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Protection by sucrose against heat-induced lethal and sublethal injury of *Lactococcus lactis*: An FT-IR study

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Abstract

The heat inactivation of *Lactococcus lactis* was studied by determination of cell counts, and by FT-IR spectroscopy recording the average structure of cell proteins. Cell counts were measured after incubation milk buffer or milk buffer with 1. 5 M sucrose, and FT-IR spectra were recorded in ^2H_2O or ^2H_2O with 1. 5 M sucrose in the range of 6–75 °C. Sucrose protected *L. lactis* against heat inactivation. The cell counts differed by up to 6-log cycles after treatment in milk buffer as compared to milk buffer with sucrose. The $^1H/^2H$ exchange in proteins, and secondary structure elements were detected by the analysis of amide I′, amide II and amide II′ bands. A reduced $^1H/^2H$ exchange as well as a lower content of disordered structural elements was observed when sucrose was present. Conformational fluctuations of native proteins as indicated by the $^1H/^2H$ exchange were apparent already at sublethal temperatures. The loss of viability of *L. lactis* occurred in the same temperature range as the loss of the protein secondary structure. These results demonstrate that sucrose protects *L. lactis* against heat inactivation, and that the increased heat stability of proteins in the presence of sucrose contributed to this enhanced heat resistance.
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Keywords: FT-IR spectroscopy; Bacterial inactivation; Protein denaturation; Heat resistance

1. Introduction

Heat treatment remains the most common processing technique to assure the microbiological safety of foods. Moreover, combined osmotic and thermal treatments are frequently applied in the stabilisation of bacterial cultures for use as starter cultures in food fermentation, or for use as probiotics. In both types of processes aiming to eliminate or to stabilise bacteria, the bacterial heat resistance must be taken into account. The heat resistance of bacteria depends on extrinsic and intrinsic factors. First, stationary cells are more resistant than cells in their exponential phase of growth due to the *de novo* synthesis of stress proteins in the early stationary phase [1–3]. Second, the medium composition with respect to the pH, water activity (a_w), and the concentration of osmolytes affect bacterial inactivation [4–6]. Bacteria exhibit a maximal resistance against heat treatment at their optimal pH of growth. Furthermore, a reduction in water activity results in a higher resistance to thermal treatment [7].

Moderately elevated temperatures cause sublethal injury, from which the organisms recover only on non-selective media [8,9]. Heat induced sublethal injury and cell death was linked to protein denaturation and the inactivation of membrane bound enzymes [10,11]. Many foods are selective media due to their particular properties such as pH-value and presence of anti-microbial compounds. In those foods, the inactivation of specific resistance mechanisms required for growth or survival in these products may be sufficient for preservation.

Bacteria adapt to hyper-osmotic conditions by accumulating organic solutes such as sugars [12–14]. Lactic acid bacteria, including *Lactococcus lactis* MG1363, react to osmotic upshock

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with sucrose by equilibration of the intracellular and extracellular sucrose concentrations [12,15]. This accumulation of osmolytes furthermore provides protection towards other lethal stressors, e.g., freeze-drying, salt, high pressure or heat [5,15,16]. FT-IR measurements demonstrated that the accumulation of osmolytes prevents membrane damage under stress conditions [15,17,18].

The heat denaturation of proteins is caused by conformational transitions in the secondary structure. Increasing temperature enhances cooperative intra-molecular motions until the melting temperature is reached. At this temperature, non-covalent forces that maintain the native structure no longer prevail against entropic forces [19,20] and the protein unfolds. The thermal stability of proteins can be changed intrinsically by modification of the primary sequence or extrinsically by the addition of suitable stabilising effectors, e.g., peptides or osmolytes [21,22]. A general mechanism proposed to explain this stabilising effect of osmolytes is their exclusion from the protein hydration shell, or preferential hydration [23].

Fourier transform infrared (FT-IR) spectroscopy is a powerful tool to study protein structure. Changes in protein conformation are recorded in the in the finger print region of the amide bands at 1500 to 1600 cm^{-1} of IR spectra [24,25]. For instance FT-IR records temperature induced changes of structural characteristics of proteins [26–28]. As a non-destructive technique, it can be used for in-vivo studies to distinguish between the native and denatured or aggregated state of intracellular proteins. FT-IR spectroscopy was previously applied to determine membrane phase transitions in bacterial cells [15,17,29], but its potential to determine structural alterations of bacterial proteins during sublethal and lethal heat treatments has not been exploited.

It was the aim of this study to investigate the protective effect of sucrose against heat inactivation in the range of 40–75 °C of *Lactococcus lactis* ssp. *cremoris* MG1363 by the determination of lethal and sublethal injury. In order to determine the protection mechanisms of sucrose, structural changes of the cytoplasmic membrane and cell proteins induced by heat were determined by recording FT-IR spectra during heat application to the population. Thermal inactivation data of bacterial cell suspensions were compared to temperature-induced conformational changes of the protein structure.

2. Material and methods

2.1. Microorganisms and media

Lactococcus lactis ssp. *cremoris* MG 1363 was grown at 30 °C in M17 broth supplemented with 1% glucose. Cells of an overnight culture were harvested by centrifugation, washed and re-suspended in buffer to cell counts of about 10^9 cells ml^{-1}. This buffer was designated as “milk buffer” because it was intentionally set up to resemble whey [30]. It contained the following compounds (g l^{-1}): KCl, 1.1; $MgSO_4 \times 7H_2O$, 0.7110; $NaH_2PO_4 \times 2H_2O$, 1.874; $CaSO_4 \times 2H_2O$, 1.0; $CaCl_2 \times 2H_2O$, 0.99; citric acid, 2.0; lactose, 52.0. The pH was adjusted to pH 6.5. When indicated, 1. 5 M sucrose (sucrose) was added to the buffer (milk buffer sucrose).

