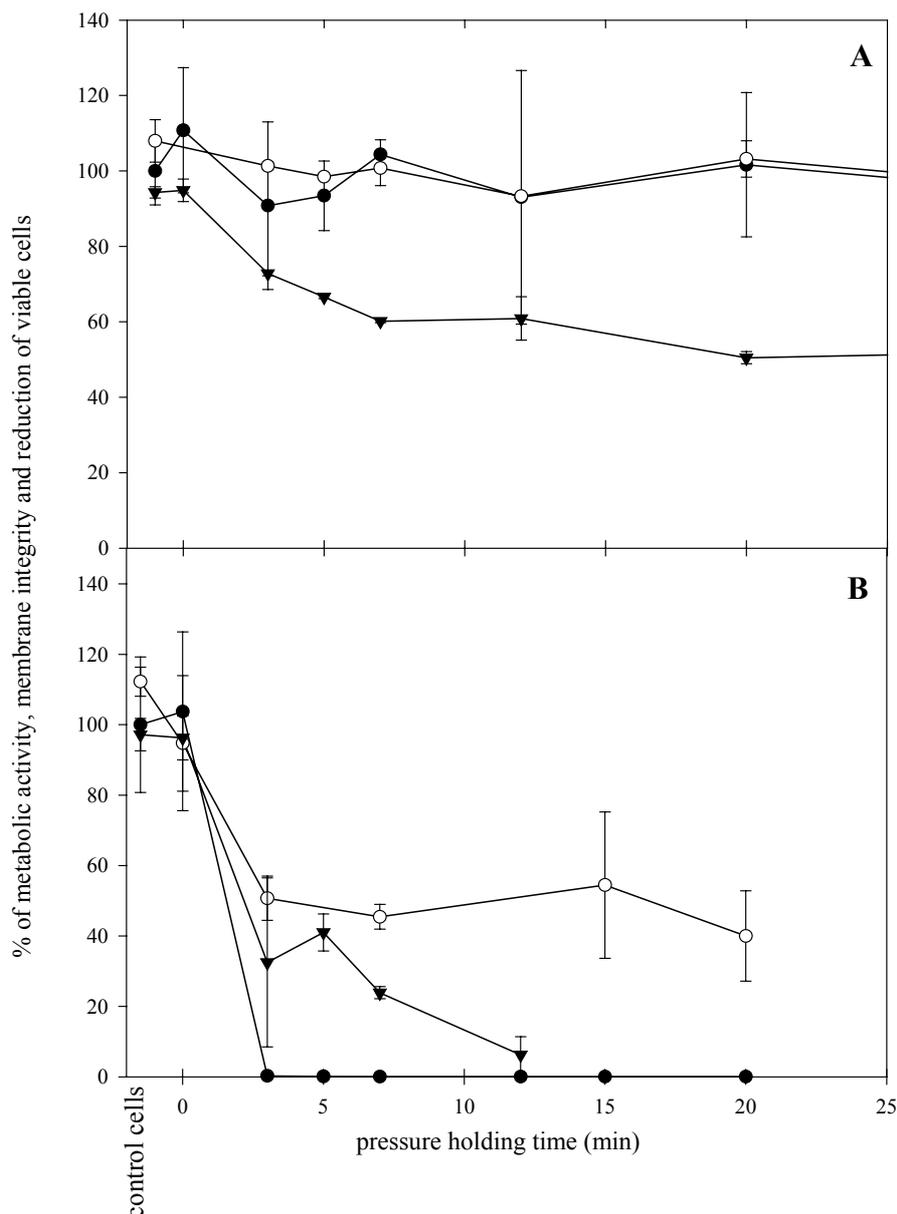


Fig. 3.17 Effect of pressure treatment at 200 MPa (A) and 300 MPa (B) on the metabolic activity (▼), membrane integrity (○) and viability (●) of *Lactococcus lactis* ssp. *cremoris* MG1363. The results show the mean \pm standard deviation of 2 or more independent experiments.

3.4.3 Inactivation kinetics of *Lactococcus lactis* in milk buffer and milk buffer with additives

The protective effect of a reduction of the water activity (a_w) against pressure inactivation was determined. Since *Lactococcus lactis* confronted with decreased a_w responds differently if the reduction was achieved with an ionic or nonionic solute, the effect of NaCl and sucrose were compared. 1 and 1.5 mol·l⁻¹ sucrose provided complete protection against cell death at either 400 or 600 MPa (**Fig. 3.18B**, **Fig. 3.19**).

No difference was observed between the baroprotective effect of 0.5 and 1 mol·l⁻¹ and 0.5 and 1 mol·l⁻¹ lactose at 300 MPa during 0, 15 and 30 min pressure holding time, respectively (**Tab. 3.1**). Due to the low solubility of lactose further experiments were carried out with sucrose.

A sudden increase in the concentration of salts of the environment results in the movement of water from the cell to the outside medium, which causes turgor pressure loss, intracellular solute concentration and cell volume changes. Such hyperosmotic conditions are detrimental to any living cell (van der Heide and Poolman, 2000). However, the addition of high concentration of salts showed a protective effect during high-pressure treatment. 2, 3 and 4 mol·l⁻¹ NaCl protected the cells against inactivation at 300 MPa (**Tab. 3.1**) as well as 400 and 600 MPa (**Fig. 3.18A**, **Fig. 3.19**). A comparison of the baroprotective effect of 2 and 3 mol·l⁻¹ NaCl and 2 and 3 mol·l⁻¹ KCl at 300 MPa during 0, 15 and 30 min pressure holding time showed no difference between these salts. The cytoplasmic accumulation of exogenous glycine betaine upon an osmotic upshift restores the cellular volume and increases the hydration of the cytoplasm of *Lactococcus lactis* (Obis *et al.*, 1999). If the compatible solute glycine betaine was added at a concentration of 2.5 mM, the baroprotective effect of 2 mol·l⁻¹ NaCl was increased in treatments at 400 MPa (**Fig. 3.18A**).

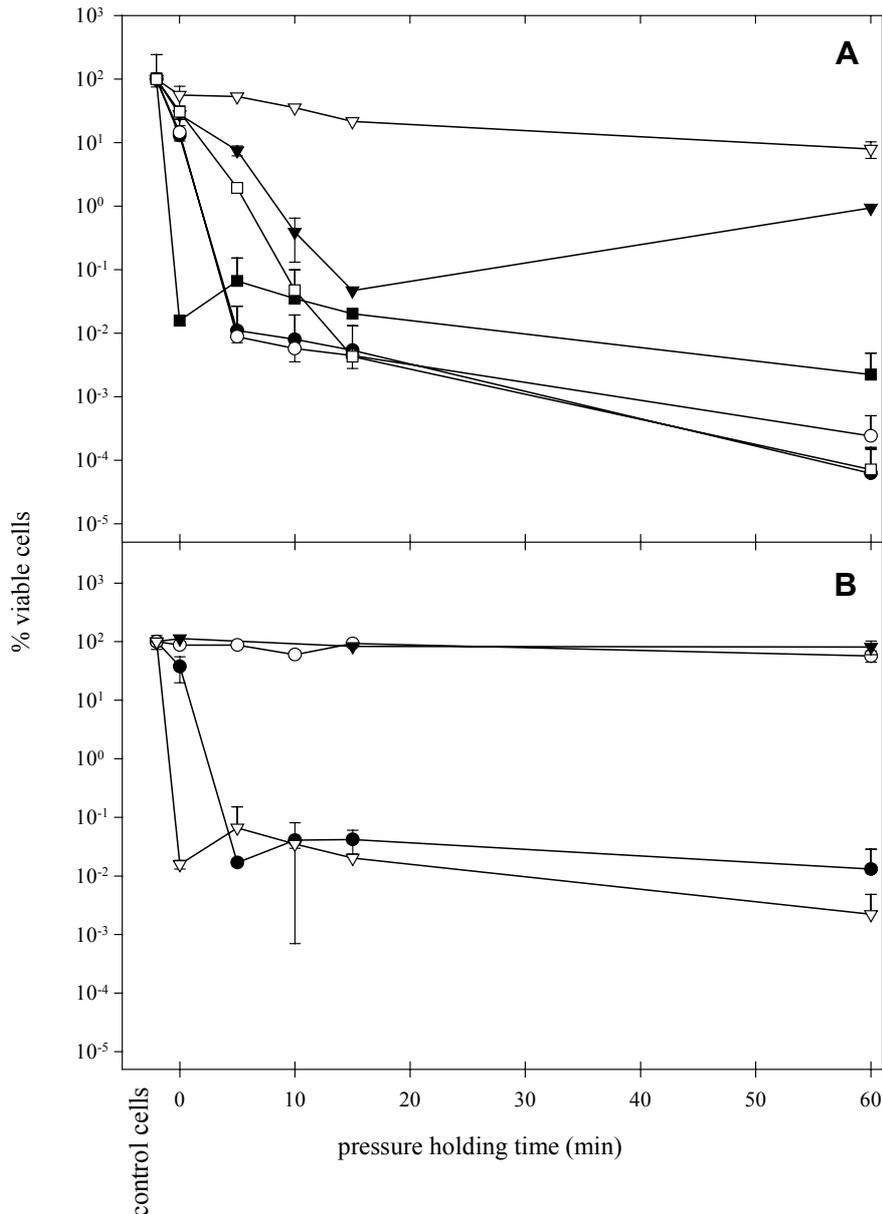


Fig. 3.18A Effects of NaCl in milk buffer at concentration of 1 M (●), 2 M (○), 2 M with 2.5 mM glycine betaine (□), 3 M (▼), 4 M (▽) and control (■), **B**. Effects of sucrose in milk buffer at concentration of 0.5 M (●), 1 M (○), 1.5 M (▼) and control (▽) on the pressure resistance of *Lactococcus lactis* ssp. *cremoris* MG1363 at 400 MPa (20°C). Symbols represent means \pm standard deviations for 2 independent experiments.

The baroprotective effect of solutes was proportional to the increase of their concentration in the buffer, but it is not proportional to the decrease of the a_w . The buffers with NaCl showed lower a_w than the buffers with sucrose, however the protective effect from sucrose at the same molar level was much higher (Tab. 3.1). In contrast to sucrose, even the highest NaCl concentration failed to achieve complete protection against cell death at 600 MPa (Fig. 3.19).

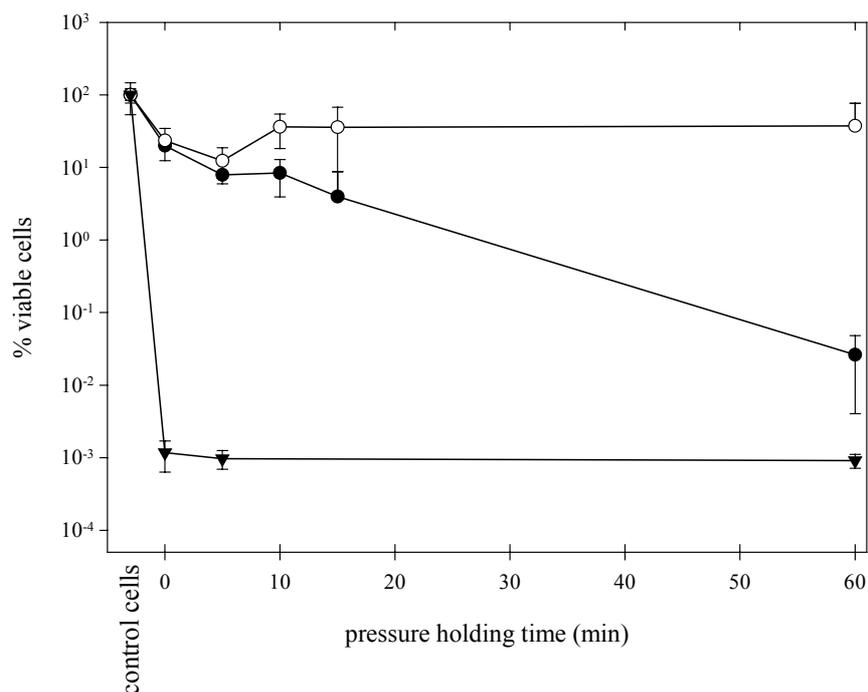


Fig. 3.19 Effect of low molecular solutes in treatment at 600 MPa (20°C). 4 M NaCl (●), 1.5 M sucrose (○), control (▼). Symbols represent means \pm standard deviations for 2 independent experiments.

Table 3.1 Comparison of water activity and baroprotective effect of solutes in milk buffer

Solute	CFU ml ⁻¹ after a treatment at 300MPa, 15 min, 20°C	a _w -Value
Milk buffer	2.0 x 10 ⁴	0.9960
NaCl		
1 mol	5.6 x 10 ⁴	0.9659
2 mol	1.0 x 10 ⁷	0.9408
3 mol	1.7 x 10 ⁸	0.9167
4 mol	2.5 x 10 ⁸	0.8954
Sucrose		
0.5 mol	1.6 x 10 ⁸	0.9849
1 mol	4.8 x 10 ⁸	0.9760
1.5 mol	2.7 x 10 ⁸	0.9663

3.4.4 Effect of low molecular solutes on metabolic activity and membrane integrity

The cells from the treatments at 300 MPa with 4 mol·l⁻¹ NaCl and 1.5 mol·l⁻¹ sucrose were analysed to determine the effect of the solutes on the metabolic activity and membrane integrity. The results with sucrose showed a protective effect on the metabolic activity and membrane integrity, which decreased to 90 and 50%, respectively within 60 min of pressure holding time (**Fig. 3.20A**). NaCl protected the membrane integrity to the same level, but the metabolic activity decreased rapidly (**Fig. 3.20B**).

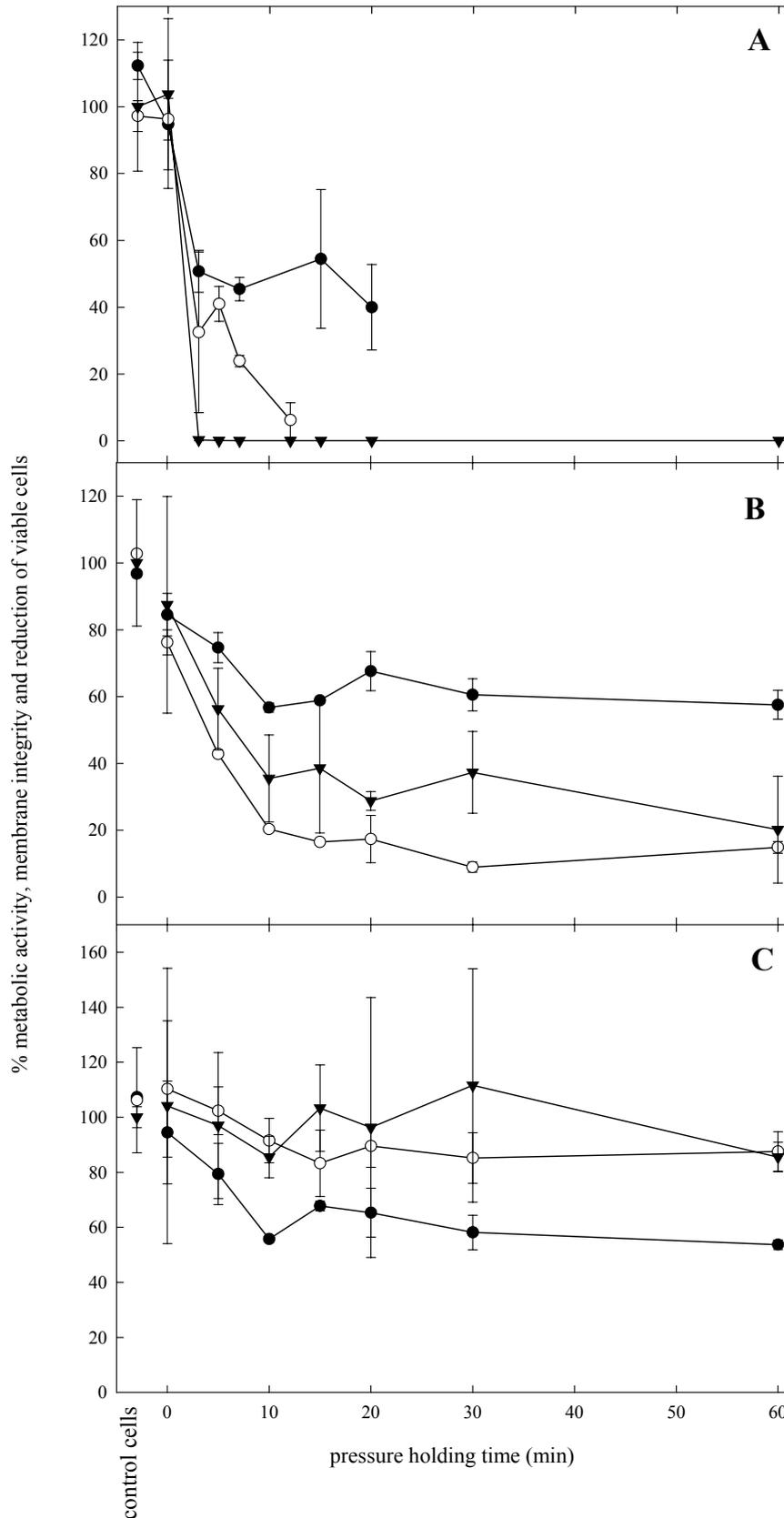


Fig. 3.20 Effect of solutes on the metabolic activity (○), membrane integrity (●) and viability (▼) of *Lactococcus lactis* ssp. *cremoris* MG1363 after treatment with 300 MPa and 20 °C. **A:** milk buffer, **B:** 4 M NaCl, **C:** 1.5 M Sucrose. Symbols represent means \pm standard deviations for 2 independent experiments.

