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Bacterial membranes: the effects of chill storage and food processing. An overview

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Abstract

The shelf life of food is extended by refrigeration because the metabolic processes of food-associated microorganisms are slowed by the lowered temperature. Nonetheless, cold-adapted psychrotrophic food-poisoning and food-spoilage bacteria remain a concern because they possess cold-adapted proteins and membrane lipids that facilitate growth at low temperatures. The use of membrane-disrupting novel preservation techniques, such as ultrasound, high hydrostatic pressure or pulsed electric field, offer the potential for an extension of shelf life. This review considers the interacting and potentially synergistic effects of chill storage or mild heat treatment on membrane properties, with the disruptive effects of membrane-targeted physical treatments.

Keywords: Membranes; Chill storage; Food-poisoning bacteria; Food spoilage; Psychrotrophs; Cold-adapted microorganisms; Cold adaptation mechanisms; Food preservation

1. Introduction: cold-adapted bacteria

Refrigeration is the most common means of preserving food, either alone or in combination with other methods such as the addition of preservatives. Therefore, an understanding of the response of food-spoilage and food-poisoning microorganisms to the stress imposed by low temperature is fundamental to the design of effective preservation strategies. This is particularly relevant in the context of modern demands for foods containing lower levels of preservatives for a more natural flavour and wholesomeness, since many of the major spoilage (e.g. *Brochothrix thermosphacta*, *Pseudomonas* spp., *Micrococcus* spp.) and poisoning (e.g. *Listeria monocytogenes*, *Yersinia enterocolitica*) microorganisms of concern are psychrotrophic (psychrotolerant) (Table 1). They are notable for having a particularly broad growth temperature range, often approaching 40 centigrade degrees, in contrast to psychrophiles which have much narrower ranges (Gounot and Russell, 1999). Although psychrotrophs cannot match the sub-zero growth of psychrophiles, they are nonetheless able to grow at low temperatures approaching 0 °C (e.g. 1–3 °C for strains of *L. monocytogenes*) as well as being capable of growing rapidly when temperatures rise to (warm) room temperatures and, for pathogens, the human body temperature of 37 °C. It is this wide thermal capability that makes them specially significant in terms of food quality and safety.

In relation to refrigeration, the temperature ranges that are most relevant are 4–6 °C (refrigerators) and 10–12 °C (open chiller display units). This thermal

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Table 1
Thermal characteristics of cold-adapted food-spoilage and food-poisoning bacteria

Bacterium	Lower growth limit (°C)	Comments
Pseudomonas fluorescens	0	Food-spoilage, nonpathogenic
Micrococcus spp.	1	Food-spoilage, nonpathogenic
Listeria monocytogenes	1	Most strains will grow at refrigeration temperatures
Clostridium botulinum	3	Only some non-proteolytic Type E strains are psychrotrophic
Salmonella spp.	5	Very slow growth of some strains at refrigeration temperatures; most species grow slowly at chilling temperatures
Lactic acid bacteria (LAB)	5	May cause unwanted deterioration of LAB-fermented foods
Staphylococcus aureus	6	Some strains will grow at refrigeration temperatures in processed foods
Bacillus cereus	10	Psychrotrophic strains can spoil milk or poison (emetic toxin) chilled foods
Clostridium perfringens	13	May grow slowly at chill-cabinet temperatures
Clostridium botulinum Type A		

Data modified from Russell and Gould (in press).

range from 4 to12 °C is one over which psychrotrophs are capable of growing at rates that may be only two- to fourfold lower than their optimum rates at 20–30 °C. Therefore, they pose a particular threat to chilled foods because bacterial populations can reach levels that are capable of serious spoilage or are above the threshold for causing food poisoning.

2. Cold-adapted enzymes

The fact that cold-adapted bacteria grow at chill temperatures at rates that are either equivalent to or not much slower than mesophiles at room or body temperatures means that they must contain proteins (enzymes) that are adapted to function at low temperatures. This adaptation has evolved over many generations and is fixed in the genome. The resulting amino acid sequence of each enzyme gives a protein folded into a three-dimensional structure that remains conformationally flexible and thus catalytically active in the cold. Different enzymes have evolved different mechanisms for achieving cold activity, but some common evolutionary adaptations have been identified: these include a reduction in the number of hydrogen bonds, salt bridges, proline and arginine contents, aromatic interactions, and hydrophobic clustering, together with increases in solvent interactions and additional surface loops. Not every kind of change is found in each enzyme, but the overall effect is to decrease the number of enthalpy-driven interactions between amino acid side chains and give a protein that is more flexible at low temperatures (Gerday et al., 1997; Russell, 2000).