2.2. Determination of viable cell counts and stress resistant cell counts

Overnight cultures of *L. lactis* were washed twice in milk buffer or milk buffer sucrose as described above, transferred to 1 ml plastic vials and exposed to temperatures ranging from 40 °C to 75 °C for 0 to 120 min. Sampling times were chosen to obtain at least 4 cell counts during incubation times that reduced cell counts by 3–4 orders of magnitude. This was not possible a very low temperatures without any inactivation and at high temperatures that reduced the cell counts by more than 6 log within few seconds. After treatment, cell suspensions were cooled rapidly on ice. Cells from each sample were diluted and plated on M17 agar supplemented with 1% of glucose, or M17 agar supplemented with 1% of glucose and 3% NaCl for determination of viable and stress resistant cell counts, respectively [30]. The plates were incubated for 24 h at 30 °C under aerobic conditions to assess viable cell counts (CFU), and for 48 h to assess stress resistant cell counts (CFUsub). All temperature inactivation kinetics were performed in duplicate, triplicate or quadruplicate independent experiments. The results are reported as means ± standard deviation.

2.3. Data analysis

Thermal inactivation data was modelled assuming first order kinetics [31] to obtain a single parameter describing the kinetics of lethal and sub-lethal injury of the bacteria:

$$\log(N/N_0) = -kt; \quad (1)$$

with N = microbial population at time t, N_0 = initial microbial population at t_0 and k = the first-order reaction rate constant.

2.4. FT-IR Spectroscopy

Conformational changes in proteins and internal vibrational modes of lipid acyl chains of the membrane of *L. lactis* were measured by Fourier transform infrared spectroscopy (FT-IR). Cells from 50 ml overnight culture were harvested by centrifugation (5 min at 10,000 × relative centrifugal force), washed twice in milk buffer or milk buffer sucrose and incubated for 45 min in the buffer system in which they were subsequently analysed. In the presence of ^1H_2O, the amide bands are obscured by the dominating spectra of ^1H_2O. Therefore, the cells were harvested after incubation and washed in triplicate in deuterium oxide (^2H_2O) or ^2H_2O-sucrose to exchange easily accessible H^+ ions by deuterium ions; the resulting sample had an optical density at 590 nm of about 10, corresponding to about 10^{10} cells per ml. Cells were poured into a 20-μm-thick infrared cell equipped with CaF_2-windows; the total volume of the cell was 20 μL. CO_2 was removed and the air moisture inside the chamber was reduced by flushing the chamber with nitrogen gas. The FT-IR spectra were recorded with an Equinox 55 spectrometer (Bruker, Ettlingen, Germany) equipped with a DTGs detector. Spectra were taken between 6 °C and 75 °C, the temperature was adjusted by an external water thermostat. The scans at a given temperature were started when the average temperature measured across the diagonal of the CaF_2-windows was within 0. 5 °C of the desired temperature. During each experiment, twenty scans were averaged for each temperature point and averaged data of at least two independent experiments are presented. The OPUS software package (Bruker, Ettlingen, Germany) was used for analysis of FT-IR spectra. Second derivative spectra were generated by using a 9-data point (9 cm^{-1}) function which is implemented in the OPUS software package.

3. Results

3.1. Temperature induced lethal and sub-lethal injury of *L. lactis*

The heat inactivation of stationary cells *L. lactis* suspended in milk buffer or milk buffer sucrose was determined. The viability and sub-lethal injury of the cultures were measured after heat treatment. A temperature range of 40–75 °C with 5 °C

increments was chosen and process times ranged from 0 min to 120 min. In Fig. 1 the thermal death time data of *L. lactis* in milk buffer are depicted. Sub-lethal injury was detected after incubation at 45 °C and lethal injury was apparent at temperature levels of 50 °C or higher. After treatment at 65 °C and 70 °C, the cell counts were below the detection limit after 4 min and less than 2 min, respectively. A process temperature of 65 °C and a process time of 2 min resulted in sub-lethal injury of all surviving cells.

Thermal death time data of *L. lactis* in milk buffer sucrose are shown in Fig. 2. In general, a protective effect for both physiological states was detected compared to the inactivation of *L. lactis* in milk buffer. For example, a difference in the cell counts of more than 6 log is apparent when treatments at 50 °C and 120 min in milk buffer and milk buffer with sucrose are compared. Nevertheless, the cell counts of *L. lactis* in milk buffer sucrose were below the detection limit after 2 min at 75 °C (Fig. 2).

3.2. Analysis of infrared spectra

To assess the suitability of FT-IR spectroscopy for analysis of structural properties of cellular proteins, spectra obtained from microbial populations were compared with literature spectra of purified proteins in aqueous solution. An overview of the bands in *L. lactis* relevant to protein or membrane structure, their assignment, the location of their absorption maxima as well as their wave number range is given in Table 1, the corresponding spectral range is shown in Fig. 3. The spectra measured with *L. lactis* suspended in ^2H_2O comprised the same characteristics as the spectra measured in ^2H_2O-sucrose (Fig. 3 and data not shown).