3.5 *In-situ* determination of intracellular pH of *Lactococcus lactis* and *Lactobacillus plantarum* during pressure treatment

3.5.1 Inactivation of *Lactococcus lactis* in milk buffer with different pH

Hydrostatic pressure may affect the intracellular pH of microorganisms by (i) enhanced dissociation of weak organic acids and (ii) increased permeability of the cytoplasmic membrane and inactivation of enzymes required for pH homeostasis (Cheftel, 1995; Wouters *et al.*, 1998; Ulmer *et al.*, 2000, 2002). Therefore, the internal pH of *Lactococcus lactis* and *Lactobacillus plantarum* during and after pressure treatment, and pH values ranging from pH 4.0 to 6.5 was determined using the internally conjugated fluorescent probe 5 (and 6)-carboxyfluorescein succinimidyl ester (cFSE) (Breeuwer *et al.*, 1996).

The influence of cFDASE incorporation on the viability of *L. lactis* was determined by comparing inactivation curves from stained and unstained cells at 300 MPa. No significant differences in the number of viable cell counts were observed in treatments carried out with stained or native cells (data not show). Further inactivation kinetics were performed with stained cells.

Cell counts of *L. lactis* were reduced by 0%, $60 \pm 40\%$, and $75 \pm 30\%$ upon treatment in milk buffer (pH 6.5) at 200 MPa for 20, 40, and 60 min, respectively. The inactivation curves at 300 MPa of *L. lactis* in milk buffer with different pH values showed the typical sigmoid asymmetric shapes when plotted in logarithmic scale (**Fig. 3.21**). The shoulder of the inactivation curves was reduced with the decrease of the pH but the level of survivors after 120 min was the same for all pH treatments. At pH 4.0 no shoulder was observed and inactivation occurred already after 1 min of treatment. After 8 min of pressure treatment, 2 log inactivation were observed at pH 6.5 whereas more than 5 log inactivation were observed at pH 4.0.

The sublethal injury of the cells is shown in **Fig. 3.21B**. Cell counts of untreated cells on GM17 agar with and without 3% NaCl were not different, therefore, failure of *L. lactis* to grow on GM17-NaCl plates indicates sublethal injury. The cell counts of pressure treated cells on GM17-NaCl were generally lower than cell counts of the same samples on non selective medium, indicating a sublethal injury prior to irreversible cell damage. This sublethal injury was most pronounced during the exponential inactivation phase. In the tailing, the counts on selective agar approached the counts on non-selective agar. As opposed to pH effects on viable cell counts on non-selective medium, the pH did not affect on the cell counts on selective agar.

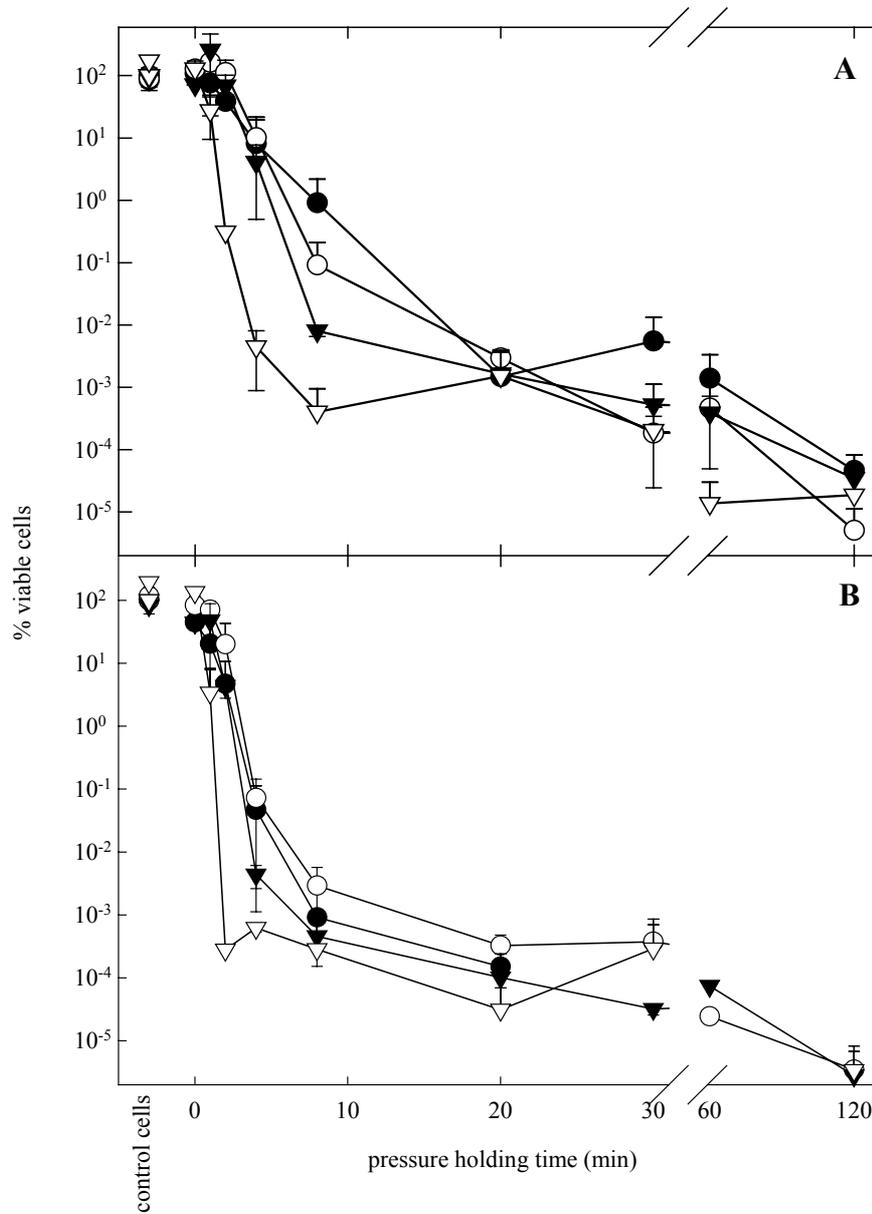


Fig. 3.21 Inactivation kinetics of *Lactococcus lactis* ssp. *cremoris* MG1363 (A) and sublethal injury (B) in milk buffer pH 6.5 (●), pH 6.0 (○), pH 5.0 (▼) and pH 4.0 (▽) after pressure treatments at 300MPa and 20°C. Shown is the viable cell count on GM17 (A) and GM17 with 3% NaCl compared to that of untreated cultures. The cell count of untreated cultures was $3.8 \pm 0.9 \times 10^9$ CFU ml⁻¹. Symbols represent means \pm standard deviations for 2 independent experiments.

3.5.2 Inactivation of *Lactobacillus plantarum* in milk buffer with different pH

No significant changes in viability were observed if *Lactobacillus plantarum* was treated in milk buffer (pH 6.5) at 200 MPa over 1 h (data not shown). Survival and sublethal injury of *Lactobacillus plantarum* during treatment at 300 MPa at various pH values is shown in **Fig. 3.22A** and **3.22B**, respectively. *L. plantarum* showed a greater resistance than *L. lactis* to pressure inactivation at 300MPa, but was more sensitive to a reduction of pH. Treatment at pH 4.0 and 5.0 showed a sharp inactivation phase, within 20 min of treatment the curves achieved a 5 log cycles reduction followed by tailing. The slope of the inactivation phase was smaller in treatments at pH 6.0 and 6.5. After 120 min of treatment at pH 6.0 a reduction of 5 log cycles was obtained, at pH 6.5 the reduction was 3 log cycles only. Ulmer *et al.* (2000) had shown that failure of *L. plantarum* TMW1.460 to grow on MRS with 4% salt indicates sublethal injury. Almost no difference was observed between selective and non-selective agar in treatment at pH 4.0 and 5.0 indicating that cells with sublethal injury were not able to survive. During the exponential inactivation phase a sublethal injury of 1 and 3 log cycles could be observed at pH 6.0 and 6.5. Independent from pH the same level of tailing on selective agar was noticed in all four treatments.

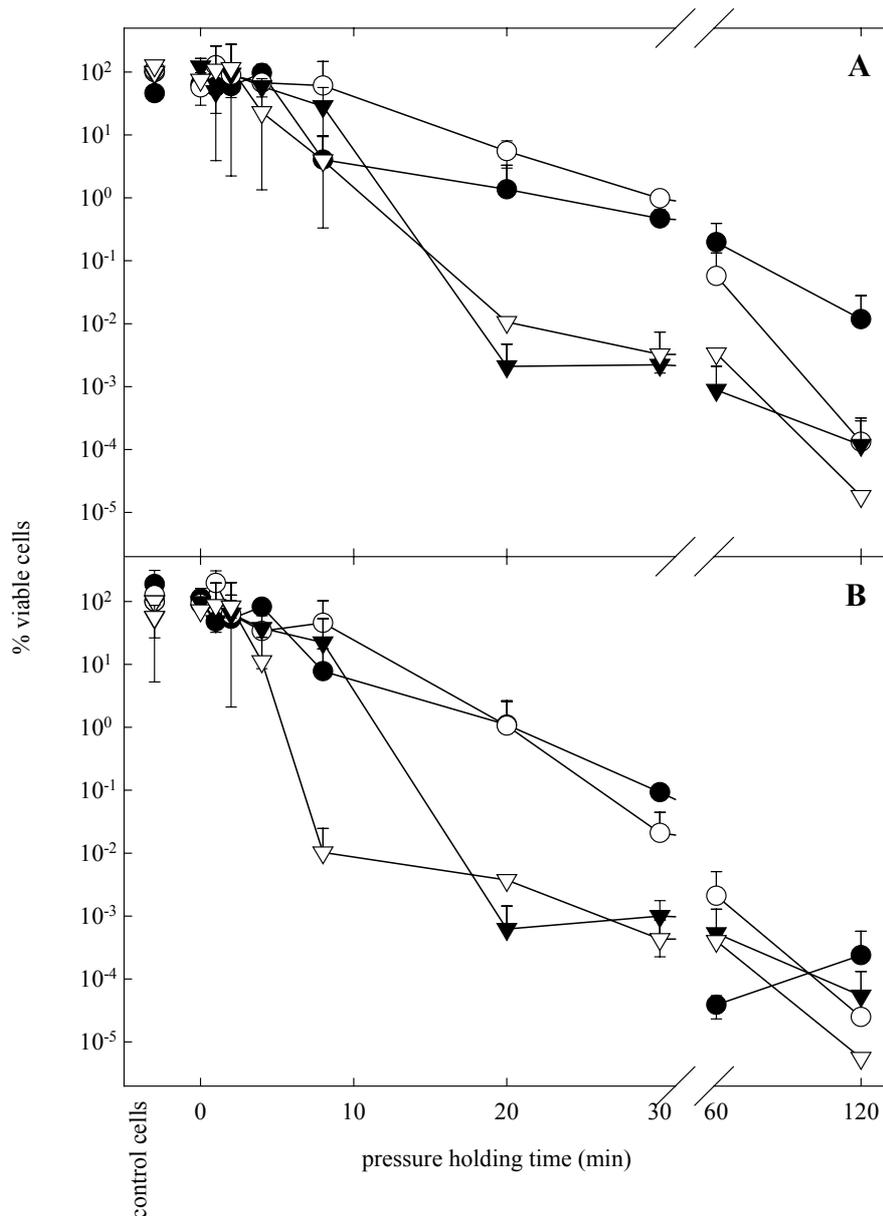


Fig. 3.22 Inactivation kinetics of *Lactobacillus plantarum* TMW1.460 (A) and sublethal injury (B) in milk buffer pH 6.5 (●), pH 6.0 (○), pH 5.0 (▼) and pH 4.0 (▽) after pressure treatments at 300MPa and 20°C. Shown is the viable cell count on MRS (A) and MRS with 4% NaCl compared to that of untreated cultures. The cell count of untreated cultures was $1.1 \pm 0.9 \times 10^9$ CFU ml⁻¹. Symbols represent means \pm standard deviations for 2 independent experiments.

3.5.3 Effect of the buffer pH (pH_{ex}) on the internal pH (pH_{in})

The pH_{in} of energized cells of *L. lactis* and *L. plantarum* were measured in HEPES, MES and citrate buffer with pH_{ex} values ranging from 4.0 to 8.0 at atmospheric pressure (Fig. 3.23). Both lactic acid bacteria decreased their pH_{in} as a function of pH_{ex}. The observed values of pH_{in} of *L. plantarum* were slightly lower than the corresponding values of *L. lactis*. Both

organisms maintained an internal pH between values of 6.3 and pH 8.0 at a buffer pH of 4.0 and 8.0, respectively. Consequently, under acidic conditions ($\text{pH}_{\text{ex}} < 5$) the ΔpH across the cell membrane was maintained at around 2.0, whereas it was dissipated at alkaline conditions.

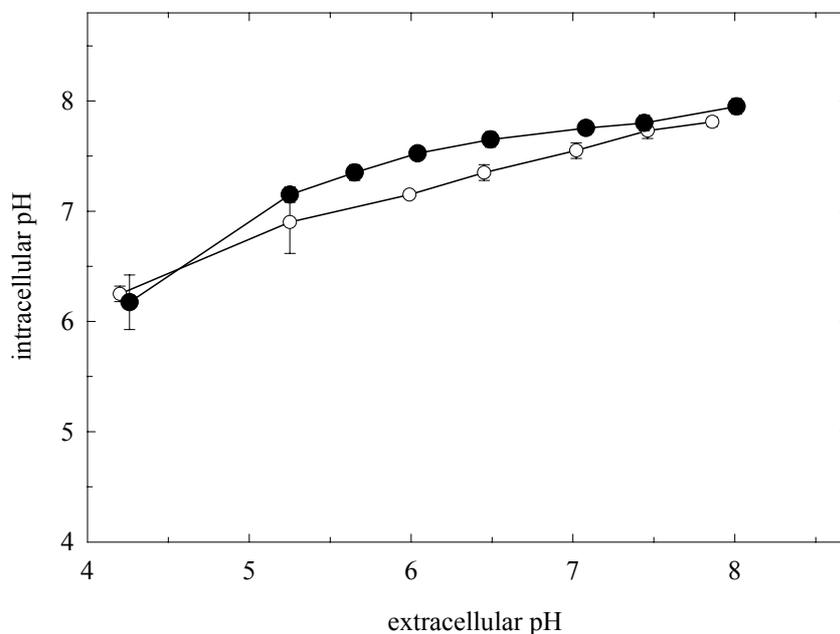


Fig. 3.23 pH_{in} of *Lactococcus lactis* ssp. *cremoris* MG1363 cells (●) and *Lactobacillus plantarum* TMW1.460 cells (○) in the presence of 10mM glucose at different pH_{ex} . The assays were performed at 30°C and 0.1MPa pressure. The buffers were prepared from citric acid (50mM), MES (50mM) and HEPES (50mM). The pH was adjusted with either NaOH or HCl. Symbols represent means \pm standard deviations for 2 independent experiments.

3.5.4 Calibration curve for measuring pH_{in} under high-pressure

For the determination of pH effects on internal pH values during high-pressure treatment it must be taken into account that both temperature and pressure cause a pH shift and each buffer exhibits a characteristic response to shifts in the p / T plane. Considering these effects, the changes of pH in buffers induced by high-pressure were calculated and taken in to account for the calibration of internal pH values at 200 and 300 MPa. The final values of the pH of the various buffers under high-pressure conditions are shown in **Table 3.2**. The calibration curves for measuring pH_{in} under high-pressure conditions were obtained by addition of valinomycin and nigericin (1 μM) to cells of *L. lactis* or *L. plantarum* suspended in milk buffer with pH 4.0, 5.0, 6.0, or HEPES buffer at pH 7.0 and 8.0. The relationship between $R_{485/410}$ and pH_{in} is non-linear. The sensitivity of the probe is greatest between pH 6.0 and 8.0. The fluorescence ratios determined at 0.1, 200 and 300 MPa for *L. lactis* and *L. plantarum* at the various pH values is indicated on the y-axes of **Figures 3.24 to 3.27**.