A corollary of the enhanced activity at low temperatures is the fact that cold-adapted enzymes are more thermolabile than their mesophilic counterparts, so that at quite moderate temperatures (typically 40–50 °C), they become too flexible, lose catalytic efficiency and eventually denature. This means that psychrotrophic bacteria are usually killed by mild heat treatment, which could be an advantage in preservation regimes such as those of sous vide foods in which mild heating is followed by refrigerated storage.

Enzymes are found either free within the cytoplasm or in the membrane, but all of the structural data on cold-active enzymes come from studies of soluble cytoplasmic ones. Nothing is known about the structure of membrane-bound cold-adapted enzymes compared to their mesophilic or thermophilic counterparts, but they will presumably also have α-helical sections that span the hydrophobic core of the membrane where they interact with the fatty acyl chains of membrane lipids. Therefore, it is certain that they too will be specifically adapted in order to function at low temperatures and that this adaptation will depend not only on their intrinsic protein structure but also on the physical properties of the surrounding lipids.

3. Temperature and lipid composition

It is well known that the lipid composition of membranes alters when microbial growth temperature alters

and that the main changes are in the fatty acyl components of membranes (Russell, 1984). Changes in the head-group composition of the lipids are much less pronounced and have much less influence on the thermal properties of the membrane (Russell, 1989). Increasing the extent of fatty acyl unsaturation, *cis*/*trans*-unsaturation ratio, methyl branching or the ratio of *anteiso*- to *iso*-branched acyl chains, or shortening the average acyl chain length, all lower the temperature of transition from a liquid-crystalline to a gel phase and so preserve membrane fluidity that is necessary for survival and growth (Russell, 1989). The term "membrane fluidity" is a convenient one to summarise a multifaceted phenomenon that has contributions from molecular packing (order) and molecular motions (viscosity). The changes in fatty acyl composition may alter either or both of these aspects of fluidity. For example, the introduction of a *cis*-unsaturated bond introduces a "kink" into the acyl chain, which therefore occupies a greater molecular profile. Similarly, a methyl group disrupts the packing of acyl chains by occupying more space, *anteiso*-branches more so than *iso*-branches. *Trans*-unsaturated double bonds alter the orientation of the acyl chain much less than do *cis*-unsaturated double bonds and so have a correspondingly smaller effect on fluidity. Disruption of acyl chain packing will not only change the packing order but also the strength of interaction between individual acyl chains. Shortening acyl chains will lessen the van der Waals intermolecular forces and therefore make the membrane more fluid, particularly if only one of the pair of acyl chains in each lipid molecule is modified, leaving more space for molecular motion of the longer acyl chain (McGibbon and Russell, 1983). The magnitude of the effect that the different temperature-dependent fatty acyl changes have on membrane fluidity is visualised in Fig. 1.

Each microorganism will have a lipid fatty acyl composition that is adapted for its particular growth temperature range. The same level of fluidity can be achieved by many combinations of different fatty acids and, hence, even though different cold-adapted bacteria may have similar low temperature growth abilities, they will almost certainly have quite different fatty acyl compositions. Differences will be influenced by phylogenetic distinctions, different metabolic capabilities and by specific protein–lipid interactions. For example, *Salmonella* adapts to temperature almost

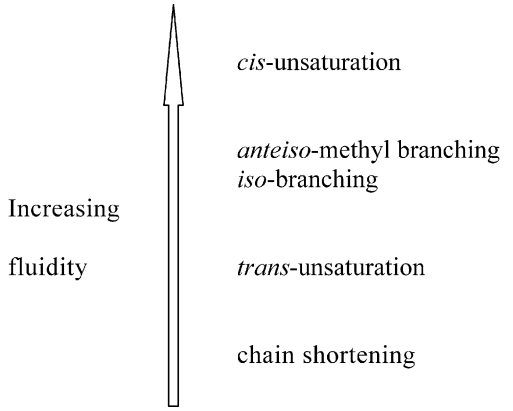

Fig. 1. A figurative representation of the proportional increases in membrane fluidity given by different fatty acyl changes relative to saturated lipids.

