Chapter 11
Manothermosonication for Microbial Inactivation

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1 Introduction

Most traditional technologies of food preservation, such as freezing, dehydration, acidification, are based on the inhibition or slowing of bacterial growth, which extends the shelf life of the product. However, the sanitary quality of the products manufactured according to the above processes can only be guaranteed if the raw materials used for their manufacture show a contamination by pathogenic microbial species lower than the infective dose. Even when starting from quality raw materials, it must be assumed that there could be low concentrations of pathogens that, though slowly, can multiply during subsequent storage, thereby jeopardizing the sanitary security of the product, which can obviously change over the course of time. Therefore, on many occasions, not only is it necessary to inhibit the microbial growth in the product to extend its shelf life, but the pathogenic species contained in it must be inactivated as well. In other words, it must be pasteurized.

Up to very recent times, heat has been practically the only method of preservation/pasteurization of food to guarantee the sanitary quality of the product, even if manufactured from raw materials of uncertain microbiological quality. The major problem of heat is its non-specificity, since heat treatments at the same time that they inactivate microorganisms can modify the nutritional value and sensorial properties of foods, thereby damaging their quality. Therefore, food technology is currently looking for alternatives and more specific methods of pasteurization and preservation, which besides guaranteeing the stability and safety of foods will not greatly modify their quality.

Ultrasound (US) is one of the new technologies of microbial inactivation that has been suggested as an alternative to current heat treatments. As a matter of fact, the bactericidal effect of ultrasound has been known since the beginning of the past century (Harvey and Loomis, 1929). Nevertheless, the first apparatus only

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allowed generation of low-intensity ultrasound (10 W/cm^2), which had low lethal efficacy, preventing their use as a food pasteurization method. The development of high-power ultrasound generators opened new ways to this technology, and this has roused a new interest to study its microbial inactivating effects.

Despite the patent improvement of current ultrasound generators, most published data indicate that the germ-killing efficacy of the process is still relatively low under room conditions, and only under special situations could ultrasound become an actual alternative to the current heat treatments. Therefore, most investigators have tried to improve the efficacy of the process, either by increasing cavitation intensity or by designing combined processes to enhance their effect (Arce-García et al., 2002; Guerrero et al., 2005; Lee et al., 2003; López-Malo et al., 2005; Ordóñez et al., 1984, 1986; Raso et al., 1998a; Sala et al., 1992).

In 1992, our research team assumed that the lethal efficacy of ultrasound was due to cavitation and that the loss of bactericidal effect with increasing temperature was due to decreased water vapour tension. Therefore, increasing the hydrostatic pressure of the system should also increase the cavitation intensity (which could be maintained even at temperatures over 100°C) and, therefore, the lethal efficacy of ultrasound. In order to check the above hypothesis, we constructed an instrument, the manothermoresistometer (Raso et al., 1998a), which allowed the application of heat, ultrasound and combined heat/ultrasound treatments under pressure, at different temperatures (up to 140°C), different pressures (up to 1,000 kPa) and different intensities of ultrasonication (up to 340 W, 170 μm of amplitude at a constant frequency of 20 kHz). In this new combined method of food preservation was called manothermosonication (MTS, Spanish Patent No. 9200686), and the first results concerning its lethal effect on microorganisms were published in the mid-1990s (Raso et al., 1994; Sala et al., 1995). In this section we review the accumulated knowledge in the last 15 years concerning the microbial lethal efficacy of manothermosonication.

2 Lethal Effect of Ultrasonic Waves Under Pressure

One of the major problems when assessing the efficacy of a new method of microbial inactivation concerns the quantification of the lethal effect on cells. In other words, the inactivation kinetics must be determined, and a mathematical equation to describe the survivor's behaviour must be found. By comparing the equation parameters, we will be able to quantify the differences of resistance between species, as well as the effect of various environmental factors. Furthermore, these parameters could be the basis for the development of secondary and tertiary models that would allow the adjustment of the treatment intensity in order to guarantee the achievement of a given inactivation level.