Temperature induced shifts of the spectra provided information about structural changes in the membrane and in proteins induced by increasing the temperature (Table 1) [17,32]. Membrane-phase transitions were detected by

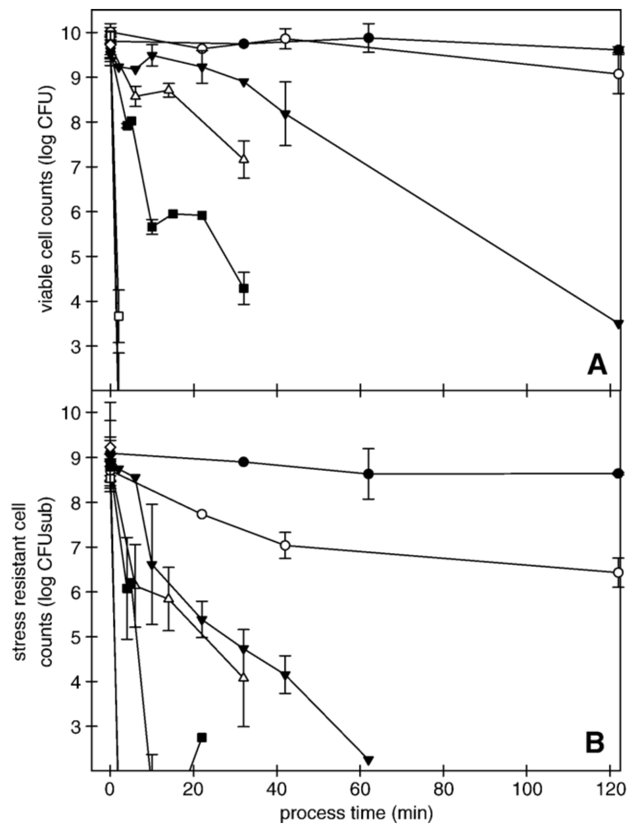

Fig. 1. Comparison of experimental and modelling results concerning lethal (CFU, A) and sublethal (CFUsub, B) injury of *L. lactis* after a treatment in milk buffer at various temperatures. 40 °C (●), 45 °C (○), 50 °C (▼), 55 °C (△), 60 °C (■), 65 °C (□), 70 °C (◆) and 75 °C (◇). Lines represent the prediction of cell counts by first order kinetics. Lines dropping below the *x*-axis indicate cell counts below the detection limit of 100 CFU ml^{-1}.

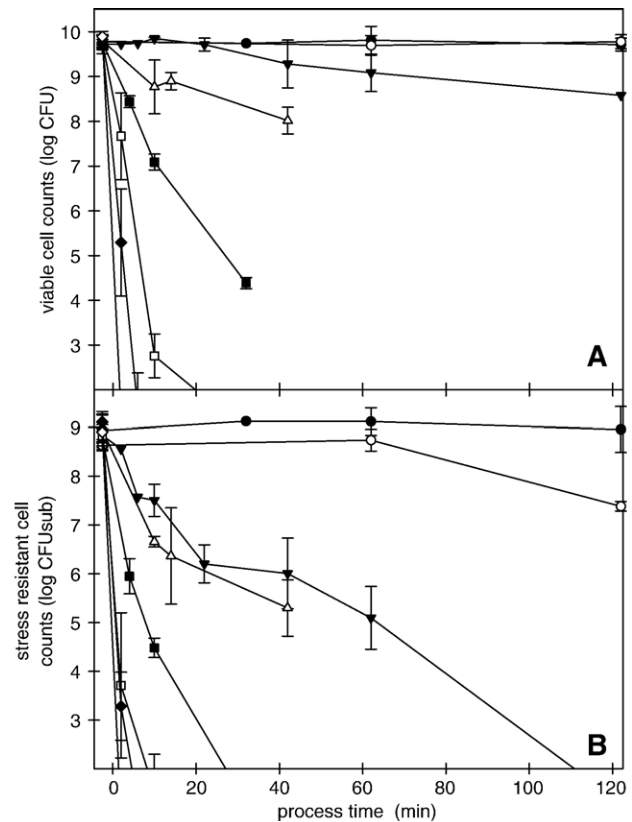

Fig. 2. Comparison of experimental and modelling results concerning lethal (CFU, A) and sublethal (CFUsub, B) injury of *L. lactis* after a treatment in milk buffer sucrose at various temperatures: 40 °C (●), 45 °C (○), 50 °C (▼), 55 °C (△), 60 °C (■), 65 °C (□), 70 °C (◆) and 75 °C (◇). Lines represent the prediction of cell counts by first order kinetics. Lines dropping below the *x*-axis indicate cell counts below the detection limit of 100 CFU ml^{-1}.

Table 1
Common range of the characteristic bands of the secondary structure of proteins detected by FT-IR in *L. lactis*

Band	Wave number (cm^{-1})	Width (cm^{-1})
Amide A+HOD	~3400	3200–3800
Symmetric CH_2	~2852	–
Amide I′	~1652	1600–1700
Amide II	~1550	–
Tyrosine	~1515	–
Side chains	–	1500–1600
Amide II′	~1455	1350–1500