entirely by changing lipid unsaturation, whereas *Listeria*, which contains predominantly branched fatty acyl chains, modifies the *anteiso*/*iso*-branched ratio and the acyl chain length; bacilli use a combination of unsaturation and changes in branching pattern. As temperature falls, membrane fluidity will decrease, and membrane-associated metabolic processes mediated by enzymes, cytochromes and permeases will slow. These events will trigger compensatory changes in fatty acyl composition so as to make the membrane more fluid. As long as the temperature change is within the normal growth temperature range, the fatty acyl changes will more or less compensate for the kinetic loss of activity. If the temperature is shifted to or just beyond the lower or upper limits, then cold shock or heat shock, respectively, will occur (see below). The rate at which the lipid changes occur on transfer to or from the cold will depend on the biosynthetic mechanisms used to modify lipid acyl composition. Changes in unsaturation brought about by desaturase enzymes are usually rapid because they generally occur in situ in the membrane through the modification of intact lipids without concomitant growth, i.e. the desaturase enzymes are located within the membrane and interact directly with lipids that are their potential substrates. In contrast, changes in methyl branching and acyl chain length take longer because they require de novo synthesis of the whole lipid molecule by cytoplasmic enzymes that are usually linked to growth (see Gounot and Russell, 1999 for details and references).

In most cases, the effect of temperature is a direct one, acting on the key regulatory enzymes to modulate the overall rate of the reaction. Induction of new enzyme synthesis is much less common, although some desaturases in bacteria and yeast are cold inducible. Activation (or inhibition) by temperature will be virtually instantaneous, since microorganisms are too small to insulate themselves against thermal effects, and enzyme induction and synthesis in bacteria take only a few minutes to complete. Therefore, the effects of changes in temperature will be reflected in an altered fatty acyl composition within minutes. However, changes that involve de novo synthesis of fatty acids will require further steps of fatty acid activation and incorporation into membrane lipids that require cellular growth. Modifications involving desaturases are an exception because existing membrane lipids are the substrate, although such changes only serve in the short term and other mechanisms based on cell growth take over after the initial adaptation phase. Therefore, whatever the strategy, the effects of membrane lipid modification require cellular growth (lipid and membrane synthesis) for their influence to be exerted and so it is significant that if the decrease in temperature is sudden, the bacteria will suffer cold shock and stop growing for a period of up to several hours due to a block in the initiation of protein synthesis. If the temperature is shifted to or just below the lower growth limit, cold shock will occur.

4. Cold shock and cold acclimation

Sudden changes of temperature will induce the synthesis of stress proteins, heat-shock proteins for a rise and cold-shock proteins for a fall in temperature (Gounot and Russell, 1999). The cold-shock response (CSR) has been identified in food-associated mesophilic and psychrotrophic bacteria that cause spoilage (e.g. *Pseudomonas fluorescens*, *P. fragi* and lactic acid bacteria) or poisoning (e.g. *L. monocytogenes*, *S. typhimurium*, *S. enteritidis*, *Staphylococcus aureus*, *Escherichia coli*, *Y. enterocolitica* and *Bacillus cereus*). Interestingly, cold-shock protein (CSP) genes are not present in *Campylobacter jejuni* (Hazeleger et al., 1998), which may explain why this food-poisoning bacterium has a very narrow growth temperature range and is unable to grow below 30 °C.

The CSR involves the differential expression of genes for up to 50 different CSPs, depending on the species (Inouye and Yamanaka, 2000). Many of these are concerned with the major functions of CSPs, given in Table 2, which reflect the importance of ensuring that protein synthesis continues at an appropriate rate at the low temperature to give balanced growth. These functions also reflect the importance of the ribosome in sensing temperature changes and the fact that the cellular function most sensitive to cold shock is the initiation of translation (Graumann and Marahiel, 1996, 1997). In addition to the common features of the CSR given in Table 2, the expression of other genes is involved and these differ between species. At present, we do not have a complete picture of CSPs in different microorganisms: we know even less about their functions.

The regulation of CSP synthesis occurs at several levels, both transcriptional and translational, involving both protein and mRNA stabilities. There are common regulatory sequences, upstream of (e.g. the "cold-shock box") and downstream within the coding region, which together coordinate the expression of cold-shock genes. Space does not permit a fuller discussion of regulation of the CSR (see Inouye and Yamanaka, 2000), but a key question in relation to psychrotrophs and food storage is "How large does the temperature fall have to be, and at what rate must it decrease, for cold shock to occur?" This is an important consideration because cold shock enables the food-associated microorganisms better able to survive and grow at the new lower temperature and, therefore, to spoil and/or poison the product.