Table 3.2 Changes of buffer pH at 20°C induced by high-pressure. The values were kindly provided by Volker Stippl¹

	0.1MPa ^{a)}	100MPa ^{b)}	200MPa ^{b)}	300MPa ^{b)}
Phosphate buffer	7.00	6.58	6.22	5.92
HEPES buffer	8.00	7.92	7.86	7.80
HEPES buffer	7.00	6.92	6.86	6.80
Milk buffer	4.00		3.64	3.49
Milk buffer	5.00		4.54	4.35
Milk buffer	6.00		5.34	5.09
Milk buffer	6.50		5.79	5.52

^{a)} reference values determined with glass electrode

^{b)} calculated values

¹⁾ TU München, Lehrstuhl für Fluidmechanik und Prozessautomation, 85350 Freising, Germany

3.5.5 Effect of high-pressure on the internal pH of *Lactococcus lactis*

Fluorescence traces of cFSE labelled *L. lactis* during pressure treatment at 300 MPa are shown in **Figure 3.24**. Shown are the fluorescence ratios of (485 / 419) nm. The fluorescence ratios of the calibration samples at pH 8.0, 7.0, 6.0, 5.0, and 4.0 at 0.1 and 300 MPa are indicated on separate y-axes to allow the conversion to pH_{in} values. The considerable base line noise of the ratio data collection was mainly caused by the rapid alteration of the emission monochromator between the two wavelengths. This mode of data collection was nevertheless preferred over a mere intensity measurement because differences in fluorescence intensity between individual samples / pressure ramps do not affect the fluorescence ratios.

The pH_{in} measured at normal pressure corresponded within experimental error to those values shown in **Fig. 3.23**. Pressure treatment at 300 MPa and pH values ranging from 3.5 to 5.5 resulted in a drop in the internal pH of *L. lactis* cells (**Fig. 3.24A-D**), which indicated that the regulation of the internal pH was impaired in these cells. The pH_{in} approached the respective values of pH_{ex} during the first 90 seconds of treatment, corresponding to the compression of the samples to 300MPa. Thereafter, the pH_{in} values remained constant. To control the reversibility of the damage, the cells from treatments at pH 6.5 were incubated with glucose (10mM) after releasing the pressure and the pH was monitored. Cells with a pressure treatment of more than 2 min were not able to restore the ΔpH. After 0, 1 and 2 min of treatment the cells were able to restore a partial ΔpH, but the value was below the value of

untreated cells. The ΔpH was restored to 63, 50 and 37 and 0% after 0, 1, 2 and 4 min of pressure treatment, respectively.

The measurements of treatment at 200MPa are shown in **Fig. 3.25**. A decrease in pH_{in} was observed during compression of the sample. As opposed to the treatments at 300 MPa, the ΔpH was not fully dissipated after the ramp up time but only after 1 min of pressure holding time. Thereafter, the values of pH_{in} corresponded to the external pH. The cells were able to restore 77, 73, 46, 44, 50 and 0% of the ΔpH after 0, 1, 4, 8, 20 and 30 min of treatment, respectively.

Despite this fast drop of pH_{in} during treatments at 200 or 300 MPa, a reduction of cell viability at pH 6.5 was not observed until 4 and 40 min of pressure holding time at 300 and 200 MPa, respectively. Mild pressure treatments (200-300MPa and 0 min) sufficed to reduce the capacity of the cells to restore a pH gradient upon pressure treatment and inflicted sublethal injury, but failed to reduce cell viability. The pressure holding time irreversibly inactivating cellular systems required for pH homeostasis at 200 and 300 MPa (4 and 30 min, respectively) correlated to the pressure holding time required for a decrease in cell viability (4 and 40 min at 300 and 200 MPa, respectively).

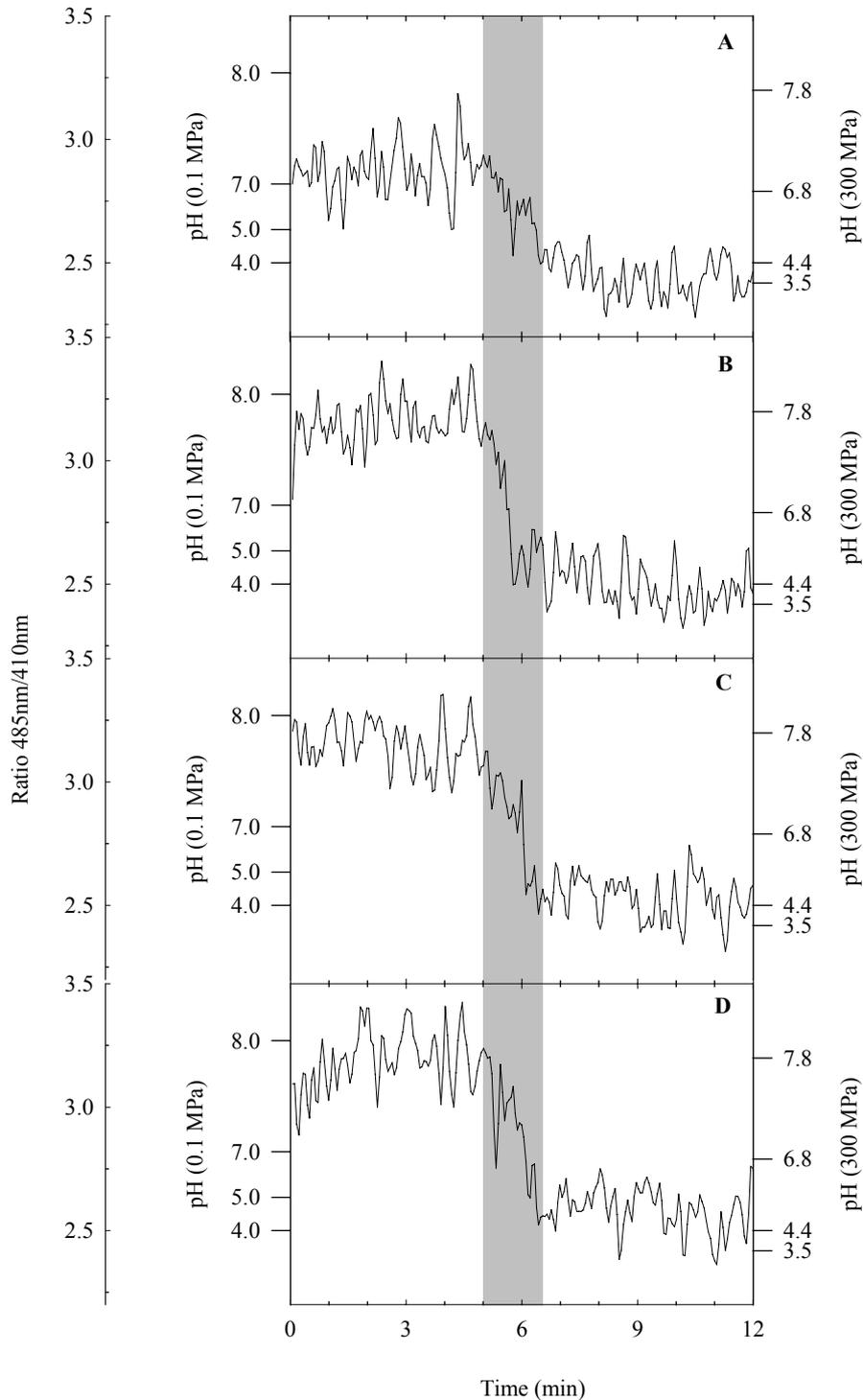


Fig. 3.24 Fluorescence trace of cFSE labelled *Lactococcus lactis* ssp. *cremoris* MG1363 during treatment at 300MPa and 20°C in milk buffer at pH values ranging from 4.0 to 6.5. Samples were energized with 10mM glucose and placed in the pressure chamber 5 min prior to treatment to equilibrate the sample temperature. **A:** pH 4.0, **B:** pH 5.0, **C:** pH 6.0, **D:** pH 6.5. The compression rate was 200MPa min⁻¹, the ramp-up time 90 sec. The shaded area indicates the ramp-up time. The pH-axes to the left and right of the panels indicate the fluorescence ratios at 0.1 and 300 MPa of cells pressure treated at the respective pH values in the presence of valinomycin and nigericin to equilibrate internal and external pH values. Data are representative for two independent experiments.

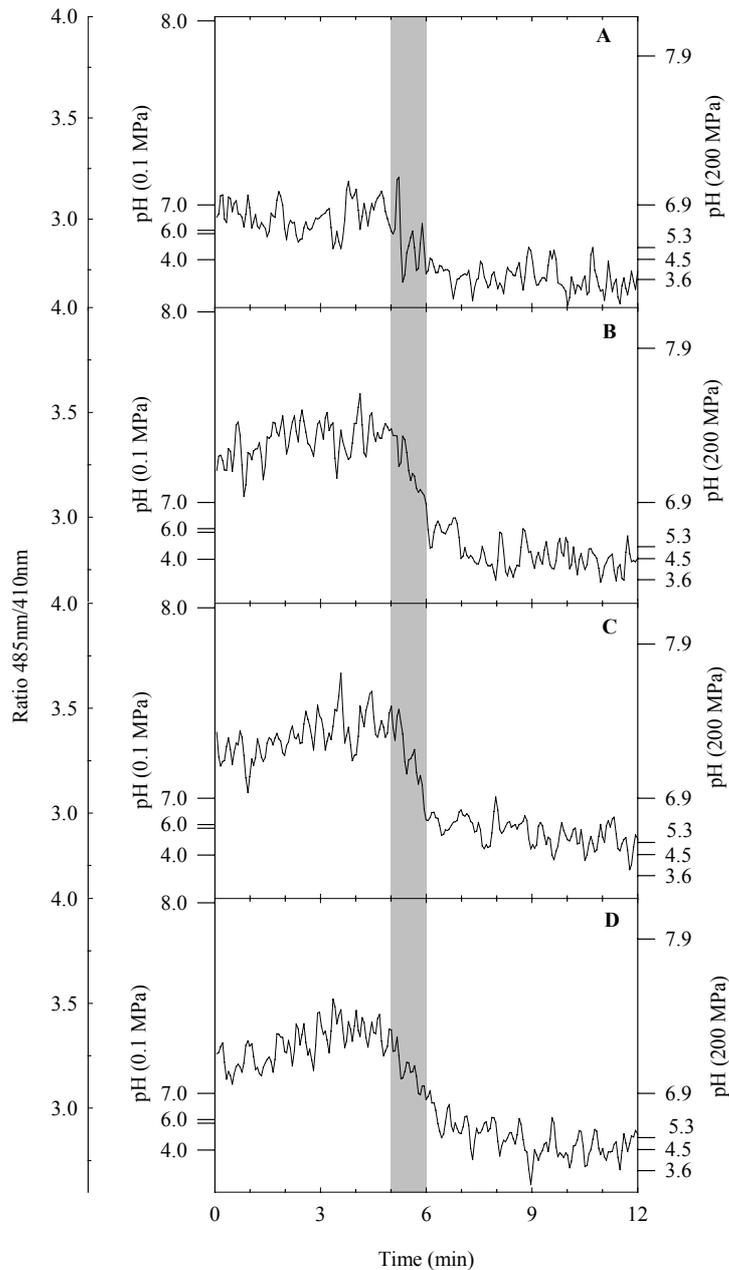


Fig. 3.25 Fluorescence trace of cFSE labelled *Lactococcus lactis* ssp. *cremoris* MG1363 during treatment at 200MPa and 20°C in milk buffer at pH values ranging from 4.0 to 6.5. Samples were energized with 10mM glucose and placed in the pressure chamber 5 min prior to treatment to equilibrate the sample temperature. **A:** pH 4.0, **B:** pH 5.0, **C:** pH 6.0, **D:** pH 6.5. The compression rate was 200MPa min⁻¹, the ramp-up time 60 sec. The shaded area indicates the ramp-up time. The pH-axes to the left and right of the panels indicate the fluorescence ratios at 0.1 and 200 MPa of cells pressure treated at the respective pH values in the presence of valinomycin and nigericin to equilibrate internal and external pH values. Data are representative for two independent experiments.

3.5.6 Effect of high-pressure on the internal pH of *Lactobacillus plantarum*

The internal pH values of *Lactobacillus plantarum* during treatments at 200 are shown in **Fig. 3.26**. In accordance with data shown in **Fig. 3.23**, values of pH_{in} prior to compression to either 200 MPa or 300 MPa (**Fig. 3.27**) were about 8 at a buffer pH of 6.5 and 6.0, and about 7.5 and 7 at a buffer pH of 5.0 and 4.0, respectively. During the pressure treatment the pH_{in} dropped with the build-up time of pressure, the final value of pH_{in} is reached after the 60 sec of the ramp. The final value of pH_{in} at pH 4.0 corresponded to the external pH. However, at a buffer pH of 5.0, 6.0, and 6.5, the internal pH remained above the external pH throughout the pressure treatment. This indicates that *L. plantarum*, as opposed to *L. lactis*, was able to maintain a transmembrane proton potential even during incubation at 200 MPa. Accordingly, the internal pH of *L. plantarum* treated in milk buffer, pH 6.5 at 200 MPa for 0, 1, 2 or 4 min corresponded to the value determined with untreated, energized cells (data not shown).

The changes of *L. plantarum* internal pH during pressure treatment at 300 MPa are shown in **Fig. 3.27**. At 300MPa, the pH_{in} dropped concomitant with compression time to 300 MPa and reached the value of the buffer pH within 90 sec of pressure holding time, i.e., during treatments at various buffer pH values, the pH_{in} was between 3.5 and 5.1. As opposed to *L. lactis*, cells of *Lactobacillus plantarum* were not able to restore a partial ΔpH after pressure treatments at 300 MPa. The results of the reversibility test did not show an increase of the pH_{in} after pressure release. Even mild treatments, 0 min at 300MPa, caused an irreversible reduction of the internal pH.

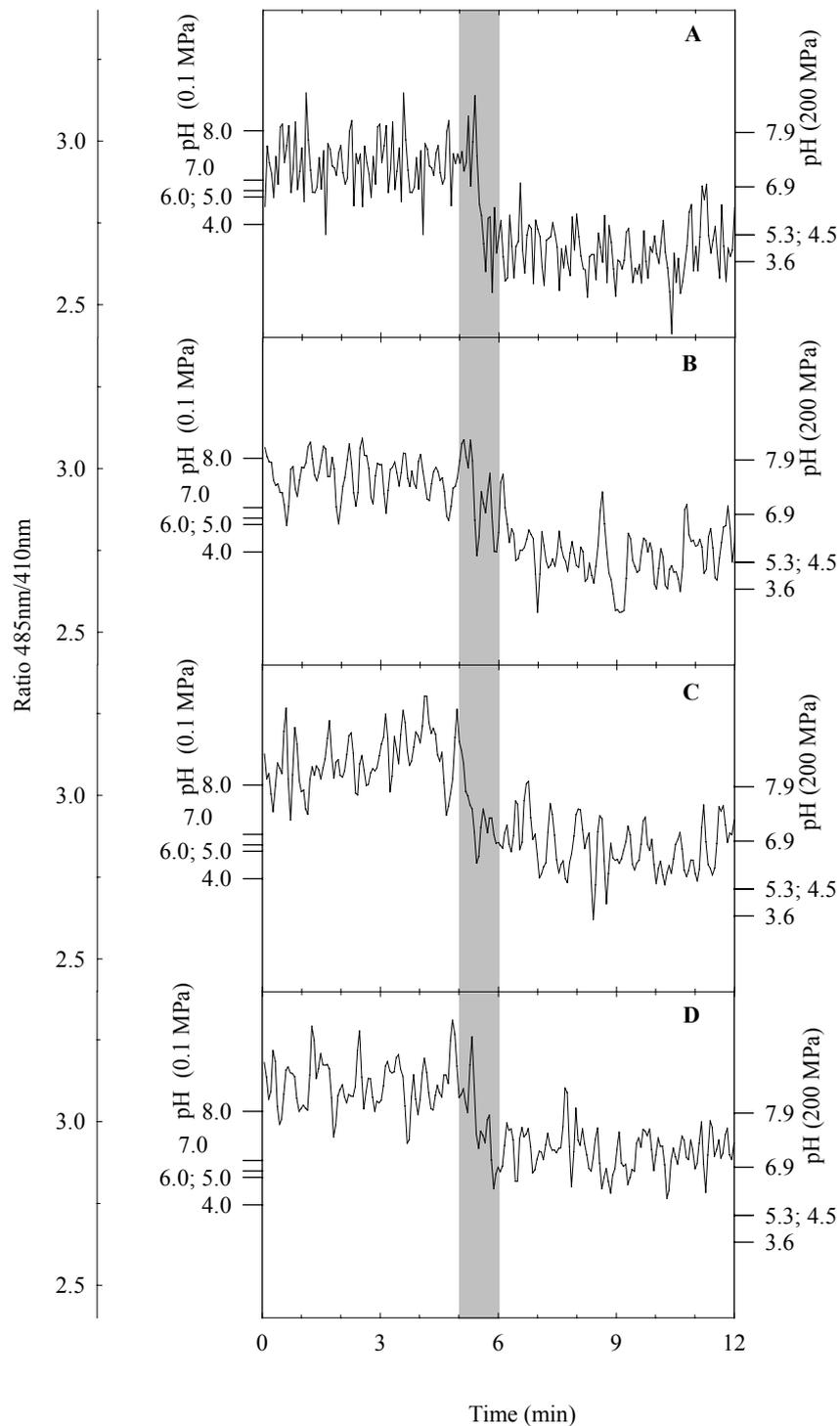


Fig. 3.26 Fluorescence trace of cFSE labelled *Lactobacillus plantarum* TMW1.460 during treatment at 200MPa and 20°C in milk buffer at pH values ranging from 4.0 to 6.5. Samples were energized with 10mM glucose and placed in the pressure chamber 5 min prior to treatment to equilibrate the sample temperature. **A:** pH 4.0, **B:** pH 5.0, **C:** pH 6.0, **D:** pH 6.5. The compression rate was 200MPa min⁻¹, the ramp-up time 60 sec. The shaded area indicates the ramp-up time. The pH-axes to the left and right of the panels indicate the fluorescence ratios at 0.1 and 200 MPa of cells pressure treated at the respective pH values in the presence of valinomycin and nigericin to equilibrate internal and external pH values. Data are representative for two independent experiments.