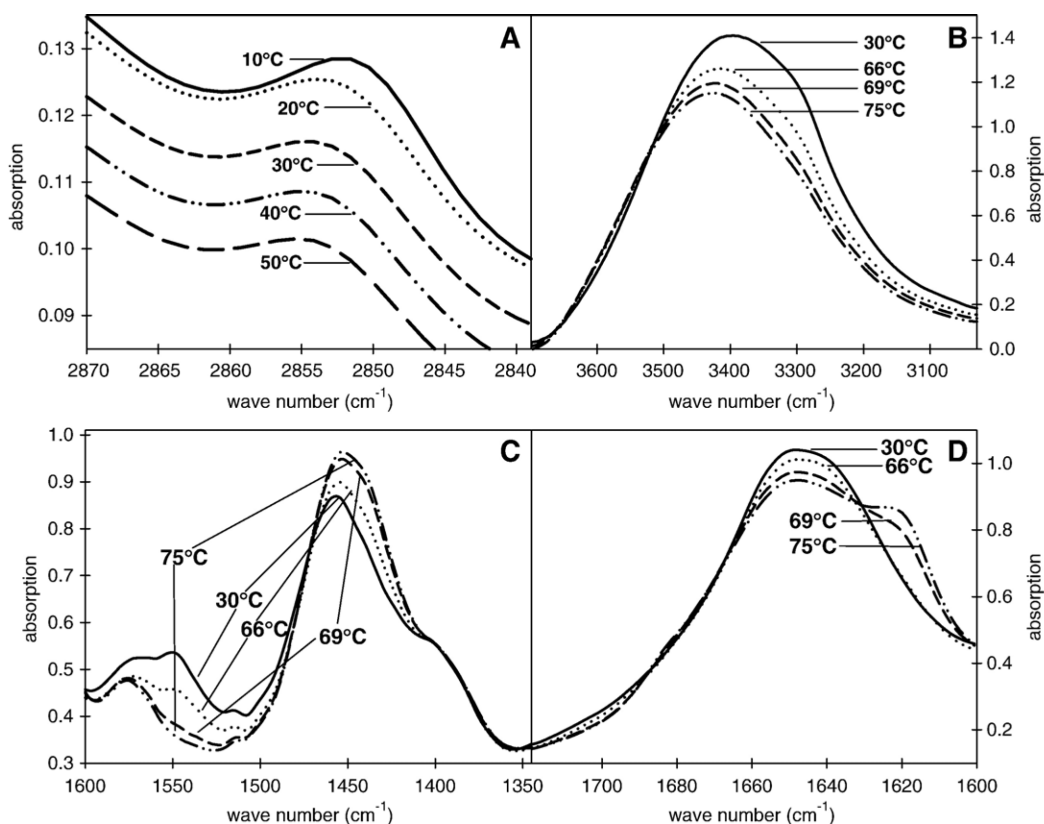

Fig. 3. Segments of the FT-IR spectra of *L. lactis* in ^2H_2O-sucrose used for the secondary structure analysis of proteins and the lipid acyl chains. A: temperature induced shift of CH_2, B: temperature induced shift of amide A + HOD, C: temperature induced shift of amide II′ and amide II, D: temperature induced shift of amide I′.

analysing the absorbance peak resulting from symmetric CH_2 stretching vibrations at wave numbers near 2852 cm^{-1} (Fig. 3 A). The internal vibrational modes of the lipid acyl chains were assigned based on studies of polymethylenes and polymethylene chain compounds [33,34]. In the region from 2800 cm^{-1} to 3100 cm^{-1}, there are infrared absorption bands due to symmetric and anti-symmetric modes of the methylene chain at about 2850 cm^{-1} and 2.920 cm^{-1}, respectively. The frequency of the absorption maximum of these bands is conformation sensitive and thus respond to temperature induced changes of the *trans*/*gauche* ratio in acyl chains. The vibrational mode (asymmetric stretch) of the terminal CH_3 group has a characteristic frequency of 2.960 cm^{-1} [15].

Fig. 3B depicts the spectral information of the amide A and HOD-stretching bands. Amide A is due to the N–H-stretching vibration band at frequencies ranging from 3300 cm^{-1} to 3400 cm^{-1} [32,35]. In aqueous D_2O solution, the HOD-stretching band is masking the amide A band. However, the maximum frequency linearly increased with increasing temperature with a slope of 0.716 $(cm \times °C)^{-1}$ in the case of ^2H_2O-sucrose and of 0.702 $(cm \times °C)^{-1}$ for ^2H_2O. Therefore, this peak served as an internal thermometer of the sample (Fig. 3B).

The amide II band (~1550 cm^{-1}, NH) and its corresponding amide II′ band (~1455 cm^{-1}, ND) record the hydrogen-3-($^1H/^2H$) exchange in proteins (Fig. 3C) [36,37]. The $^1H/^2H$ exchange provides information about the stability and flexibility of proteins because the $^1H/^2H$ exchange rates strongly depend on the local environment of the protons. The hydrogen ions of an un-structured part of the protein exchange faster than those protons protected from the aqueous environment [38]. The spectral range shown in Fig. 3C is sensitive to conformational transitions in proteins as reflected by the H–D exchange [17,39,40]. The intensity of the amide II′ band at ~1455 cm^{-1} grows with increasing H–D exchange, and a corresponding decrease of the intensity of the amide II peak at ~1550 cm^{-1} is generally observed. Corresponding shifts in the amide II and amide II′ bands with increasing temperature were also apparent in the IR spectra of *L. lactis* (Fig. 3C).

Fig. 3D depicts the amide I′ band (1600 cm^{-1} to 1700 cm^{-1}) measured at different temperatures. Spectral data in this range provide information on the secondary structure of a protein. The C═O-stretching vibrations contribute 70–85% to the amide I′ intensity, 10–20% involve the C–N-stretching vibration [35]. The frequency of amide I′ decreases with the strength of hydrogen bonding of the C═O acceptor [41,42]. Unfolded proteins exhibit a broad amide I′ band at approximately 1645 cm^{-1}. In contrast, spectra from aggregated proteins display a strong absorption band near 1620 to 1615 cm^{-1} and a minor component at 1683 to 1680 cm^{-1} reflecting anti-symmetric β-sheets. Often protein unfolding at high temperature will result in extensive aggregation accompanied by the formation intermolecular β-pleated sheets [43]. Thus, aggregated proteins can be distinguished from unfolded ones. With the second derivative of the displayed absorbance spectra, a classification of the secondary structural changes in proteins

is possible (Table 2). As a result of heat induced conformational changes, ordered structures are shifted to unordered structures by thermal treatment [42].