Table 2
Major characteristics of the cold-shock response

Effect of low temperature	Cellular response
Block in initiation of protein synthesis	Synthesise proteins to stabilise interaction of mRNA with 30S ribosomal subunit
Disruption of ribosome structure	Synthesise proteins to stabilise protein–protein and protein–rRNA interactions within the ribosome
Formation of secondary structures	Synthesise RNA chaperones to maintain mRNA in linear form ("hairpins") in mRNA
Increased negative supercoiling of DNA	Induction of DNA-unwinding enzymes and stabilising (histone-like) proteins

Significantly, compared to mesophiles, after cold shock in psychrotrophs, there is no concomitant suppression of the expression of so-called housekeeping genes that encode, for example, enzymes of central metabolic pathways, so growth lag times are likely to be shorter or nonexistent. Moreover, the number of CSPs and the extent of their synthesis depend on the depth of the cold shock, and one particular class of proteins (the cold-acclimation proteins) is permanently induced during constant growth at low temperature (summarised in Gounot and Russell, 1999). For example, the food-spoilage bacterium *P. fragi* makes 15 CSPs on shifting from 20 to 5 °C, but 24 CSPs when shifted from 30 to 5 °C. It is capable of growing after 3–5 h lag following such temperature shifts (Hébraud and Potier, 2000). We do not know the functions of the extra CSPs, or whether they increase the ability to grow and survive in foods, but significantly, the temperatures and time scales involved are ones that might be relevant. For instance, consider the retail purchase and transfer of foods from a supermarket chill cabinet (10–12 °C) to a car that might then left in the sun (30 °C) before the food is placed in a domestic refrigerator (5 °C). Another practical scenario might be cooked food left inadvertently overnight in a warm kitchen (20 °C) before refrigeration. The time scales involved could easily be a few hours, which would be sufficient to give several generations of growth and large enough numbers of warm-adapted spoilage psychrotrophic bacteria that would then experience cold shock on being placed in the refrigerator and subsequently would cold-adapt and grow. Whether the same considerations apply to a food-poisoning bacterium such as *L. monocytogenes* is arguable, as there is disagreement about the length of the lag times following cold shock (Hébraud and Potier, 2000).

Thus far, changes in lipid composition and cold shock have been discussed separately. However, in the cell, they must be linked because balanced growth requires coordination of intracellular and extracellular events, as well as those occurring within the membrane matrix. The membrane is likely to have a role in the sensing of temperature, and a number of two-component signalling systems involved in global regulatory phenomena are well known (Hoch and Silhavy, 1995). An inducible desaturase has been identified as a cold-shock protein in *B. subtilis* (Aguilar et al., 1998), thus linking the cold-stress response to lipid changes in the membrane. The links between lipid changes mediated by the cytoplasmic fatty acid synthetase and those of the CSR are more difficult to determine.

5. Temperature and lipid phase behaviour

A second aspect of membrane structure that is less commonly taken into account in discussions of thermal adaptation is the need to preserve the bilayer (lamellar) phase, i.e. prevent the formation of non-bilayer phases such as hexagonal, which destroy the selective permeability properties of the membrane (Russell, 1989). Even more so than changes of temperature, the presence of salt(s) has a large effect on the transition between bilayer and non-bilayer phases, and there is an interplay of effects between temperature and solute concentration, which is relevant to products such as minimally processed foods that rely heavily on chilling for extension of their shelf life.

Taking into account what we know about the membrane lipid changes triggered separately by low temperature and the presence of (preservative) salts, in combination they may act antagonistically, since for a given lipid composition, lowering growth temperature will reduce the likelihood of formation of non-bilayer phases whereas lowering the water activity (i.e. raising the salt concentration) will have the opposite effect. Therefore, at lower temperatures, bacteria should grow better in salt (i.e. have higher optimum salt concentrations). Just such an effect has been demonstrated for a moderately halophilic bacterium (Adams and Russell, 1992), but has not been explored for relevant food-spoilage or food-poisoning bacteria. Ignoring any other growth inhibitory effects of the preservative, the reduction of salt in chilled foods would have a beneficial effect as far as changes in membrane lipid composition are concerned. In practice, the effect is likely to be small and it would be more effective to find a means of disrupting membrane stability so that lipid changes were less effective in adapting the spoilage/poisoning microorganism to growth at low temperatures. One approach is to combine physical methods of membrane disruption with cold storage. The available methods include treatment with ultrasound, high pressure or pulsed electric fields.