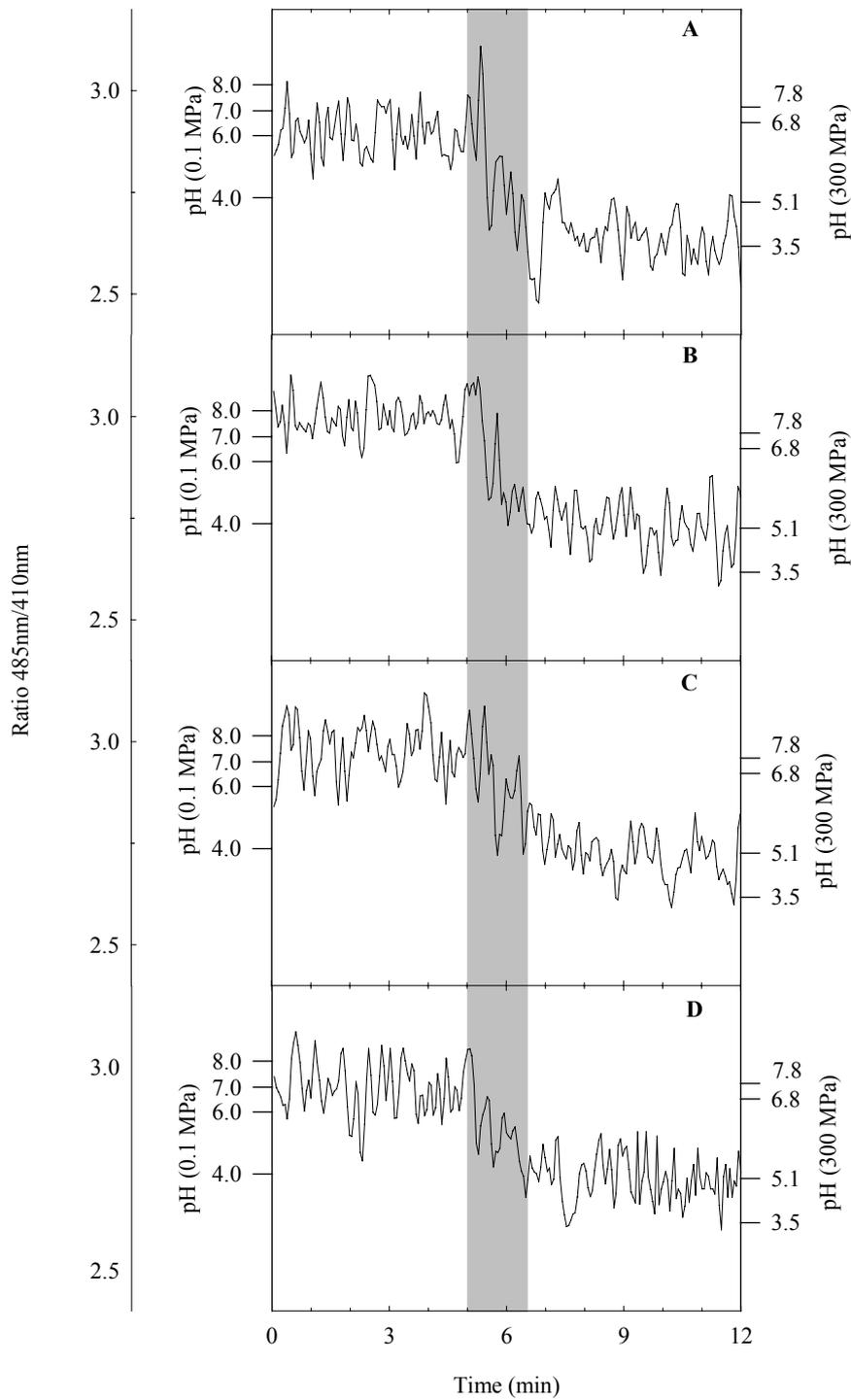


Fig. 3.27 Fluorescence trace of cFSE labelled *Lactobacillus plantarum* TMW1.460 during treatment at 300MPa and 20°C in milk buffer at pH values ranging from 4.0 to 6.5. Samples were energized with 10mM glucose and placed in the pressure chamber 5 min prior to treatment to equilibrate the sample temperature. **A:** pH 4.0, **B:** pH 5.0, **C:** pH 6.0, **D:** pH 6.5. The compression rate was 200MPa min⁻¹, the ramp-up time 90 sec. The shaded area indicates the ramp-up time. The pH-axes to the left and right of the panels indicate the fluorescence ratios at 0.1 and 300 MPa of cells pressure treated at the respective pH values in the presence of valinomycin and nigericin to equilibrate internal and external pH values. Data are representative for two independent experiments.

3.5.7 Effect of high-pressure on the intracellular pH of *Lactococcus lactis* in presence of osmolytes

The effect of high-pressure treatment at 200 MPa on the intracellular pH (pH_{in}) of *Lactococcus lactis* in milk buffer, milk buffer with 4M NaCl and milk buffer with 0.5M sucrose are shown in **Fig. 3.28A-C**.

Comparing the initial pH_{in} of the cells, the addition of sucrose and NaCl caused a decrease of the pH_{in} reducing the ΔpH of the cells. The addition of glucose increased the ΔpH of cells in milk buffer with sucrose, but not to the level of cells in milk buffer without additives. In milk buffer with NaCl the addition of glucose did not show to have any effect.

A drop of the pH_{in} occurred immediately within the raise of pressure reaching the final value after 120 sec in treatments in milk buffer and milk buffer with sucrose. In treatment with 4M NaCl the pH_{in} reached the final value after 60 sec corresponding to the build-up time of pressure. The end value of pH_{in} was for all treatments about 4.5 and correlated to the pH of buffer at 200 MPa. The dissipation of ΔpH indicated that the regulation of the pH_{in} was impaired in these cells.

The reversibility test of cells in milk buffer indicated that upon 20 min of treatment the cells were able to restore a partial ΔpH . The build-up time of pressure caused a reduction of the capacity to restore a ΔpH , the cells were able to restore just 77% of ΔpH . After 30 min of treatments the cells were not able to restore the ΔpH . The loss of capacity to restore a ΔpH was prevented with the addition of sucrose to milk buffer. After 120 min of treatment in milk buffer with 0.5M sucrose the cells were still able to restore around 60% of ΔpH . The addition of 4M NaCl dissipated the ΔpH of the cells. Untreated and pressurized cells in milk buffer with NaCl were not able to increase the pH_{in} after addition of glucose.

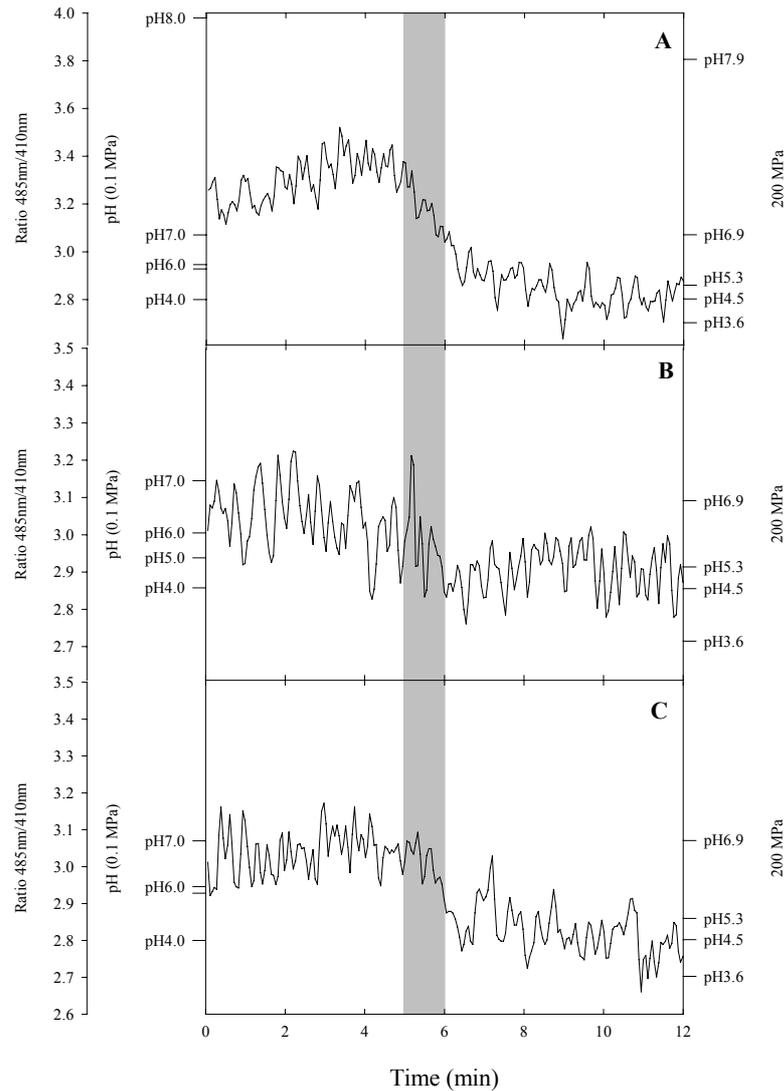


Fig. 3.28 Change in the pH_{in} of *Lactococcus lactis* ssp. *cremoris* MG1363 during treatment at 200MPa and 20°C in milk buffer with additives. Samples were energized with 10 mM glucose and placed in the pressure chamber 5 min prior to treatment to equilibrate the sample temperature. **A**: milk buffer without additives, **B**: milk buffer with 4 M NaCl, **C**: milk buffer with 0.5 M sucrose. The compression rate was 200 MPa min^{-1} , the ramp-up time 60 sec. The shaded area indicates the ramp-up time.

3.6 Investigation of mechanisms of baroprotection of sodium chloride and sucrose

3.6.1 Comparison of inactivation kinetics of *Lactococcus lactis* in presence of different solutes

The addition of ionic and nonionic solutes has shown to protect *Lactococcus lactis* cells against high-pressure inactivation. The results of metabolic activity, membrane damage and internal pH showed differences in the mechanism of baroprotection. Therefore, the effects of sucrose and NaCl were studied at the level of viability, membrane phase transition and inactivation of the membrane-bound enzyme LmrP. Molar concentration and a_w failed to give a basis to compare the baroprotective effect of osmolytes. Further comparative experiments

were realised using ionic and non-ionic osmolytes in approx. the same weight/volume concentration. Inactivation kinetics of *Lactococcus lactis* in milk buffer, milk buffer with 0.5 mol·l⁻¹ sucrose (171.15 g·l⁻¹), 4 mol·l⁻¹ NaCl (233.76 g·l⁻¹) and 1 mol·l⁻¹ (182.17 g·l⁻¹) mannitol were performed.

Treatments at 200MPa (**Fig. 3.29A**) did not show to be effective for the inactivation of *Lactococcus lactis*. The first reduction of CFU in milk buffer was observed after 40 min of treatment, after 120 min the reduction was 2 log cycles. The addition of 0.5 M sucrose, 4 M NaCl and 1 M mannitol protected the cells against inactivation. For all treatments with solutes the reduction of CFU was not significant after 120 min of treatment. The inactivation curves of the bacteria at 300MPa in milk buffer and milk buffer with 0.5 M sucrose showed the typical sigmoid asymmetric shape (**Fig. 3.29B**). In milk buffer the inactivation phase started after 2 min of treatment. The effectivity of the treatment is reduced after 20 min reaching a tailing. Increasing the time of pressurization to 120 min enhanced the inactivation just in 1 log cycle. The results with sucrose showed a protective effect on the viability of *Lactococcus lactis*. The shoulder of the sigmoid asymmetric shape was extended until 8 min and the inactivation phase was retarded. After 20 min of treatment the level of survivors in milk buffer with sucrose was 2 log cycles above the level in milk buffer without additives. An increase of pressurization time dissipated the difference between inactivation in milk buffer and milk buffer with sucrose. The addition of 4M NaCl showed a strong protective effect in treatment at 300MPa. Treatments upon to 60 min did not show a significant reduction of CFU. 120 min of treatment achieved 1 log cycle reduction of viable cells. The inactivation curves at 600MPa are shown in **Fig. 3.29C**. Treatments in milk buffer showed a rapid inactivation. The build-up time of pressure achieved a 5 log cycles inactivation. Increasing the time of treatment to 60 min did not enhance the inactivation. Treatments in milk buffer with 0.5M sucrose also showed a rapid inactivation. However, the level of survivors after the build-up time of pressure was 1 log cycle above the level in milk buffer. In accordance with our previous results, an increase of the pressurization time reduced the difference between curves. After 10 min of treatment there was not difference between levels of survivors in milk buffer and milk buffer with sucrose. The protective effect of 4M NaCl was reduced at 600MPa. A reduction of about 1 log cycle was achieved with the build-up time of pressure. Contradictory to the results in milk buffer and milk buffer with sucrose, the slope of the inactivation phase was rather small. Just 0.5 log cycle further inactivation was reached after 15 min. 60 min of treatment did not reach the same level of inactivation as treatment in milk buffer and milk buffer with sucrose.

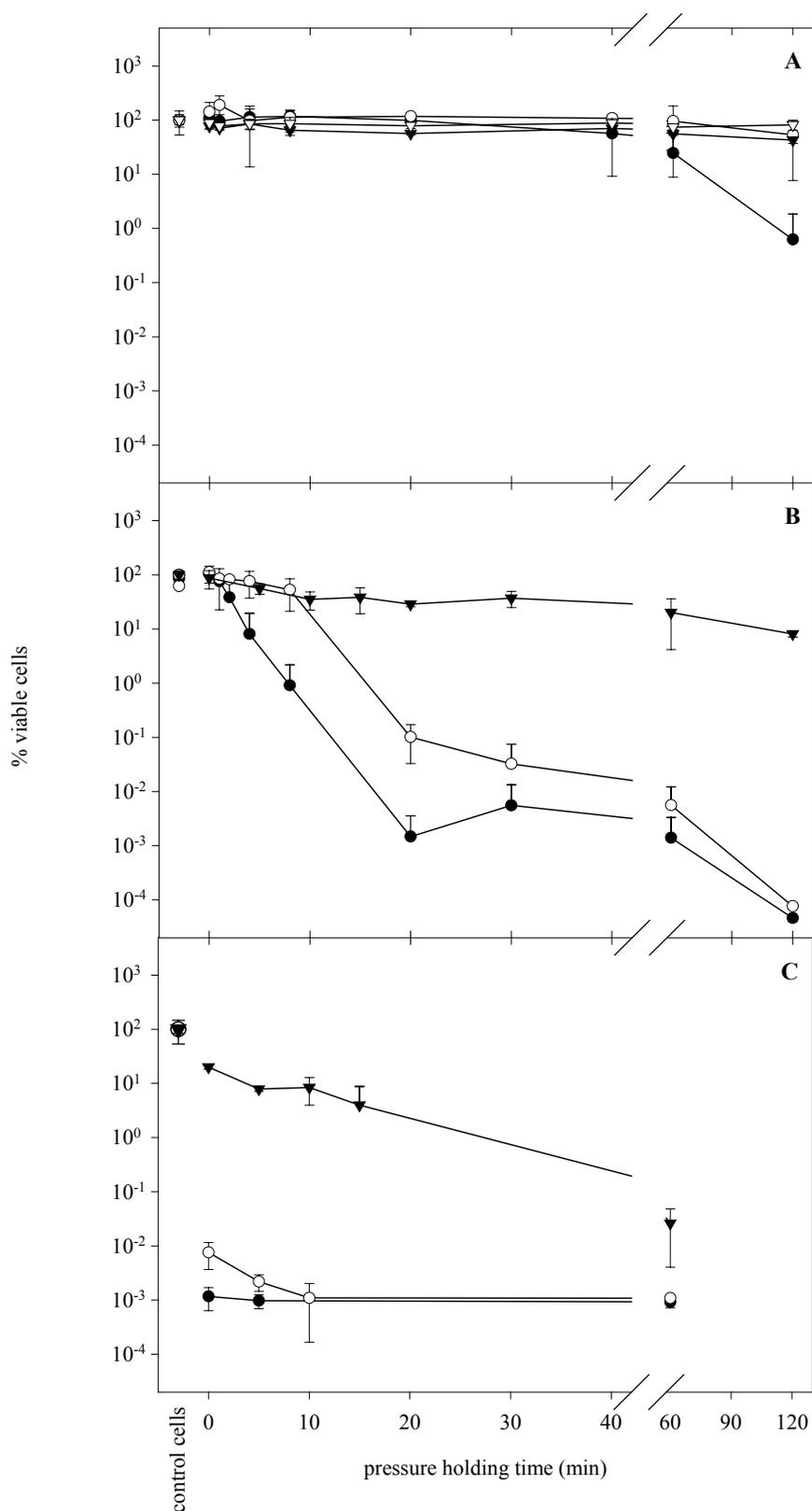


Fig. 3.29 Inactivation kinetics of *Lactococcus lactis* ssp. *cremoris* MG1363 in milk buffer (●), milk buffer with 0.5 M sucrose (○), milk buffer with 4M NaCl (▼) and milk buffer with 1M Mannitol (▽) after pressure treatments at 200 MPa (A), 300MPa (B), 600MPa (C) and 20°C. Shown is the viable cell count on GM17 compared to that of untreated cultures. The cell count of control cultures was $(2.4 \pm 0.8) \times 10^9$ CFU ml⁻¹. Symbols represent means \pm standard deviations for 2 independent experiments.