Once the inactivation kinetics has been established, it is important to quantify the efficacy of the new technology in regard to microbial species of reference, which must include both pathogenic and spoilage species. The study of the efficacy

on bacterial spores will allow establishment of the actual possibility of the new technology as a food sterilization method.

2.1 MS/MTS Microbial Inactivation Kinetics

In general, when the kinetics of microbial inactivation by a new technology is first approached, it is intended to adjust the equations of first-order reaction kinetics by using the models already developed for heat. Works carried out by Bigelow (1921) led him to conclude that the number of survivors following heat treatment was an exponential function of time. Therefore, by representing the logarithm of the number of survivors against time, a straight line should be obtained, the so-called survival curve.

The negative inverse of the survival line slope is called D_t, and it is defined as the treatment time at a constant temperature "t" that must be applied to the population in order to reduce the count to a tenth. By representing the logarithm of the D_t value against the corresponding treatment temperatures, a straight line is again obtained. In our research group we denominate this curve the decimal reduction time curve (DRTC), in order to distinguish it from the so-called thermal death time curve (TDT), which was obtained by representing the logarithm of time necessary to achieve a fixed number of logarithmic cycles of inactivation (F value) at each temperature. In any case, if the inactivation follows a strictly exponential course, the DRTC and TDT lines are parallel and the negative inverse of their slope corresponds to the z value. The z value is indicative of the thermodependence of the reactions leading to microbial inactivation and quality loss, and it allows calculation of treatments of similar lethal efficacy at different temperatures.

If one assumes that the microbial inactivation rate follows a first-order kinetics, one must admit that the inactivation is due to the alteration of a unique key molecule that, in the case of heat, has been traditionally associated with DNA denaturizing (Gould, 1989). In this case, the exponential course of the inactivation may be due either to a normal distribution of the resistance of the key molecule against the inactivating agent or to the existence of a distribution of energies in the treatment medium. Thus, in the latter case the quantity of heat received by each molecule would be different. If we assume that the heat resistance of the key molecule is similar in all cells, and that the number of molecules with enough energy to produce the inactivation is kept constant, under this circumstances microbial death would be a probabilistic phenomenon, which explains the fact that a constant percentage of the population becomes inactivated at similar time intervals.

Although the exponential model has been used for years for the calculation of heat treatments, and D_t values are widely used to compare microbial heat resistance, nowadays a number of experimental data showing the existence of linearity deviations of the survival curves have accumulated. The occurrence of such deviations, which are known as shoulder and tail phenomena, does not necessarily mean that the inactivation course does not follow an exponential kinetics, but they may reflect the addition of different events, such as the activation of spores, their simultaneous

inactivation or even their adaptation to heat (Palop et al., 1997). The reader will find further information about survival curve deviations in the classical works by Rahn (1945), Shull et al. (1963), Moats et al. (1971) and Cerf (1977). In the last 20 years, considerable efforts have been made to find alternative mathematical approaches to describe these non-linear kinetics, even to predict the effect of anisothermal heating (Hassani et al., 2005, 2006; Mafart et al., 2002; Peleg, 1999; Peleg and Cole, 1998), but it is difficult to find an unique and final model, since the causes of the deviations largely depend on the microbial species and, above all, on the physiological state of the cells (vegetative cells vs. bacterial spores). No matter the cause of the occurrence of these deviations, their practical consequences are evident: the use of the equations of survival curves and DRTC for the calculation of survival probability of the microbial population will lead to an overestimation of the lethal efficacy of the treatment, with subsequent increased risks for public health. Furthermore, the direct comparison of D_t and z values will not be sufficient to predict which microbial species will limit the intensity of the heat treatment. For similar reasons, it is essential to precisely establish the inactivation kinetics of any food preservation technology.