6. Ultrasound and membranes

Ultrasound disrupts biological membranes, probably by a combination of cavitation phenomena and associated shear disruption, localised heating and free radical formation (Sala et al., 1995). Typical treatments are for 1–30 s using 20–40 kHz ultrasound. Ultrasonication in combination with mild heating (e.g. 50–60 °C) is more effective at inactivating a range of vegetative food-spoilage and food-poisoning microorganisms, as well as spores, but no satisfactory explanation exists for the synergy of so-called thermosonication. If the rapid pressure changes that occur during cavitation are responsible for the lethal effect of ultrasound, then raising the temperature and hence membrane fluidity (i.e. weakening the intermolecular forces) would enhance the disruption. However, it is not known if membrane lipid composition of the target organisms is a determining factor in ultrasound sensitivity.

7. High pressure and membranes

High hydrostatic pressure, in the order of 100–1000 MPa (i.e. 1–10 kbar), inactivates enzymes and causes a variety of structural changes in the morphology, cell wall and membranes of microorganisms; and membrane fluidity may be a factor in the pressure-sensitivity of an organism (Knorr, 1995; Ledward et al., 1995). Gram-positive bacteria are less sensitive than are Gram-negative bacteria, probably due to the thicker cell wall of the former. It has also been noted for *L. monocytogenes* (Simpson and Gilmour, 1997) and *E. coli* (Benito et al., 1999) that there can be considerable differences in sensitivity between strains of the same organism. For *E. coli*, it has been suggested that the differences in resistance between strains are related to their susceptibility for membrane damage, but the relationships between pressure sensitivity, phase of growth of batch cultures and whether the applied pressure is high or low are complex (Benito et al., 1999; Pagan and Mackey, 2000). It had been suggested previously that bacteria with a more fluid membrane are more resistant to high pressure (Smelt, 1998), but no direct membrane fluidity measurements were made by Mackey and coworkers. Membrane fluidity may exert its influence through control of the ion pumps in the membrane that are essential for maintaining pH homeostasis. This is consistent with the fact that pressurisation is more effective at inactivating microorganisms when it is combined with mild heat treatment, since membrane repair of pressure-induced pores would be harder to accomplish if intermolecular forces are weakened by warming. Such co-treatment may further be combined with ultrasound treatment, in a process known as manothermosonication.

8. Pulsed electric field and membranes

A drawback of high pressure or ultrasound processes is that they all give inactivation curves with "tails" of surviving microorganisms, and the use of higher temperatures or other operational parameters to reduce the number of survivors would have adverse effects on the organoleptic qualities of the food. In contrast, we have demonstrated that treatment of either *L. monocytogenes* or *S. typhimurium* with pulsed electric field (PEF) is an "all or nothing" effect in that the bacterial cells are either killed or survive normally following exposure to effective doses (Simpson et al., 1999). This is in marked contrast to other novel physical preservation methods, such as high-pressure treatment, which result in a proportion of bacteria that are damaged but are capable of recovery under favourable growth conditions. Thus, predictions of food shelf life should be more reliable after PEF treatment compared with ultrasound or high-pressure treatments.

It is generally agreed that PEF treatment leads to the permeabilisation of biological membranes. We found that increasing the applied electric field gave greater microbial inactivation, which was matched by increased leakage of UV-absorbing cellular material and loss of the ability to maintain pH homeostasis, although, in contrast with high-pressure treatment (Wouters et al., 1998), PEF efficacy did not correlate with the inhibition of membrane H^+-ATPase activity (Simpson et al., 1999).

Pulsed electric field is a modification of the original "Electropure process" in which an alternating current was used to pasteurise milk (Palaniappan and Sastry, 1990). The current was not pulsed and the lethal effect derived from the heating that occurred. If, instead, the electric field is delivered in pulses with proper control of their strength, number and format (e.g., bipolar, square waves are better than monopolar, exponential

waves), then microbial inactivation is not due to thermal effects (Jeyamkondan et al., 1999). A number of theories have been put forward to explain the membrane-disrupting action of PEF, but they are similar and all are based on the fact that lipid molecules are dipolar and the membrane bilayer has a net electric charge (Barbosa-Cánovas et al., 1999). Application of a PEF causes reorientation of the lipids, stressing the lipid bilayer and eventually causing pores to form. This is consistent with the observation that electric field strength has a more profound influence than treatment time on the lethal effect, because it is the field strength that overcomes the intermolecular forces responsible for maintaining the lipid bilayer core of the membrane. It is also consistent with the fact that raising the treatment temperature increases the efficacy of PEF.