3.3. Sucrose effects on membrane phase transitions

FT-IR spectra of *L. lactis* were recorded using stationary phase cells re-suspended in ^2H_2O or ^2H_2O-sucrose. Membrane phase transitions were detected by measuring the temperature induced shift of the symmetric CH_2 absorbance peak maxima in the presence of ^2H_2O or ^2H_2O-sucrose (Fig. 4). If sucrose was added to ^2H_2O, the peak was shifted to lower frequencies. Therefore, a difference in the frequency response was observed between ^2H_2O and ^2H_2O-sucrose. The osmolyte induced a difference of 1 cm^{-1} and 2. 5 cm^{-1} at low and high temperatures, respectively, as was previously reported [17,18]. The temperature-induced shift of the peak maxima to higher frequencies corresponds to “melting” of the acyl chains of the phospholipids and the high conformational disorder of the phospho-lipids at high temperatures [44]. For *L. lactis* suspended in ^2H_2O, the phase transition was detected between 7 °C (gel phase) and 35 °C (liquid crystalline phase). If sucrose was added to ^2H_2O, the phase transition occurred between 8 °C and 30 °C (Fig. 4).

3.4. Detection of the temperature-dependent conformational changes in the protein structure by the use of FT-IR spectroscopy

FT-IR spectra were recorded in the temperature range from 30 °C to 65 °C and from 30 °C to 75 °C in ^2H_2O and ^2H_2O-sucrose, respectively. The temperature range was chosen to include the optimal temperature of growth of *L. lactis*, and the temperatures that allowed a complete inactivation of the population in less than 2 min (Figs. 1 and 2).

3.5. Analysis of the amide II′- and the amide II band

The amide II band and its corresponding amide II′ band are sensitive indicators of hydrogen-deuterium ($^1H/^2H$) exchange in proteins. To highlight the temperature induced $^1H/^2H$ exchange in *L. lactis* proteins, difference spectra were generated by subtracting data obtained at 30 °C from those obtained at elevated temperatures (Fig. 5). The central frequency corresponding to the amide II and amide II′ bands changed significantly with increase in temperature. The protonated fraction (1550 cm^{-1}) decreased with increasing temperature. The amide II′ band at 1450 cm^{-1}, representing the deuterated fraction of proteins, increased with increasing temperature. These spectral changes were observed in both

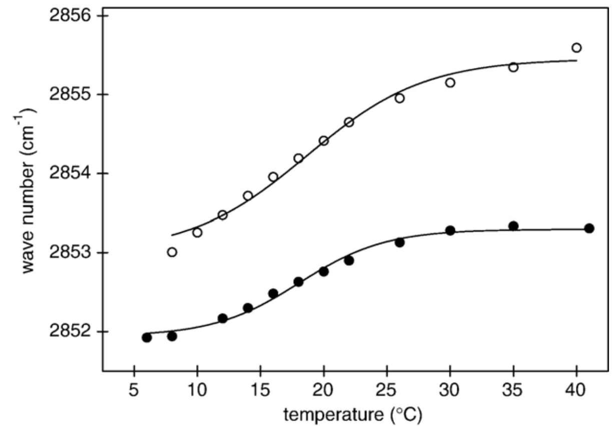

Fig. 4. Vibrational frequencies for the CH_2 symmetric stretch of membrane lipids of *L. lactis* cells in ^2H_2O (○) and ^2H_2O-sucrose (●) as a function of temperature. The solid line represents the fit using a sigmoidal function with 4 parameters to describe the temperature shift of the membrane from the gel to the liquid crystalline phase.

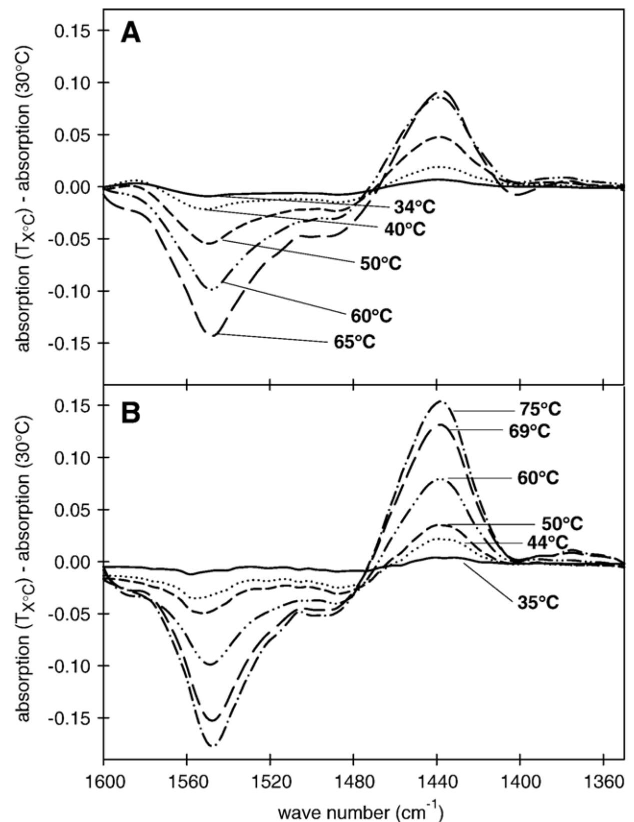

Fig. 5. Difference FT-IR spectra of amide II and amide II′ region (1360 cm^{-1} to 1600 cm^{-1}) generated by subtraction of the spectrum at 30 °C from the spectra at elevated temperatures. A: data generated in ^2H_2O. B: data generated in ^2H_2O-sucrose.