B3.6.2 LmrP activity of pressurized cells in milk buffer and milk buffer with additives

In the gram-positive bacterium *Lactococcus lactis* at least four drug extrusion activities have been detected (Molenaar *et al.*, 1992; Bolhuis *et al.*, 1994). One of these systems, LmrP, is a secondary drug transporter located in the membrane. This system utilises the membrane potential and the transmembrane proton gradient mediating an electrogenic $n\text{H}^+$ /drug antiport reaction ($n \geq 2$) to drive the extrusion of drugs of the cell (Bolhuis *et al.*, 1996; Putman *et al.*, 1999a-b). The extrusion of ethidium bromide in the wild-type strain of *Lactococcus lactis* MG1363 occurs predominantly by the LmrP transporter being inhibited by ionophores that dissipate the proton motive force (Bolhuis *et al.*, 1994, 1995, 1996). According to the HorA assay by Ulmer *et al.* (2000, 2002) the effect of pressure on LmrP activity can be determined comparing the ethidium bromide efflux of pressurized cells with the efflux of starved and untreated cells. **Fig. 3.30** shows the comparison of LmrP activity after treatments at 200 MPa in milk buffer and milk buffer with additives. Cells in milk buffer and milk buffer with sucrose did not show a significant difference of activity in untreated cells. High-pressure impaired the activity of the enzyme. In treatments in milk buffer the LmrP activity was reduced after 8 min and reached the level of starved cells after 40 min. This effect correlated with the loss of capacity to restore a ΔpH and the first reduction of CFU. With the addition of 0.5M sucrose the activity of LmrP was protected. The pressure inactivation of the enzyme was retarded, cells showed activity after 40 and 60 min of treatment, but lost the activity after 120 min. However, no reduction of CFU was reported and the cells were able to restore a partial ΔpH after 120 min of treatment. Untreated cells in milk buffer with 4M NaCl showed reduced LmrP activity. The activity was reduced already during the build-up time of pressure, after 1 min of treatment the activity reached the level of starved cells. These results agreed with the lower pH_{in} and the absence of ΔpH , but the viability of the cells was not affected. Untreated cells in milk buffer with 1M mannitol showed also a reduced LmrP activity. The build-up time of pressure was sufficient to reduce the activity, but the cells retained a partial activity upon 60 min of treatment. After 120 min of treatment the cells lost the LmrP activity without reduction of CFU.

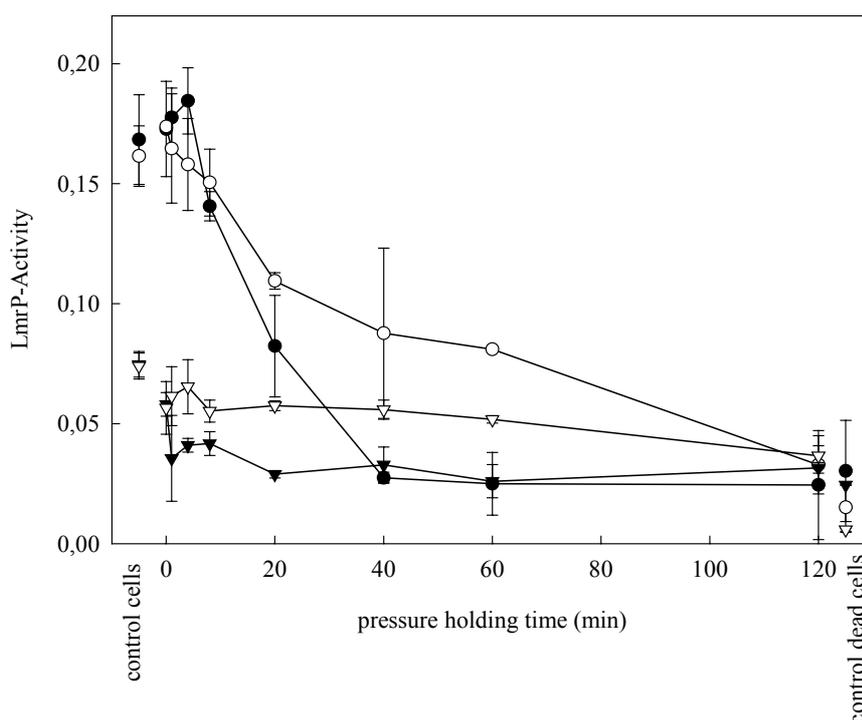


Fig. 3.30 Baroprotective effects of additives on the LmrP activity during high-pressure treatment at 200MPa and 20°C. Shown is the activity of LmrP of pressurized cells compared to that of untreated in milk buffer (●), milk buffer with 0.5 M sucrose (○), milk buffer with 4M NaCl (▼) and milk buffer with 1M Mannitol (▽). Symbols represent means \pm standard deviations of 2 independent experiments.

3.6.3 Intracellular accumulation of osmolytes by osmotic upshock

Bacteria respond to changes in medium osmolarity by varying the intracellular concentrations of specific solutes in order to maintain constant turgor. Sugar- and salt-stressed *Lactococcus lactis* cells accumulate different osmolytes (Molenaar *et al.*, 1993). We determined qualitatively the main accumulated osmolytes in cells exposed to 30 min incubation in milk buffer, milk buffer with 0.5M sucrose and milk buffer with 4M NaCl. Furthermore, we determined the effect of the addition of 2.5mM glycine betaine to the 4M NaCl milk buffer.

The HPLC detection of sugars showed that sucrose, glucose and fructose were the major osmolytes in cells incubated in milk buffer with sucrose. The cells exhibited a sucrose uptake as physiological response against the nonelectrolyte stress. Inside the cells the sucrose was cleaved into glucose and fructose. None of these sugars was present in cells incubated in milk buffer or milk buffer with 4M NaCl. The level of lactose did not show significant difference between cells incubated in milk buffer and milk buffer with 0.5M sucrose. However, cells incubated with NaCl without glycine betaine contained a higher level of lactose. Glycine betaine was accumulated if it was added to the buffer, cells incubated in milk buffer with 4M

NaCl and glycine betaine showed a high level of this osmolyte and lower level of lactose than cells incubated just with 4M NaCl. The accumulation of an unknown osmolyte was apparent in cells incubated in milk buffer with 4M NaCl.

3.6.4 Effect of osmolytes on the membrane phase state at different temperatures measured by FT-IR spectroscopy

The presence of sugars aids to protect cell membranes against stresses. Adding disaccharides to cells before drying lowers the transition temperature (T_m) of the dry membranes preventing the phase transition and its accompanying leakage upon rehydration (Crowe *et al.*, 1988, 1997). Since one of the principal targets of high-pressure inactivation is the membrane, we determined and compared the effect of adding osmolytes on the membrane phase state at different temperatures and pressure levels.

The T_m of the phase transition was determined by measuring the wave number of the symmetric CH_2 absorbance peak at wave numbers around 2,850 at various temperatures and then plotting the wave number against temperature (**Fig. 3.31**). The internal vibrational modes of the lipid acyl chains are assigned on the basis of the well-known studies of polymethylenes and polymethylene chain compounds (Reis *et al.*, 1996; Snyder, 1961). In the 2,800- to 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 wave numbers of these bands are conformation sensitive and thus respond to temperature- and pressure-induced changes of the *trans/gauche* ration in acyl chains. The vibration mode (antisymmetric stretch) of the terminal CH_3 group occurs at about 2,960 cm^{-1} . The FT-IR melting curves were fitted to the following logistic function:

$$y = y_o + \frac{A}{1 + e^{-(T-T_m)/b}}$$

where y_o 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. All of the liquid crystalline or gel phase regions are characterised by a nearly horizontal slope. This method has been used successfully to measure the T_m s of *Escherichia coli* (Leslie *et al.*, 1995) and *Lactobacillus plantarum* (Ulmer *et al.*, 2002).

Lactococcus lactis cells in milk buffer have a T_m of 21.4°C. Addition of sucrose and NaCl to the buffer lowers the T_m of the phase transition. The sucrose lowered the T_m of the cells from 21.4 to around 16.8°C, while NaCl lowered to 16.6°C.

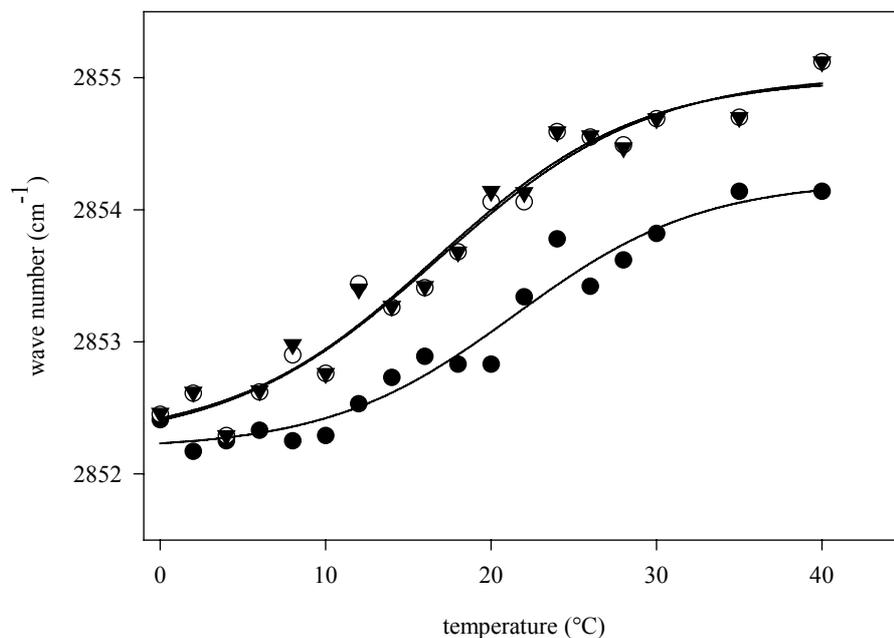


Fig. 3.31 Vibrational frequencies for the CH₂ symmetric stretch from *Lactococcus lactis* cells in milk buffer (●), milk buffer with 0.5 M sucrose (○) and milk buffer with 4 NaCl (▼) as a function of temperature. Data are representative for two independent experiments. Every experiment was done by heating the cells from 0 to 40°C. The curves represent the best fit of the logistic function ($r^2 \geq 0.96$).

3.6.5 Effect of osmolytes on the membrane phase state at different temperatures and pressures as measured by Laurdan fluorescence spectroscopy

The temperature-dependent membrane phase behaviour obtained by FT-IR spectroscopy was confirmed by Laurdan fluorescence spectroscopy. The Laurdan fluorescence spectra depend on changes in environmental polarity induced by membrane phase transitions (Bagatolli *et al.*, 1999; Parasassi *et al.*, 1995). The temperature dependence of the GP values of cells in milk buffer and milk buffer with additives is shown in **Fig. 3.32**. With decreasing the temperature, the polarity around the fluorophor in fluid membranes decreases, resulting in higher GP values. The change can be interpreted as temperature-induced phase transition from liquid crystalline to gel phase. Cells in milk buffer without additives showed the higher values of GP at 10°C indicating a rigid state of the membrane. At the same temperature the values of GP in presence of additives were lower indicating the coexistence in the membrane of the liquid crystalline and gel phases.

The pressure-dependent membrane phase behaviour was determined also by Laurdan fluorescence spectroscopy (**Fig. 3.33**). An increment of pressure decreases the polarity around the fluorophor in fluid membranes increasing the GP values. By analogy to temperature assay, the change can be interpreted as pressure-induced phase transition from liquid crystalline to

coexistence of liquid crystalline and gel phase regions. Cells in milk buffer without additives showed the greatest increase of GP values with pressure indicating a rapid phase transition. Cells in milk buffer with sucrose or NaCl did not show an increase of GP values exhibiting no significant changes of membrane phase state with pressure. Up to 300 MPa their membranes stayed in a liquid crystalline phase.

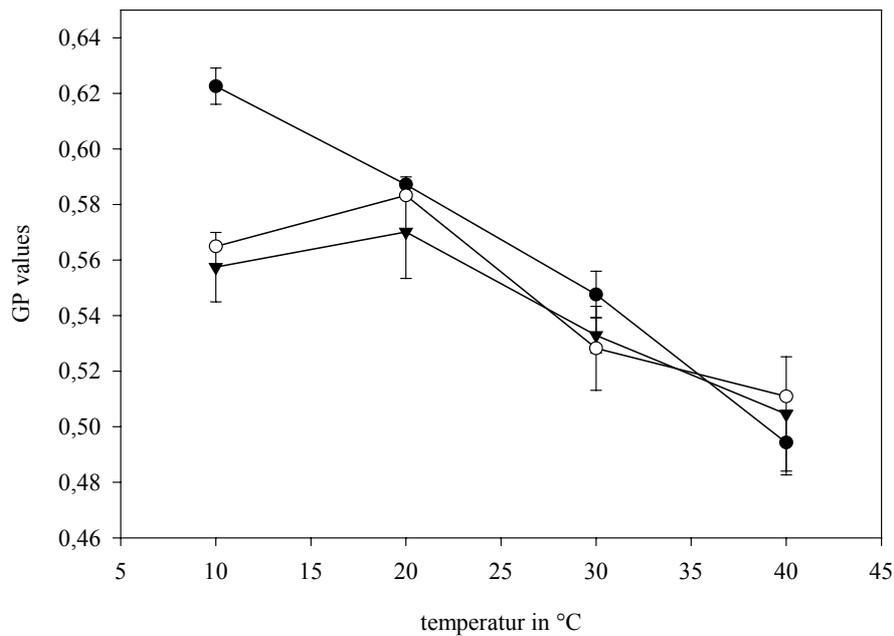


Fig. 3.32 GP values of *Lactococcus lactis* cells stained with Laurdan in milk buffer (●), milk buffer with 0.5 M sucrose (○) and milk buffer with 4 NaCl (▼) as a function of temperature. Data are representative for two or more independent experiments. Symbols represent means \pm standard deviations of 2 measurements.

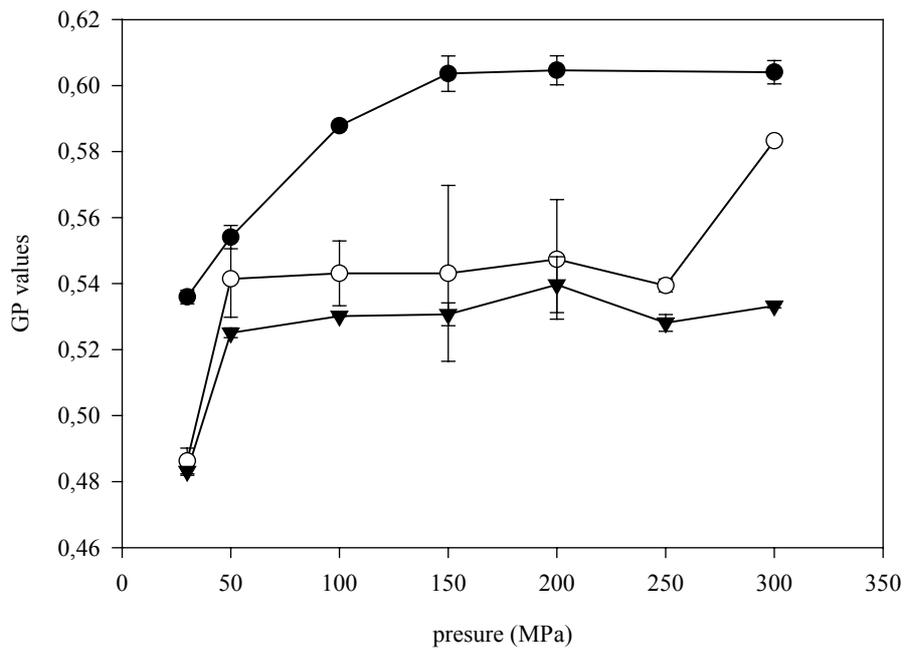


Fig. 3.33 GP values of *Lactococcus lactis* cells stained with Laurdan under pressure conditions. The treatments were performed in milk buffer (●), milk buffer with 0.5 M sucrose (○) and milk buffer with 4 NaCl (▼). Data are representative for two or more independent experiments. Symbols represent means \pm standard deviations of 2 measurements.