The viscoelastic properties of the bilayer oppose these disruptive forces, and the pores will cyclically reseal themselves as soon as they are formed, unless the PEF treatment is sufficient in strength to overcome the repair process. Therefore, membrane lipid composition will influence this balance so as to make the cells more or less sensitive to PEF treatment. Growth at low temperatures will give bacteria with a membrane lipid composition that will be more fluid at room temperature than the membranes of bacteria grown at moderate temperatures. Therefore, one can predict that cultures grown in the cold would have increased PEF sensitivity (at room temperature) compared with those grown at room temperature, because the cold-adapted membranes have less ability to repair electropores.

Indeed, we have found that cultures of *L. monocytogenes* or *S. typhimurium* grown near the upper limit of their temperature ranges (37 and 45 °C, respectively) were more resistant to PEF treatment than those grown near the lower limit (4 and 10 °C, respectively), as measured by the number of bacterial survivors and loss of UV-absorbing material, but not by the ability to maintain pH homeostasis (N.J. Russell and co-workers, in preparation). It was shown that the growth-temperature-triggered alteration in PEF sensitivity was correlated with changes in membrane lipid composition, particularly the lipid fatty acyl composition. In *L. monocytogenes*, the major growth temperature-dependent alteration was in the ratio of the two major *anteiso*-branched fatty acids, *anteiso* 15:0 and *anteiso* 17:0, whereas in *S. typhimurium*, it was a change in the proportion of total unsaturated fatty acids (mainly 16:1Δ9 and 18:1Δ11).

It is hypothesised that alterations in membrane lipid fatty acyl composition give a membrane with changed viscoelastic properties, which modify the ability of the bacterial cell to immediately repair damage due to the formation of PEF-induced electropores. Support for this hypothesis comes from our observation that ethanol, which is a membrane-fluidising agent, increases microbial inactivation; and phenethyl alcohol, a more potent membrane fluidiser, has a greater effect. Further support comes from a comparison of different strains of *L. monocytogenes*, including two nisin-resistant strains, which have altered lipid compositions and PEF sensitivities.

Most recently, using defined growth media as an independent means of giving membranes that have altered membrane fluidity, we have been able to manipulate the ratio of the major *anteiso*-branched fatty acids in *L. monocytogenes*, for example, having membranes in which the *anteiso*-branched fatty acyl content is reduced from 78% to 51% and the *anteiso*/*iso*-branched ratio is lowered from 7.1 to 1.4 (N.J. Russell and J.K. Coleman, in preparation). One would predict that such a change in lipid composition should give a membrane that was less fluid and therefore less sensitive to PEF, if our hypothesis is correct. However, the opposite result was found. Therefore, either the fluidity prediction is wrong or the role of membrane fluidity in PEF sensitivity is more complex. Other features of lipid organisation may be involved that, in order to elucidate them, will require direct biophysical examination of the modified membranes.

9. Concluding comments

In conclusion, refrigeration remains one of the most effective means of extending the shelf life of fresh and processed foods, because lowering temperature reduces the growth rate of even cold-adapted microorganisms, particularly if they have been damaged by use of a physical pretreatment. In order to optimise the efficacy of such combined preservation regimes and provide a synergistic extension of the safe shelf life of the food, a better understanding of the molecular basis of the lethal action of the physical treatments is needed. In particular, the role of mem-

brane lipid physicochemical properties and organisation require investigation.

This review has considered how mild heating enhances the efficacy of such physical treatments, and how that might be related to changes in membrane physicochemical properties such as fluidity, which can also be modified by changes in bacterial growth temperature that alter lipid composition. It appears that increasing membrane fluidity makes membranes more sensitive to disruption using ultrasound or high-pressure treatments, possibly by reducing the ability to repair damage, although in neither case has this been proven by direct biophysical measurements. For PEF treatment, the relationship between membrane fluidity and sensitivity is more complex, and other organisational aspects of the membrane lipids might be involved. Whatever the underlying explanation, subsequent storage of treated foods at low temperature will reduce metabolic rates and, therefore, the ability of surviving food-associated microorganisms to grow to troublesome population sizes.

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