Table 2
Protein secondary structure elements and the corresponding peak frequencies in absorption FT-IR spectra

Structure element	Wave number (cm^{-1})	Width (cm^{-1})
α-helix	~1652	1649–1653
β-sheet	~1681	1680–1683
β-sheet	~1636	–
β-sheet	~1618	–
Turn	~1687	–
Random coil	~1658	–
Unordered	~1642	–

solvents, ^2H_2O and ^2H_2O-sucrose; however, in the presence of sucrose, higher temperatures were needed to induce the hydrogen exchange. To visualise the effect of sucrose on the $^1H/^2H$ exchange in *L. lactis* proteins, the area ratio of the amide II band (1510 cm^{-1} to 1590 cm^{-1}) relative to the corresponding band areas at 30 °C is shown in Fig. 6. The amide II peak area is decreasing due to the increasing $^1H/^2H$ exchange and a corresponding increase of the amide II′ peak areas (1410 cm^{-1} to 1490 cm^{-1}) was apparent (data not shown). The difference in the relative peak areas suggests an enhanced $^1H/^2H$ exchange in ^2H_2O compared to ^2H_2O-sucrose. For example, in ^2H_2O, a decrease of the protonated fraction of about 33% is observed at 65°C, whereas only a reduction of about 20% is seen with cells suspended in ^2H_2O-sucrose.

3.6. Analysis of the amide I′ band

The amide I′ region detected in *L. lactis* shows a broad and asymmetric band with the maximum around located near 1645 cm^{-1} (Figs. 3D and 8). To emphasise temperature dependent changes, difference FT-IR spectra were calculated by subtracting the spectrum measured at 30 °C from the spectra obtained at elevated temperatures (Fig. 7). For *L. lactis* suspended in ^2H_2O, spectral changes were detected at temperatures above 45 °C, whereas temperatures above 50 °C were required to induce spectral changes with *L. lactis* in ^2H_2O-sucrose (Fig. 7). This result suggests sucrose enhances the thermal stability of intra-cellular proteins.

The peak at ~1645 cm^{-1} derives from to α-helices and polypeptide chains with random structure (Table 2) [32,35,41,42,48]. The temperature-induced changes of the spectra suggest a decrease of the structural elements detected within this range of frequency with increase in temperature. The emerging new bands around 1620 cm^{-1} and 1683 cm^{-1} at high temperatures reflect the formation of a hydrogen-bonded

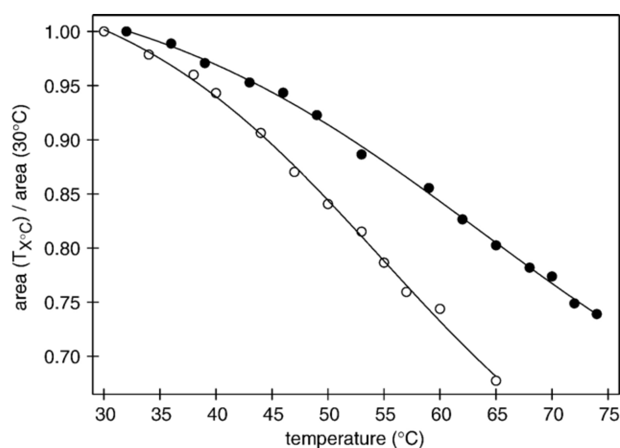

Fig. 6. Temperature effect on the amide II peak areas at elevated temperature relative to the amide II peak area at 30 °C. Amide II peak areas were calculated for wave numbers ranging from 1510 cm^{-1} to 1590 cm^{-1} for data generated in ^2H_2O (○) and ^2H_2O-sucrose (●).

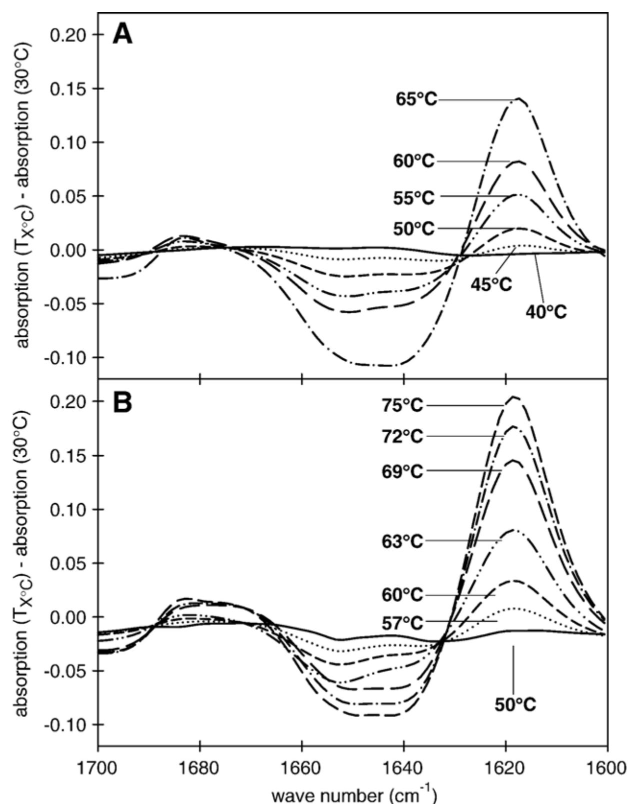

Fig. 7. Difference FT-IR spectra of the amide I′ region (1600 cm^{-1} to 1700 cm^{-1}) generated by subtraction of the spectrum at 30 °C from the spectra at elevated temperatures. A: measurement data generated in ^2H_2O. B: measurement data generated in ^2H_2O-sucrose.

β-sheet structure, leading to an irreversible re-association of unfolded peptide segments (Table 2) [45–47].

A second-derivative-analysis of the amide I′ region provided a more detailed decomposition of various secondary structure elements in *L. lactis* proteins (Table 2). A comparison of the spectra of the amide I′ region and the corresponding second derivative spectra recorded at 32 °C and 74 °C is shown in Fig. 8. Conformational changes that were induced by a temperature up-shift indicate the formation of β-sheet-structures at ~1683 cm^{-1} (Fig. 8). The new bands at 1658 cm^{-1} and 1642 cm^{-1} emerging at high temperatures suggest a growing fraction of disordered segments. Moreover, the second derivative analysis reveals a loss in α-helical structures (1649 cm^{-1}) and a gain in (intermolecular) β-sheet-structures (1618 cm^{-1}).