4. Discussion

This study provides insights in the mechanism of high-pressure induced growth inhibition and cell inactivation of *Lactococcus lactis*. This was achieved by using newly developed and adapted *in-situ* measurement techniques distinguishing between primary and secondary pressure effects, reversible and irreversible reactions as well as responses at organizational and physiological level.

Fig. 4.1 summarises the methods used in this work to assess various aspects of the physiology of bacterial cells after and during high-pressure treatment. Fluorescent determination of microbial physiology enables *in-situ*, kinetic determination of pressure inflicted injury and thus enables the distinction between reversible and irreversible pressure effects (Gänzle and Vogel, 2001).

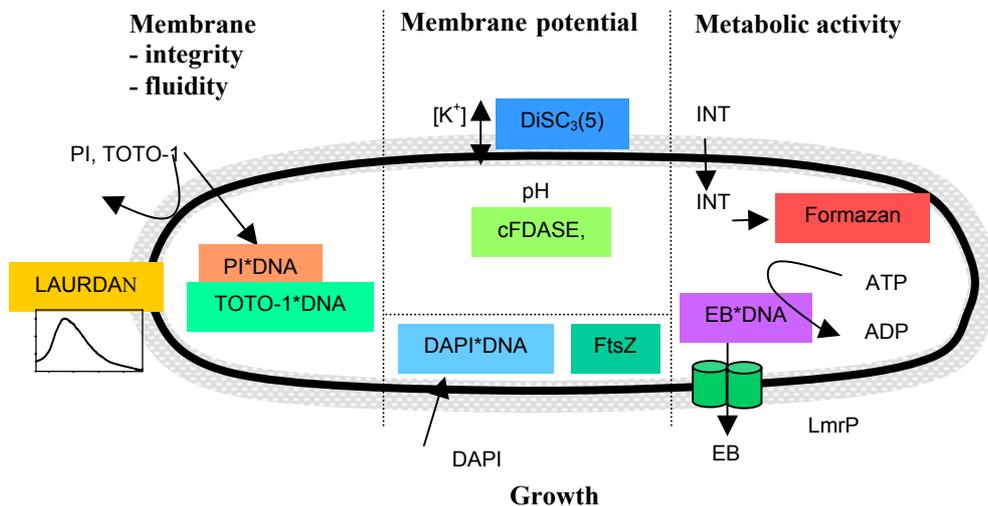


Fig. 4.1 Methods to characterise the high-pressure induced responses of *Lactococcus lactis*. This figure was kindly provided by Michael Gänzle.

The examination of the metabolic activity after pressure treatment 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 membrane-bound enzyme LmrP is an unspecific substrate transporter. The extrusion of the fluorescent dye ethidium bromide in the wild-type strain of *Lactococcus lactis* occurs predominantly by the LmrP transporter. This enabled to measure LmrP activity after pressurization by fluorescence kinetic experiment (Ulmer *et al.*, 2000, 2002).

The internal pH of the cells was determined with the fluorescent probe cFSE, which conjugates with the aliphatic amines of intracellular proteins. Changes of the membrane phase state during pressure treatment were measured by Laurdan fluorescence spectroscopy.

Additionally, the growth under sublethal pressure was characterised with respect to chromosomal replication by DAPI-staining and FtsZ ring formation by immunofluorescence.

4.1 Effect of high-pressure during growth

High-pressure can be used for cold pasteurization of foods as well as in biotechnological food processes. Casal *et al.* (1999) suggested the use of lactic acid bacteria cells attenuated by high-pressure to accelerate cheese ripening. The use of sublethal pressure on cells lowered the lactic-acid producing activity without causing damage to the peptidolytic activity (Casal *et al.*, 1999). On account of this, we investigated the effect of pressure and temperature on mesophilic bacteria used in food biotechnology, i.e. *Lactococcus lactis* and *Lactobacillus sanfranciscensis* at the level of growth rate, cell morphology and cellular organization.

Non-adapted (mesophilic) microorganisms commonly show growth inhibition at about 40-50 MPa (Fujii *et al.*, 1996; Gross and Jaenicke, 1994). This study has shown that at optimal temperature of growth the mesophilic strains *Lactococcus lactis* MG1363 and *Lactobacillus sanfranciscensis* were able to grow at pressures up to 50 and 40 MPa, respectively. According to the definition of Abe and Horikoshi (2001) both strains are piezosensitive. *Lactobacillus sanfranciscensis* failed to grow at 50 MPa and the growth rate of *Lactococcus lactis* at 50 MPa was less than 30% of the growth rate at atmospheric pressure. The cell morphology was affected during the growth under pressure conditions, the cells were elongated and the cell division was affected. This is in accordance with findings for the gram-negative bacterium *Escherichia coli* for which it was shown that its growth under pressure conditions is accompanied by formation of filaments (Sato *et al.*, 2002). Sharma *et al.* (2002) observed residual formate oxidation by *Escherichia coli* and *Shewanella oneidensis* at pressures ranging up to 1600 MPa and suggested that any pressure encountered on earth is not a limiting factor for microbial life. However, comparison of the data presented in this work with *Lactococcus lactis* and *Lactobacillus sanfranciscensis* (Korakli *et al.*, 2002) and *E. coli* (Sato *et al.*, 2002) and *S. cerevisiae* (Abe and Horikoshi, 1997) clearly demonstrate that multiplication of organisms is inhibited at much lower pressures that overall metabolic activity or even the activity of a single enzyme.

The morphologic changes observed under pressure conditions suggest that some of the cytoskeleton or autolytic proteins were affected by high-pressure. McDonald (1971) noted

that in *L. lactis*, filament formation was associated with decreased autolysin activity, an observation later confirmed by Langsrud *et al.* (1987). Furthermore, autolytic activity in *L. lactis* is involved in cell separation. Buist *et al.* (1995) showed that the muramidase AcmA is required for cell separation of *L. lactis* as an *acmA* deletion mutant grew in long chains. AcmA is essential for cell separation and is the only autolysin in *L. lactis*. Therefore, our microscopic observation of *L. lactis* suggests that the biosynthesis or the activity of AcmA enzyme was impaired by high-pressure.

Chromosomal DNA stained by DAPI was observed in all cells indicating that DNA was replicated and segregated independently of morphological changes and high-pressure conditions. The inhibitory effect of pressure on the cell division was also found in *Lactobacillus sanfranciscensis*, cells under pressure formed filaments.

Protein-protein interactions are important in various biological systems including multimeric enzymes, ribosomes, cytoskeleton proteins and proteins that act in signal transduction pathways; such interactions are thought to be sensitive to increasing pressure. Hydrostatic pressure causes the dissociation of numerous multimeric proteins because the processes are typically accompanied by negative volume changes (Abe and Horikoshi, 2001; Gross *et al.*, 1993; Gross and Jaenicke, 1994). Ribosome dissociation has been demonstrated using light-scattering analysis *in vitro* under high-pressure (Gross *et al.*, 1993; Gross and Jaenicke, 1994). This has led to the suggestion that ribosome dissociation may be one of the principal causes of growth inhibition at high-pressure. Gross *et al.* (1993) investigated the effects of high-pressure on elongation-cycle intermediates and observed ribosome dissociation starting at 40-60 MPa. They suggested that the post-translational complex is the most pressure-sensitive component of the elongation cycle and is possibly the limiting factor for the pressure-induced block of protein biosynthesis and bacterial growth. Ribosome dissociation was observed *in vivo* during incubation of *Escherichia coli* at pressures ranging from 50 – 250 MPa (Niven *et al.*, 1999). Because levels of extracellular Mg^{2+} influenced ribosome conformation after pressure treatment, Niven *et al.* (1999) suggested that, in addition to effects of pressure on multimeric proteins only, the stability of ribosomes *in vivo* is affected by the loss of ion gradients resulting from a permeabilised cytoplasmic membrane.

Immunofluorescence microscopy indicated that aggregation of the FtsZ protein also was affected by high-pressure. The images of IFM demonstrated a reduction of the number of FtsZ rings in exponential cells of *Lactococcus lactis* MG1363 at high-pressure conditions. These observations correlate with the work of Sato *et al.* (2002) with *Escherichia coli*. They reported almost no FtsZ rings in the elongated cells under high-pressure and suggested that

the cells under pressure might be “frozen” at early stage in the cell cycle, since the FtsZ ring does not form just before septation, rather it is assembled very early in the cell cycle (Erickson, 1997; Losick and Shapiro, 1999; Sun and Margolin, 1998). Whether the presence of ions also affects FtsZ ring formation and thus depends on intact membrane remains to be elucidated.

The physiological adaptation of microorganisms from the deep-sea involves the regulation of the membrane fluidity by incorporation of increased proportions of unsaturated fatty acids (Allen *et al.*, 1999; Delong and Yayanos, 1985; Marteinson *et al.*, 1997; Somero, 1992; Tanaka *et al.*, 2001). High hydrostatic pressure has been shown to enhance the growth rate and produce a shift of the optimal temperature of growth from piezophilic bacteria (Canganella *et al.*, 1997; Kato *et al.*, 1995, 1997, 2000; Marquis, 1984). This extension in growth pressure with temperature could be explained by mechanisms involving membrane viscosity in cells, since pressure and heat are parameters, which interact with membrane fluidity. However, this adaptation response seemed to be reduced in mesophilic bacteria, since the piezoresistance of *Lactococcus lactis* MG1363 and *Lactobacillus sanfranciscensis* was not improved at temperatures above their optimal range. Whereas piezophilies regulate their synthesis of poly- and monounsaturated fatty acids depending on pressure and temperature conditions of growth, lactic acid bacteria tend to accept a variety of fatty acids present in the media rather than synthesising them being e.g. oleic acid-auxotrophic microorganisms (Allen *et al.*, 1999; Foucaud *et al.*, 1997; Sugihara and Kline, 1975).

High-pressure induces unique stress responses in mesophilies, such synthesis of specific proteins, accumulation of osmolytes and changes in metabolism (Abe and Horikoshi, 2000; Fujii *et al.*, 1996; Iwahashi *et al.*, 1997a-b, 2000; Korakli *et al.*, 2002; Welch *et al.*, 1993). Our work has shown that pressure also alters morphology and cytoskeleton proteins. This study provides insights into pressure-induced response of lactic acid bacteria.

4.2 Effects of high-pressure during inactivation

High-pressure has been investigated as new promising technique for sterilizing and processing food. As a non-thermal process it inactivates microorganisms and enzymes with reduced adverse effects on taste, flavour and nutrient content. However, microbial inactivation by high-pressure is a complex phenomenon, which involves more than one physiological reactions and is still not completely understood. Inactivation of microorganisms is initiated by membrane damage, but the changes observed after a pressure treatment, further involve ribosome denaturation, coagulation of cytoplasmic protein, enzyme inactivation, inhibition of

transcription and protein synthesis (Cheftel, 1995; Kalchayanand *et al.*, 1998b; Pagan and Mackey, 2000).

In this study the effect of high-pressure inactivation on *Lactococcus lactis* cells was evaluated. The pressure-treated cells were characterised with respect to viability, metabolic activity, membrane integrity, detection time and inactivation of the membrane-bound enzyme LmrP. The effects of external factors such as addition of ionic and nonionic solutes and changes of external pH on the pressure inactivation were determined. Furthermore, the baroprotective mechanisms of salt and sucrose were studied.

The composition of the medium where the microorganisms are dispersed in at the moment of pressurization influences significantly the efficiency of inactivation, the loss of viability is lower in food systems than in phosphate buffer (Kalchayanand *et al.*, 1998a-b). The milk buffer was selected to achieve i) a food-like system and ii) a chemical defined buffer without variations. Our results indicated that the inactivation kinetic in milk buffer correlated with the inactivation kinetic in milk permeate, allowing to use the buffer as chemically defined milk model system.

The patterns of pressure-inactivation kinetics have been frequently discussed. Some investigators indicate first-order kinetics [$\log(N/NO)$ being linear as a function of time] in the case of yeast and bacteria (Butz *et al.*, 1990; Carlez *et al.*, 1992; Ludwig *et al.*, 1992; Raffalli *et al.*, 1994). Other investigators observed a change in slope and a two-phase inactivation phenomenon, with a shoulder and tailing (Cheftel, 1995; Heinz and Knorr, 1996). In this case, the first fraction of the population is quickly inactivated, whereas the second fraction appears to be much more resistant to pressure. Our results corroborate these observations and thus provide further evidence that first order kinetics does not adequately describe pressure inactivation time.

The inactivation kinetics of all strains had the same rate of inactivation and the same level of baroresistent bacteria (tailing of 10^5 cfu). The difference between them was the length of the shoulder. This shoulder with approximately no inactivation suggests a certain resistance mechanism. This phase was explained by phenotypic heterogeneity in the population for the transition from “stable” to “metastable” state during pressure treatments (Oxen and Knorr, 1993). Aside from this difference, a treatment at 300MPa for 12 min at 20°C is sufficient for a microbial reduction of 4 log cycles of all tested strains. Studies with bacteria have revealed that the loss of cell viability increased with increase in pressure and time. Treatments up to 200 MPa inactivated, even after 30 min, less than 1 log cycle (Kalchayanand *et al.*, 1998a-b; Oxen and Knorr, 1993). This correlate with our finding that treatments at 200 MPa up to 60

min did not reduce the number of microorganisms. An increase of pressure reduced the length of the shoulder and increased the rate of inactivation, but it did not affect the tailing.

High-pressure inactivation kinetics are in many cases characterised by tailing, suggesting a more barotolerant fraction of the initial population. The growth of “tailing” cells during storage represents a risk for the consumers. Accordingly to this, one aim of this work was the characterization of the “tailing” cells of *Lactococcus lactis*.

Modelling of bacterial growth using viable counts is time consuming, therefore researchers have explored more simple and rapid methods. One of the simplest methods to detect bacterial growth is the use of optical density (OD) measurements, where growth can be related to the increase in turbidity of a bacterial culture (McKellar and Knight, 2000). The detection time (t_d) for a turbidimetric instrument can be defined as the time required for an initial measurable increase in OD (Wu *et al.*, 2000). Baranyi and Pin (1999) have proposed a method for calculating lag-phase (λ) and specific growth rate (μ) from t_d . Their method is based on the biological interpretation that lag is a period of adjustment of the initial physiological state of the cells to the new environment during which only the intracellular conditions changes (Baranyi and Roberts, 1995; Baranyi, 1998; Baranyi and Pin, 1999). Buchanan *et al.* (1997) proposed that the time required for the transition between lag and exponential phases reflects biological variation among individual cells of the bacteria population. Assuming pure exponential growth until the time of detection, preceded by the lag period only, the distribution of the detection times reflects the distribution of the population lag times which is more and more scattered for lower inoculum levels (Baranyi, 1998). Measurement of lag or detection times of individual cells allows useful and convenient comparison of heterogeneity of cultures.

The characteristic tailing effect of pressure inactivation can be related to heterogeneity in the cells of the same population corresponding to the existence of a more pressure-resistant subfraction (Earnshaw, 1995). When this apparent subfraction was isolated by culturing the surviving cells after extended pressure exposure, no significant difference in pressure sensitivity to the original culture was observed (Metrick *et al.*, 1989; Ulmer *et al.*, 2000). This indicates that the barotolerant fraction is determined rather to physiological heterogeneity than to genetical diversity.

The extension of lag phase as result of physical injury of bacterial cells has been frequently reported (Mackey and Derrick, 1982, 1984; Stephens *et al.*, 1997; Augustin *et al.*, 2000; Smelt *et al.*, 2002). Mackey and Derrick (1982) indicated that sublethally injured bacteria inoculated into growth media undergo a prolonged lag phase during which time injury is

repaired and the ability to grow restored. Populations of injured cells are heterogeneous and contain individuals with varying degrees of injury (van Shothorst & van Leusden, 1975). The measured lag time of such populations may therefore reflect the lag times of the least severely injured individuals which are first to repair injury and to begin multiplication.