4. Discussion

The thermal stability of proteins can be modified intrinsically by altering the sequence amino acids or extrinsically by suitable stabilising effectors, e.g., osmolytes. The general mechanism of protein stabilisation by solutes and carbohydrates in particular, is the exclusion of solute from the protein domain [23]. The ability of carbohydrates to stabilise proteins has been attributed to this preferential hydration of proteins [21,22,49]. Bacteria are required to

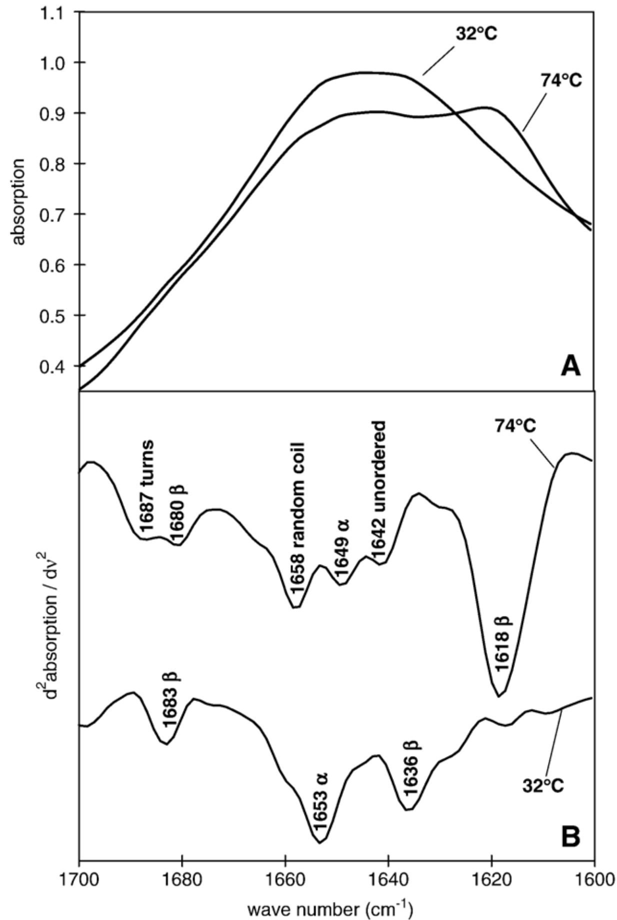

Fig. 8. Amide I′ band of the infrared spectra obtained from *L. lactis* at 32 °C and 75 °C corresponding to viable and inactivated cells, respectively. The FT-IR spectra (A) and their second derivatives (B) were recorded in $^{2}H_2O$-sucrose.

adapt to osmotic up-shift by accumulating compatible solutes [12,13]. This accumulation of solutes equilibrates the intracellular osmotic pressure to the environmental osmotic pressure and additionally provides protection to other stress conditions such as heat, freezing, drying, and high hydrostatic pressure [5,6,15,17,18]. The protective effect of compatible solutes on bacterial survival was previously correlated with the protective effect of solutes on the bacterial membrane [15,17,29]. This study confirmed a protective effect of sucrose on the bacterial survival and cytoplasmic membranes, but the membrane phase transition occurred in the temperature range of 5–30 °C and thus appeared to be unrelated to the heat inactivation of *L. lactis*.

Conformational changes in the proteins of *L. lactis* were determined with FT-IR spectroscopy. The data obtained in this work with *L. lactis* are in excellent agreement with the spectra of purified proteins [32] and correspond to previous FT-IR measurements on bacterial cells [17].

The analysis of heat induced conformational changes of the proteins of *L. lactis* enables a comparison of the temperature effects on protein structure with bacterial survival and sublethal injury (Fig. 9). The difference in the intensities of the amide II and II′ peaks (1550 cm^{-1} and 1440 cm^{-1}, respectively) at elevated temperature compared to the peak intensity at 30 °C were used as an index for heat-induced $^{1}H/^{2}H$ exchange. The buried residues of the protein structure became accessible to deuterium upon temperature up-shift and were exchanged by the surrounding $^{2}H_2O$ (Figs. 5, 9A) [36,37,39,40]. Modifications of the secondary structure of the proteins were detected by analysis of the amide I′ band (Figs. 7 and 9B). To obtain a rate constant as an indicator for the temperature effects on bacterial viability, the experimental data on bacterial inactivation was fitted to a first order equation (Fig. 9C). Although first order models may be inadequate to describe inactivation curves with pronounced non-linearities, they have been widely used to describe

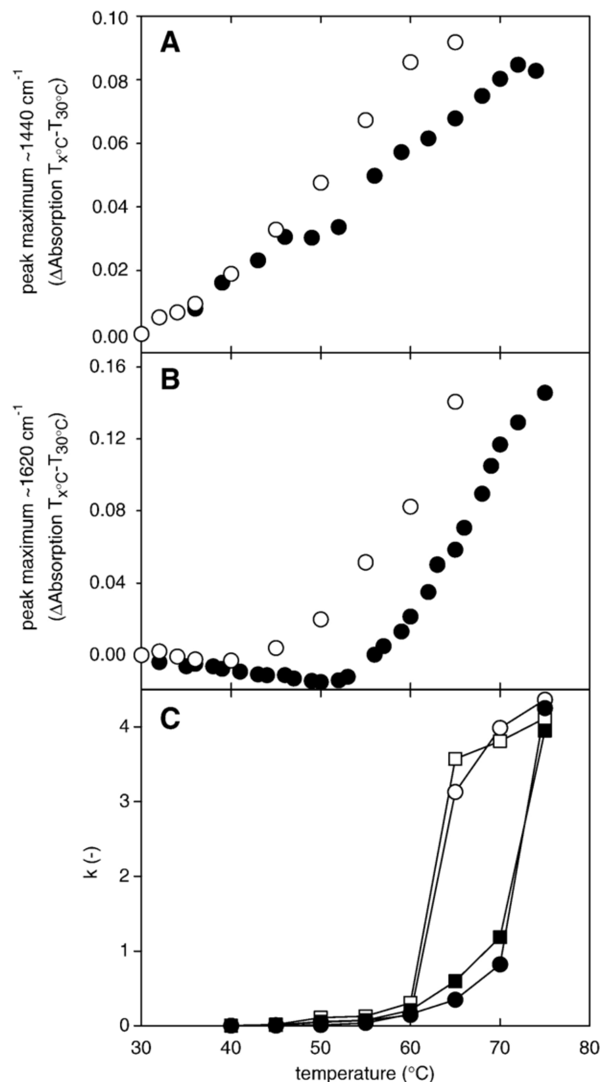

Fig. 9. Temperature effect on the different parameters describing thermal inactivation data of bacterial cell suspensions and temperature induced conformational changes of the protein structure. A: intensity of the amide II′ band (1440 cm^{-1}) at elevated temperatures corrected for the peak intensity at 30 °C. B: intensity of the amide I′ band (1620 cm^{-1}) at elevated temperatures corrected for the peak intensity at 30 °C. C: rate constants k describing lethal (○) and sublethal (□) injury of the bacteria. The filled symbols represent data generated in $^{2}H_2O$-sucrose, the empty symbols represent data generated in $^{2}H_2O$.

thermal death time data and provided a reasonable agreement to our experimental data (correlation coefficient $r^2>0.87$). In contrast to more elaborate models that take into account non-linear survivor curves, a simple first order model allows the representation of the inactivation kinetics by a single parameter, the rate constant k, and thus greatly facilitates the comparison with the FT-IR data on protein structure. However it should be stressed that the extensive washing steps that were required to replace ^1H_2O by ^2H_2O for FT-IR spectroscopy of the cultures likely affected various aspects of bacterial physiology.

In the presence of sucrose, the heat-induced structural changes in *L. lactis* proteins detected by the amide I′ and the amide II′ bands were shifted to higher temperatures by about 10 °C. Inhibition of the $^1H/^2H$ exchange by sucrose was more prominent at lethal temperatures above 50 °C compared to ambient temperatures of 30–45 °C (Figs. 6 and 9). The presence of high (intracellular) sucrose concentration thus increased the stability of *L. lactis* proteins at denaturing temperature. Furthermore, the bacterial viability was protected by sucrose. A temperature increase of about 10 °C was required to achieve the same lethal effect in milk buffer sucrose compared to milk buffer (Fig. 9C).

The heat induced conformational changes of the *L. lactis* proteins are related to the survival of this organism at lethal temperatures. No conformational changes in the *L. lactis* proteins were detected by analysis of the amide I′ band at sublethal temperatures. The onset of conformational changes could be located between 50 and 60 °C in ^2H_2O and ^2H_2O-sucrose, respectively. These temperatures correspond to the lowest temperatures at which substantial inactivation of *L. lactis* were detected in milk buffer and milk buffer with sucrose, respectively (compare Fig. 9B and C). Temperature up-shift induced an increase of structural disorder and intermolecular β-sheet-structures in *L. lactis* proteins, a clear sign of irreversible protein aggregation and a decrease of ordered structural components, mainly α-helices. A more detailed interpretation of the conformational changes of protein secondary structures is not feasible because the combined spectra of the more than 2000 proteins expressed by *L. lactis* [50] were determined and the structural changes of individual proteins overlap. These results indicate heat-induced protein denaturation to an extent, which is beyond the cellular capacity of protein repair or *de-novo* synthesis, and hence contributes to cell death. The presence of intracellular unordered or denatured proteins is attributable to heat denaturation of existing proteins or mis-translation occurring at the ribosome. Ribosome denaturation in *Escherichia coli* occurs in the range of 50–90 °C and was previously identified as a direct cause for heat-induced cell death [51].

The $^1H/^2H$ exchange was more susceptible to temperature than the spectral bands relating to protein secondary structure. Remarkably, an increased $^1H/^2H$ exchange was observed already in the range of 32–42 °C, which is above the optimal temperature of growth but below the maximum growth temperature of *L. lactis*. This indicates that conformational instabilities of proteins that may interfere with their respective functions occur already at temperatures permitting growth of the organisms. In keeping with this interpretation, the primary response of *L. lactis* to temperature upshift is the overexpression of heat shock proteins, including the DnaK-GrpE-DnaJ and the GroELS chaperone complexes [52,53]. In *Lactobacillus sakei*, the induction of heat shock proteins was apparent already at a temperature a few degrees above the optimum temperature of growth [54]. The molecular chaperones are highly conserved families of proteins that maintain or restore native protein conformation even at high temperatures, or assist in the degradation of unfolded and aggregated proteins [55]. As the ribosome has been described as (heat) stress sensor and ribosome function is coupled to protein quality control via *trans*-translation, the expression of chaperons is also a reaction to impaired translation by stalled ribosomes at sublethal stress temperatures [3,56].

In conclusion, in this work demonstrated that molar concentrations of sucrose increase the heat resistance of *L. lactis*. This finding extends previous findings on the role of compatible solutes for the bacterial resistance to freezing, drying, and heat. Additionally, it demonstrates that high intracellular sucrose concentrations prevent the denaturation or misfolding of *L. lactis* proteins. Remarkably, the use of FT-IR spectroscopy enabled the detection of protein conformational instabilities occurring already at elevated temperatures within the growth range of *L. lactis*. It is anticipated that these results will facilitate the process design for thermal treatments to eliminate or stabilise bacterial cells in food and biomedical applications.

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