Our results show a considerable heterogeneity of the distribution time of untreated cultures, suggesting that physiological variation within the initial population may account for tailing. Furthermore, a significant difference between detection time of cells from original culture and from the pressure-resistant fraction was observed. This difference can be explained by sublethal injury of surviving cells or by survival of those cells of the initial population with an extended detection time. Allowing 30 min of incubation for repair of sublethal injury or activation of resting cells, a rapid inactivation upon a second pressure cycle was observed. Therefore, we favour the interpretation of presence of resting cells, which were activated during the first cycle of treatment.

Although screening by enumeration of the survivors e.g. by plate counts is a quite acceptable selection method for the high-pressure inactivation, it is labour intensive and it requires some days before the results can be read. Additional screening methods have shown to assess in more detail the physiological state of the cell (Smelt *et al.*, 2002). Methods involving dyes have been successfully used to determine the loss of membrane integrity after treatment with high-pressure. Pagan and Mackey (2000) used propidium iodide to determine the loss of membrane integrity in pressure-treated *Escherichia coli*, in our laboratory Ulmer *et al.* (2000) used propidium iodide to analyse *Lactobacillus plantarum* after high-pressure treatment. The LIVE/DEAD[®]BacLight[™] Bacterial Viability Kit (Molecular Probes) utilise mixtures of their SYNTO[®] 9 green fluorescent nucleic acid stain and the red fluorescent nucleic acid stain, propidium iodide. With an appropriate mixture of both stains, bacteria with intact cell membrane stain fluorescent green, whereas bacteria with damaged membrane stain fluorescent red (Anonymous, 1999).

Tetrazolium salt offers a rapid, culture-independent means to determine viability and relative rate of reducing equivalent production in microorganisms (Thom *et al.*, 1993). The use of the redox dye 2-(p-iodophenyl)-3-(p-nitrophenyl)5-phenyltetrazolium chloride (INT) as an indicator of microbial metabolic activity has become a standard laboratory method. This compound is reduced by the electron transport system in active bacteria to an insoluble purple tetrazolium crystal within the cell which can be visualised using bright field microscopy (Thom *et al.* 1993; Walsh *et al.*, 1995). This method has been used to determine the metabolic activity of pressure-treated *Lactobacillus plantarum* (Ulmer *et al.*, 2000).

Treatment of *Lactococcus lactis* at 200 MPa did not affect viability but reduced metabolic activity to 50%. Cell death observed during treatment at 300 MPa was followed closely by loss of metabolic activity whereas dead cells conserved membrane integrity. These results suggested that the high-pressure treatment of *Lactococcus lactis* affects metabolic activity and subsequently damages membrane integrity.

The addition of NaCl and sugars protects microorganisms during high-pressure treatments. This increased barotolerance was attributed to cell shrinkage, which probably causes a thickening in the cell membrane reducing membrane permeability and fluidity (Hayakawa *et al.*, 1994; Knorr, 1994; Oxen and Knorr, 1993; Takahashi *et al.*, 1993). However, the effect of NaCl or sugars on the osmotic pressure of foods or suspension media is not sufficient to explain the marked baroprotective effects of these solutes on microbial inactivation (Cheftel, 1995). Our work supports this statement because 1.5 M sucrose with a higher water activity had a stronger baroprotective effect than 4 M NaCl.

In general, sugars had a stronger protective effect than salts on the inactivation of *Lactococcus lactis* and this effect did not only rely on the lowering of a_w . High concentrations of sugars (lactose or sucrose) impose only a transient osmotic stress in *Lactococcus lactis*, because the cells are able to equilibrate the extra- and intracellular concentrations of sucrose and lactose (Glaasker *et al.*, 1998). Disaccharides stabilize proteins in their native state and preserve the integrity of membranes during stresses (Leslie *et al.*, 1995). Adding a disaccharide lowers the transition temperature T_m of the membranes by replacing the water between the lipid headgroups, preventing the phase transition, and inhibiting the fusion between the liposomes due to glass formation (vitrification) (Crowe *et al.*, 1997). Furthermore, the addition of sucrose raises the internal pressure of the solvent, forcing the protein to overcome a greater energy barrier in unfolding (Timasheff *et al.*, 1976) and stabilizes the proteins because of preferential exclusion of sucrose. As our results show that the high-pressure inactivation of the membrane-bound enzyme LmrP was retarded with the addition of 0.5 M sucrose to the buffer. The results of the HPLC indicate an internal accumulation of sucrose and glucose/fructose produced by its hydrolysis during hyperosmotic conditions with 0.5 M sucrose. Therefore, we suggest that the intracellular sucrose accumulation protected the metabolic enzyme and the lipids of the membrane during the high-pressure treatment.

High salt concentration is detrimental for microorganisms, however the addition of NaCl was shown to protect the cells during high-pressure treatments. Microorganisms accumulate compatible solutes upon challenge by decreased water activity. The intracellular accumulation of compatible solutes maintains the osmotic balance with the extracellular environment, the

integrity of biological membranes and enhances the stability of enzymes (Kets *et al.*, 1997). *Lactococcus lactis* accumulates glycine betaine when subjected to salt stress. However, since *Lactococcus lactis* is unable to synthesize this compound, it is replaced by unknown osmolytes, unless it is added to the medium. The increase of the baroprotective effect of 2 NaCl in presence of glycine betaine indicated that the intracellular accumulation of compatible solutes played a major role in the baroprotective effect of NaCl. In the treatments with NaCl without glycine betaine the baroprotective effect could be explained by an accumulation of unknown osmolytes from the milk buffer. The results of the HPLC support this, since cells accumulated glycine betaine by osmotic upshock with NaCl if glycine betaine was added to the buffer, whereas cells without glycine betaine accumulated lactose and unknown osmolytes.

Hydrostatic pressure affects the intracellular pH of microorganisms by (i) enhanced dissociation of weak organic acids and (ii) increased permeability of the cytoplasmic membrane and inactivation of enzymes required for pH homeostasis. In this study it was shown by *in-situ* determination of pH_{in} values that sublethal high-pressure treatments decreased the internal pH of *Lactococcus lactis* and *Lactobacillus plantarum* cells to the level of the external pH. A reversible reduction of the pH_{in} occurred concomitantly with compression prior to a reduction of viable cell counts. Furthermore, the regeneration of the ΔpH upon pressure release was monitored.

The cFSE method for pH determination is based on the internal conjugation of cFSE in the cytoplasm of cells, followed by the elimination of free probe by an incubation in the presence of a fermentable sugar. Efflux of fluorescent probe during high-pressure treatment, that may permeabilize the cells envelope were avoided because the succinimidyl group of cFSE is conjugated with the aliphatic amines of intracellular proteins (Haugland, 1992). The useful range of fluorescent pH indicators is determined by their pK_{a} , cFSE has a pK_{a} of 6.5, compatible to work in the range expected for the pH_{in} of our microorganisms. For lower pH_{in} the cFSE may be replaced with 5-(and 6-)-carboxy-2',7'-dichlorofluorescein succinimidyl ester, which has a pK_{a} of 3.9 in buffer and 4.9 when incorporated in bacterial cells (Breeuwer *et al.*, 1995; Siegumfeldt *et al.*, 2000). The effects of cFSE incorporation on the cells prior to high-pressure treatments were negligible, since the viable counts of cells stained with cFSE were not different from those observed with unstained cells. Furthermore, the external pH did not affect either cell viability or the internal pH during storage of cells for up to 3 h at ambient pressure. The values for the internal pH at atmospheric pressure of *L. lactis* and *L. plantarum* at external pH values ranging from 4.0 to 8.0 determined in this work were comparable to

those reported previously for *L. lactis* and *L. plantarum* (Belguendouz *et al.*, 1997; Breeuwer *et al.*, 1996; Siegumfeldt *et al.*, 2000; Wouters *et al.*, 1998). In both species investigated in this work, the internal pH decreased with decreasing buffer pH, resulting in ΔpH values between 1.9 and 2.1 pH unit at pH 4.0.

It is well established that a reduction in the pH of the suspending medium causes a progressive increase in the sensitivity of bacteria to pressure (Alpas *et al.*, 2000; Garcia-Graells *et al.*, 1998; Mackey *et al.*, 1995; Nannen and Hutkins, 1991; Rademacher, 1999; Ritz *et al.*, 2000). For example, pressurization at 345 MPa and a pH values of 4.5 increased the viability loss of *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enteritidis* by an additional 1.2-3.9 log cycles compared to pressurization at pH 6.5 (Alpas *et al.*, 2000). The data on pressure inactivation kinetics at acidic and near neutral pH values presented in this study are consistent with these literature data. Both *L. lactis* and *L. plantarum* were less pressure sensitive at pH 6.5 than at pH 4.0. However, independent of the buffer pH, a fraction of about 1 in 10^6 cells withstood pressure treatment for up to 120 min. For either strain, a greater degree of sublethal injury was observed at pH values of 6.5 and 6.0 compared to pressure treatment at pH 4.0 and 5.0. This indicates that at a pH value near neutral, sublethally injured cells survive and recover eventually upon transfer to a rich medium. At acidic pH values, sublethally injured cells are inactivated during pressure treatment.

Wouters *et al.* (1998) reported a decrease on pH_{in} of *Lactobacillus plantarum* after high-pressure treatment. Our results confirmed and extended these observations by pH measurements during pressure treatment, which allow the distinction between reversible and irreversible effects of pressure treatment on pH homeostasis. Compression of the samples resulted in a fast and reversible decrease of the internal pH. At 300 MPa, the internal pH was equal to the external pH with completion of the pressure up ramp, prior to a reduction of viable cell counts. The observation that the decrease of internal pH occurred virtually in equilibrium with the pressure build up suggests that both the membrane permeability and the activity of proton pumps were affected by high-pressure treatment. Wouters *et al.* (1998) also found that cells grown at lower pH were more resistant to pressure than cells grown at optimal pH. This difference in resistance may be explained by a higher F_0F_1 ATPase activity, better ability to maintain a ΔpH , or a higher acid efflux of cells grown at low pH. A correlation between acid stress and barotolerance was also observed in our laboratory with sublethal acid shock on cells of *Lactobacillus sanfranciscensis* indicating that pressure stress may involve acid stress (Scheyhing, 2002).

Membrane and membrane-bound enzymes are a major target for the pressure inactivation of microorganisms (Smelt *et al.*, 1994). Under high-pressure the membrane lipids undergo a phase transitions from the liquid crystalline phase to the gel phase. This phase transition enhances the permeability of the membrane to ions, and inflicts cell injury because some transport proteins only function in a liquid crystalline membrane (Beney and Gervais, 2001). The sensitivity of bacteria is markedly affected by membrane properties (ter Steeg *et al.*, 1999; Ulmer *et al.*, 2002).

In *L. plantarum*, pressure treatment caused partial inactivation of the F₀F₁ ATPase such that the ability of cells to maintain a Δ pH was reduced, and the acid efflux mechanism was impaired (Wouters *et al.*, 1998). Direct denaturation, dissociation of the quaternary structure, or the dislocation of the enzyme from the membrane may account for this effect. Ulmer *et al.* (2000, 2002) reported that inactivation of transport proteins is an early event during pressurization of microorganisms.

The findings in this work confirmed the pressure inactivation of the membrane-bound multidrug transporter LmrP in *L. lactis* before a reduction on the CFU was observed.

Remarkable differences in barotolerance pH homeostasis during pressure treatment were observed between *L. lactis* and *L. plantarum*. The minimum pH permissive for growth of *L. lactis* is 4.0, whereas *L. plantarum* is a highly acid tolerant beer spoiling organism growing at pH values of 3.5 or lower (Gänzle *et al.*, 2001). In *L. lactis*, pH homeostasis was irreversibly disturbed after 4 min of pressure holding time at 300 MPa and after 30 min at 200 MPa. The addition of sucrose to milk buffer increased the capacity of the cells to restore a Δ pH, whereas the addition of NaCl dissipated the transmembrane proton potential prior pressure treatment. The loss of viability related not to the decrease of the internal pH but to the loss of ability to restore the Δ pH after pressure treatment. *L. plantarum* was more pressure tolerant than *L. lactis* at any pH value. The transmembrane proton potential was not fully dissipated by treatments at 200 MPa. The sensitivity of *L. plantarum* to treatments at 300 MPa was affected to a greater extent by low pH conditions than that of *L. lactis*. This observation may relate to the inability of *L. plantarum* to restore a Δ pH upon pressurization at 300 MPa even after 0 min treatment time. Our data thus support the hypothesis that pressure treatment restricts the pH range tolerated by bacteria as a consequence of the irreversible inhibition of ATPase-dependent transfer of protons and cations (Ritz *et al.*, 2000). Pagán *et al.* (2000, 2001) reported a sensitisation of *E. coli* cells to acid by a pressure treatment. Because the lowering of the internal pH upon pressure treatment did not explain the death of pressure damaged cells under acidic conditions, the authors suggested that this acid sensitivity is caused by the loss of

protective or repair functions rather than to the loss a transmembrane ΔpH per se. Garcia-Graells *et al.* (1998) reported a secondary inactivation of *E. coli* during storage in fruit juices at low pH after a primary pressure treatment inflicting sublethal injury to a large proportion of cells. This observation was confirmed for pressure resistant strains of *E. coli* O157-H7 and *Listeria monocytogenes* (Jordan *et al.*, 2001). Survival and growth of lactic acid bacteria in beer was prevented by pressure treatments that resulted in a reduction of 50 – 99% of viable cell counts only but fully eliminated hop resistance of lactobacilli (Gänzle *et al.*, 2001; Ulmer *et al.*, 2002). Schweininger (2002) also reported an inactivation of lactic acid bacteria during storage at low pH in apple juice, yoghurt and milk buffer after a sublethal pressure treatment. The observation that the ability of microorganisms to survive in harsh environments is eliminated by pressure treatment allows the distinction between primary and secondary effects of high-pressure treatments. The reversible or irreversible loss of concentration gradients across the cytoplasmic membrane may be considered as a primary effect of pressure treatment. Cell death may be considered a secondary event depending not primarily on pressure or pressure holding time, but on the environmental conditions during and after pressure treatment. This concept may prove useful to study the relevance of membrane properties in the bacterial response to environmental stress.

4.3 General remarks

In conclusion, evidence is provided that primary pressure effects are related to or initiated by changes of the phase behaviour and permeability of cytoplasmic membranes. These changes occur fast during the build-up time of pressure and are often reversible at low pressures. They cause the inactivation of multi-drug transport enzymes and dissipate pH gradient, which are important for the survival of microorganisms under adverse conditions encountered in food (Gänzle *et al.*, 2001). The inactivation of MDR transport activity by high-pressure can be measured before the loss of viability, indicating that survival or inactivation of microorganisms will depend on further environment conditions. The baroprotective effect of salts and sugars rests on their properties to retard the changes of membrane fluidity during high-pressure treatment and the protection against protein denaturation. A model for the pressure induced bacterial injury based on the results presented in this thesis and literature data is shown in **Fig. 4.2**. The high-pressure induced membrane effects occur concomitant to build-up time of pressure and reversible effects, e.g. dissipation of ΔpH , are observed prior to irreversible cellular damage. The reversibility of the effects will depend on holding time of treatment, and the lethal effects result from the combination of damaged membrane transport

systems and detrimental environmental parameters. High-pressure treated cells are impaired to maintain vital concentration gradients of ions and metabolites. Therefore, cell death will occur as secondary effect of high-pressure depending on the environmental conditions.

These results provide insights into the mechanism of high-pressure inactivation that is especially relevant for the design of high-pressure processes for food preservation. Furthermore, the study of the cellular responses under growth at sublethal pressure conditions can be used for novel biotechnological processes and possibly the synchronisation of cultures.

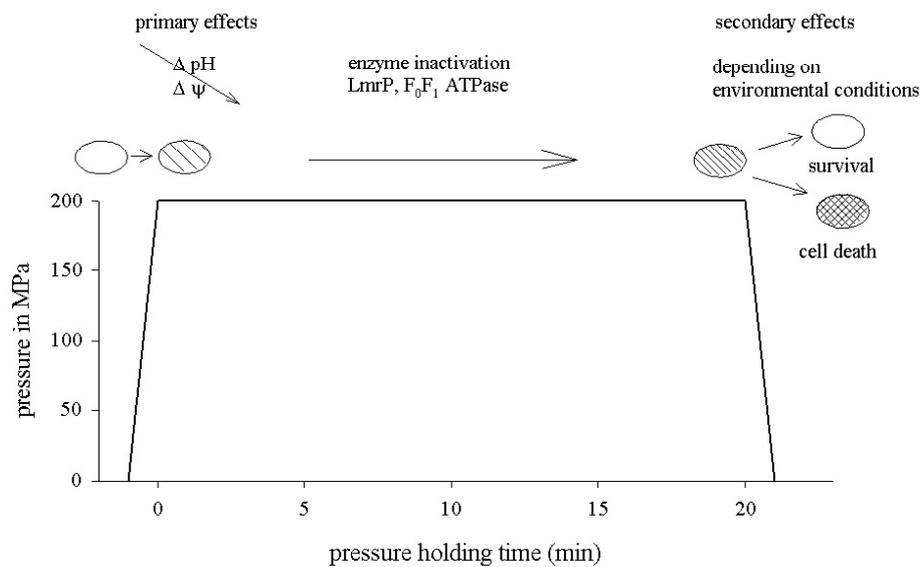


Fig. 4.2 Pressure ramp profile is shown for 20 minutes at 200 MPa (—) distinguishing primary from secondary effects.

5. Summary

The aim of this thesis was to study the mechanism of high-pressure induced growth inhibition and cell death distinguishing primary from secondary pressure effects. The lactic acid bacterium *Lactococcus lactis* was chosen as a model system and its responses to high-pressure were characterised during growth and after pressure-induced inactivation.

The piezosensitivity of *Lactococcus lactis* grown under sublethal pressure was characterised with respect to chromosomal replication by DAPI-staining and FtsZ ring formation by immunofluorescence. The cell morphology was affected during the growth under pressure conditions, the cells were elongated and the cell division was affected. These morphological changes suggest that some of the cytoskeleton or autolytic proteins are affected by pressure. Since the muramidase AcmA is essential for cell separation and is the only autolysin in *Lactococcus lactis*, we suggest that the biosynthesis or the activity of AcmA enzyme was impaired by high-pressure. The aggregation of FtsZ protein required for cell division was also affected by pressure, whereas DNA replication and segregation were independent of morphological changes. An increase of growth temperature did not improve the piezotolerance of *Lactococcus lactis*. The adaptation mechanisms to higher temperature and pressure observed in deep-sea microorganisms is reduced in lactic acid bacteria, because they are not able to regulate the synthesis of poly- and monounsaturated fatty acids.

The selection of a specific model for the study of high-pressure kinetics is necessary to facilitate the identification of future optimised preservation strategies and will provide a basis for estimates of microbial behaviour outside the measured data points. The milk buffer developed for these experiments allowed to investigate the impact of single factors without losing the similarity to a food system, facilitating the observation of pressure induced inactivation under well-defined conditions.

The inactivation kinetics of different strains of *Lactococcus lactis* were qualitatively similar in their sigmoid asymmetric shapes with an initial shoulder and an inactivation phase followed by tailing. Pressures above 200 MPa were effective for inactivating *Lactococcus lactis*. An increase of pressure reduced the length of the shoulder, but it did not affect the tailing. The tailing effect of high-pressure inactivation was characterised by means of detection time. The physiological variation of the original population was demonstrated by a considerable heterogeneity of the distribution time of untreated cultures. The detection time of pressure treated cells was extended, this effect can be explained by sublethal injury in surviving cells or presence of resting cells within the initial culture. The baroresistance of the tailing showed

to be rather physiologically determined than genetically, since the pressure sensitivity of cultures generated by cells isolated from tailing was not different from the original culture.

The methods involving dyes and absorbance measurements were successful to identify the effect of high-pressure on *Lactococcus lactis* cells. *Ex-situ* and *in-situ* methods were adapted to characterise the physiological changes of the cells. These methods provided evidences to the mechanism of pressure induced inactivation distinguishing primary and secondary pressure effects, reversible and irreversible reactions as well as responses at organizational and physiological level. It was shown that a loss of metabolic activity, membrane damage, reduction of internal pH, dissipation of K⁺-membrane potential and inactivation of the membrane-bound enzyme LmrP occurred prior to cell inactivation. Therefore, cell inactivation may be considered a secondary event not only depending on pressure or pressure holding time, but on the environmental conditions during and after pressure treatment.

Addition of ionic and nonionic osmolytes exhibited a baroprotective effect against the loss of viability of *Lactococcus lactis*. The baroprotective effect of sucrose is stronger compared on a molar basis. While sucrose protected the metabolic activity, membrane integrity and LmrP activity, salt did not show any protection on metabolic activity and LmrP activity. This work suggested the accumulation of compatible solutes to understand the baroprotective effect of low molecular solutes. Intracellular sucrose accumulation protected the metabolic enzyme and the lipids of the membrane during the high-pressure treatment. Sucrose protects enzyme by preferential exclusion of water, whereas it lowers the transition temperature of membrane. NaCl protects by an accumulation of lactose and unknown compatible solutes from the milk buffer. Addition of glycine betaine to the milk buffer with NaCl improved the baroprotection because of its functionality as compatible solute.

A reduction in the pH of the milk buffer increased the cell sensitivity to pressure. With a decrease of external pH the shoulder of inactivation kinetics was reduced and the inactivation phase was accelerated. The first *in-situ* measurement of internal pH during high-pressure treatment was performed in this work. Sublethal high-pressure treatments decreased the internal pH of the cells to the level of external pH. The reduction occurred concomitantly with the compression prior to a reduction of viable cells counts. A reduction of viable cells counts was related to the loss of capacity to restore a Δ pH. The addition of sucrose improved the ability to restore the Δ pH. These findings suggested that both the membrane permeability and the activity of proton pumps were affected by high-pressure treatment.

Membrane and membrane-bound enzymes are a major target for the pressure inactivation of microorganisms (Smelt *et al.*, 1994). The reduction of internal pH, dissipation of K⁺-

membrane potential and inactivation of the membrane-bound enzyme LmrP corroborated this hypothesis. The effects can be explained by direct denaturation, dissociation of the quaternary structure or the dislocation of the enzyme from the membrane. Pressure causes phase transitions of membrane lipids from the liquid crystalline phase to the gel phase (Ulmer *et al.*, 2002). Changes of membrane fluidity affect the activity of membrane-bound enzyme because some transport proteins only function in a liquid crystalline membrane. In this case, cell inactivation will occur if those changes are irreversible and the environmental conditions are adverse.

This work provides evidence that primary pressure effects are related to or initiated by changes of the phase behaviour and permeability of cytoplasmic membranes. These changes occur fast during the build-up time of pressure and are often reversible at low pressures. They cause the inactivation of multi-drug transport enzymes and dissipate the pH gradient, which are important for the survival of microorganisms under adverse conditions encountered in food (Gänzle *et al.*, 2001). These insights into the mechanism of high-pressure inactivation are especially relevant for the design of high-pressure processes for food preservation.

6. Zusammenfassung

Das Ziel der Arbeit war die Untersuchung der Mechanismen der druckinduzierten Wachstumshemmung und Zellinaktivierung von *Lactococcus lactis*. Während des Wachstums, sowie während und nach der Hochdruckinaktivierung, wurden die Reaktionen dieses Organismus auf Hochdruck charakterisiert. Dies ermöglichte die Unterscheidung primärer und sekundärer Druckeffekte, sowie reversibler und irreversibler Reaktionen.

Die Empfindlichkeit unter subletalem Druck wurde bezüglich der chromosomalen Replikation durch DAPI-Färbung und der FtsZ-Ringbildung durch Immunofluoreszenz untersucht. Während des Wachstums unter Hochdruck traten Veränderungen der Zellmorphologie auf. Die Zellen verlängerten sich, und die Zellteilung wurde beeinträchtigt. Die morphologischen Veränderungen lassen vermuten, dass der Druck Auswirkungen auf einige der Cytoskelett- oder Autolyse-Proteine hat. Da die Muramidase AcmA wesentlich zur Zellteilung beiträgt und das einzige Autolysin in *Lactococcus lactis* ist, ist davon auszugehen, dass die Biosynthese oder die Aktivität des AcmA-Enzyms beeinflusst wird. Die für die Zellteilung notwendige Aggregation des FtsZ-Proteins wurde ebenfalls beeinträchtigt, während die DNA-Replikation und Segregation unabhängig von morphologischen Veränderungen waren. Eine Erhöhung der Wachstumstemperatur bewirkte keine Verbesserung der Drucktoleranz des *Lactococcus lactis*. Der bei Tiefseemikroorganismen beobachtete Adaptionsmechanismus für höhere Temperaturen und Drücke wurde bei Milchsäurebakterien nicht beobachtet. Diesen Bakterien fehlt die Fähigkeit, die Synthese von mehrfach gesättigten und einfach gesättigten Fettsäuren zu regulieren.

Die Auswahl eines spezifischen Modells für die Untersuchung der Hochdruckinaktivierungskinetik ist notwendig, um zukunftsweisende Konservierungsstrategien in Lebensmitteln abzuleiten und das mikrobiologische Verhalten zu ermitteln. Der für diese Arbeit etablierte Milchpuffer ermöglichte die Untersuchung von Einzelfaktoren in einem lebensmittelähnlichen System unter definierten Bedingungen.

Drücke höher als 200 MPa waren effektiv für die Inaktivierung von *Lactococcus lactis*. Die Inaktivierungskinetiken unterschiedlicher Stämme von *Lactococcus lactis* waren qualitativ ähnlich. Alle Kurven zeigten eine sigmoide Form mit einer Schulter und einer Inaktivierungsphase gefolgt von einem „Tailing“. Ein Anstieg des Druckes reduzierte die Dauer der Schulter, hat aber keine Auswirkung auf das „Tailing“. Dieser „Tailing“-Effekt der Hochdruckinaktivierung wurde durch die Untersuchung der Detektionszeit charakterisiert. Die physiologische Variation der ursprünglichen Population wurde durch eine heterogene Verteilung der Detektionszeit von unbehandelten Zellen nachgewiesen. Die Zeit, um die

behandelten Zellen zu detektieren, verlängerte sich. Dieses Phänomen kann durch subletale Schädigung oder die Anwesenheit von ruhenden Zellen in der ursprünglichen Kultur erklärt werden. Die Baroresistenz der Zellen in „Tailing“ weist eher einen physiologischen als einen genetischen Charakter auf, da die von Zellen aus dem „Tailing“ gezüchteten Kulturen keine erhöhte Baroresistenz zeigten.

Die fluoreszenz-basierten und optischen Methoden waren gut geeignet für die Kennzeichnung der Hochdruckauswirkungen auf Zellen von *Lactococcus lactis*. *Ex situ* und *in situ* Methoden wurden angepasst, um die physiologischen Veränderungen der Zellen zu charakterisieren. Mit Hilfe dieser Methoden gelang die Unterscheidung von primären und sekundären Druckeffekten, reversiblen und irreversiblen Reaktionen, sowie von Reaktionen der intrazellulären Organisation und der Physiologie. Es wurde gezeigt, dass Phänomene wie die Reduzierung der Stoffwechselaktivität, die Membranschädigung, die Senkung des intrazellulären pH-Wertes, der Verlust des Kaliummembranpotentials und die Inaktivierung des membrangebunden Enzyms LmrP vor dem Zelltod auftreten. Deswegen kann die Zelinaktivierung als sekundärer Effekt betrachtet werden, die nicht nur von Druck und Druckhaltezeit, sondern auch von Milieubedingungen während und nach der Hochdruckbehandlung abhängig ist.

Die Zugabe von ionischen und nicht-ionischen Osmolyten ergab eine Schutzfunktion gegen die Hochdruckinaktivierung von *Lactococcus lactis*. Bei gleicher Molarität sind die baroprotektiven Effekte von Saccharose stärker als die von Kochsalz ausgeprägt. Während Saccharose die Stoffwechselaktivität, die Membranintegrität und die LmrP-Aktivität schützte, trat für Salz keine Schutzfunktion hinsichtlich der metabolischen und der LmrP-Aktivitäten auf. Aus dieser Arbeit ergaben sich Hinweise auf den Schutzmechanismus nicht-ionischer Osmolyten: die intrazelluläre Akkumulation von Saccharose schützt die metabolischen Enzyme und die Membranlipide während der Hochdruckbehandlung. Saccharose setzt die Phasenübergangstemperatur der Membran herab. Die Vermutung liegt nahe, dass die Schutzfunktion der Saccharose auf die Proteine auf den Ausschluss von Wasser zurückzuführen ist. Die baroprotektiven Effekte von NaCl beruhen auf der Akkumulation von Laktose und unbekanntem „compatible solutes“ des Milchpuffers. Zugabe von Glycin Betain zu dem Milchpuffer mit NaCl verbesserte den Schutzeffekt durch dessen Funktionalität als „compatible solute“.

Eine Absenkung des pH-Wertes des Milchpuffers erhöhte die Druckempfindlichkeit der Zellen. Mit der Abnahme des externen pH-Wertes wurde die Schulter der Inaktivierungskinetik reduziert und die Inaktivierungsphase beschleunigt. In dieser Arbeit

wurde die erste *in situ* Messung des internen pH-Wertes während der Hochdruckbehandlung ausgeführt. Subletale Hochdruckbehandlungen senkten den intrazellulären pH-Wert auf den externen pH-Wert ab. Die Absenkung des intrazellulären pH-Wertes erfolgte rasch bereits während der Rampe und trat vor der Abnahme der Lebendkeimzahl auf. Die Abnahme der Lebendkeimzahl war mit der Fähigkeit der Wiederherstellung des Membranpotentials (ΔpH) verbunden. Die Zugabe von Saccharose verbesserte diese Fähigkeit. Diese Erkenntnisse legen nahe, dass sowohl die Membranpermeabilität als auch die Aktivität der Protonenpumpen durch die Hochdruckbehandlung beeinflusst werden.

Membran- und membranengebundene Enzyme sind wichtige Wirkungszentren der Hochdruckinaktivierung von Mikroorganismen. Die Reduzierung des intrazellulären pH-Wertes, der Verlust des Kaliummembranpotentials und die Inaktivierung des membrangebundenen Enzyms LmrP unterstützen diese Hypothese. Auswirkungen lassen sich durch unmittelbare Denaturierung, Dissoziation der quartären Struktur oder die Verlagerung der Membranenzyme erklären. Druckbehandlung führt bei Membranlipiden zu Phasenübergängen von der flüssig-kristallinen zur Gel-Phase. Veränderungen der Membranfluidität beeinflussen die Aktivität membrangebundener Enzyme, da die Funktionalität einiger Membrantransporter an einen flüssig-kristallinen Membranzustand gebunden ist. In diesem Fall tritt die Zellinaktivierung bei irreversiblen Veränderungen ein, wenn die Milieubedingungen ungünstig sind.

Diese Arbeit beweist, dass das Auftreten von primären Druckeffekten mit Veränderungen im Phasenverhalten und der Permeabilität von Zytoplasmamembranen verbunden ist oder dadurch initiiert wird. Die Veränderungen vollziehen sich schnell während der Zeit des Druckaufbaus und sind bei niedrigem Druck oft reversibel. Sie führen zur Inaktivierung von Multi-Drug-Transport-Enzymen und dem Verlust des pH-Gradienten der Zytoplasmamembran. Beides ist bedeutend für das Überleben von Mikroorganismen unter ungünstigen Bedingungen, wie sie in Lebensmitteln vorhanden sein können.

Diese Einblicke in den Mechanismus der Hochdruckinaktivierung sind von besonderem Interesse für die Entwicklung von Hochdruckverfahren für die Konservierung von Lebensmitteln.

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geboren am: 21.04.1972 in Oaxaca, Mexiko
Familienstand: verheiratet mit Erik Höppner

Schulbildung:

1978-1990 Instituto Carlos Gracida, Oaxaca, Mexiko

Studium:

Seit 10.1999 Promotion mit dem Thema „Physiological response of *Lactococcus lactis* to high-pressure“ am Lehrstuhl für Technische Mikrobiologie der TU-München in Freising
3 Monate Forschungsaufenthalt am Japan Marine Science and Technology Center in Yokosuka, Japan
2000-2002 Aufbaustudium Biotechnologie an der TU-München
1997-1999 Dipl.-Ing. Verarbeitungs- und Verfahrenstechnik mit Vertiefungsrichtung Lebensmitteltechnik an der TU-Dresden
Diplomarbeit mit dem Thema „Lebensmitteltechnische Untersuchungen zur Herstellung von Russisch Brot für Diabetiker“
1991-1995 Food Industries Engineering am Instituto Tecnológico y de Estudios Superiores de Monterrey (ITESM), Monterrey, Mexiko
2 Semester Austauschstudentin an der University of Nebraska, Lincoln, Nebraska, USA

Beruflicher Werdegang:

02.98-06.99 Arbeit als studentische Hilfskraft am Institut für Verfahrens- und Umwelttechnik der TU-Dresden
10.97-06.99 Arbeit als studentische Hilfskraft am Institut für Lebensmittel- und Bioverfahrenstechnik der TU-Dresden
08.98-10.98 Praktikum bei Grumbacher Kartoffeln Spezialitäten, Grumbach
09.97-02.98 Praktikum bei der Dr. Quendt Backwaren GmbH, Dresden
02.-05.1995 Arbeit als Physik- und Chemielehrerin an der Madison- Schule in Monterrey, Mexiko
01.-06.1995 Praktikum am Obst- und Gemüsetechnologielaboratorium des ITESM
08.-12.1994 Praktikum am Fleischtechnologielaboratorium des ITESM

Freising, 19.11.2002

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