Our investigations with this technology in the last 15 years allow us to state that the microbial inactivation by MS/MTS fits a first-order kinetics more closely than that of heat, at least for 99.9% of the cell population. Figure 11.1 exemplifies some survival curves obtained with bacterial spores and vegetative cells of gram-positive and gram-negative species. Linearity deviations are very rare and, in general, both the shoulders and the tails shown in the diagrams of survival to heat are minimized when ultrasound under pressure is applied. This effect is well illustrated in works by Sala et al. (1995) and Pagán et al. (1999b). These results are logical if, as discussed

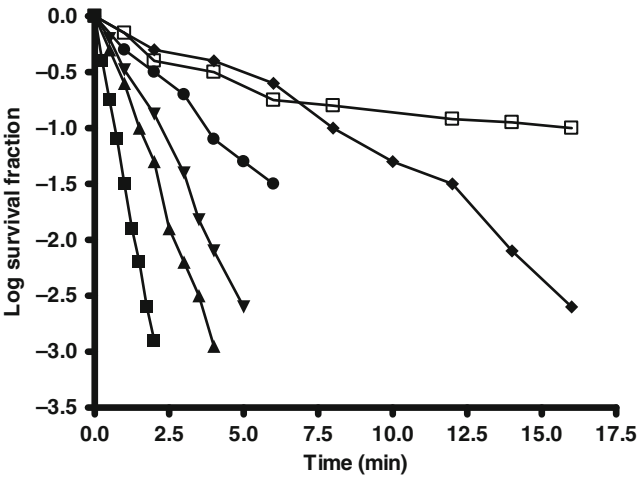

Fig. 11.1 Survival curves for ultrasonic under pressure treatments (40°C, 200 kPa, 117 μm) of *B. subtilis* (□), *B. circulans* (♦), *E. faecium* (●), *S. aureus* (▼), *L. monocytogenes* (▲) and *A. hydrophila* (■)

below, we consider that inactivation by manosonication meets the two necessary requirements so that the inactivation may follow an exponential order: the microbial inactivation by ultrasound under pressure is an "all or nothing" event, with the cell envelopes as the key target; and, the inactivation is due to transitory cavitation, which occurs in specific points of the media and whose intensity is kept nearly constant throughout the treatment, at least in our experimental conditions. Under these circumstances, it is reasonable to think that the percentage of living cells affected by the treatment remain constant throughout time. However, recent investigations have shown that tailing effects may appear when higher levels of inactivation are reached (Lee et al., 2009).

We have found small but frequent shoulders in the survival curves to manosonication with some strains of *Staphylococcus aureus*. In this case, shoulders occur as a result of the initial disintegration of the naturally present cell aggregates, as produced by the shock waves generated by cavitation. Sonication at atmospheric pressure under sublethal conditions provokes an increase in the microbiological count of the suspension, up to the expected N_0 value estimated with the equation of the straight stretch of the survival curve. The appearance of tails is also quite rare, at least when 99.9% of cells of the initial population are inactivated. Under our usual experimental conditions, the counts for less than the 0.1% of the initial cell population have little statistical reliability (usually less than 300 ufc/ml). Occasionally, we have found a tailing phenomenon corresponding to a suspension of *Bacillus cereus*, which cannot be assigned to a methodological device (Sala et al., 1995). Perhaps this suspension contained two sub-populations of the same strain with different resistance to ultrasound, as we have demonstrated with spores of other microbial species against heat (Palop et al., 1997).

In short, microbial inactivation by MS/MTS is an exponential function of the treatment time. Therefore, the $D_{\mathrm{MS/MTS}}$ parameter can be defined as the treatment time necessary so that the survival line goes through a logarithm cycle. Similar to heat treatments, the time of an industrial treatment can be calculated by multiplying the $D_{\mathrm{MS/MTS}}$ value by the number of inactivation cycles we intend to achieve.

As described below, D_{MS} values are influenced by a number of physical parameters of the treatment, whereas heat resistance depends only on temperature. Therefore, up to now, we had not tried to develop a DRTC for manosonication. The data by Raso et al. (1999) prove the existing relationship between the amplitude of ultrasonic waves, the hydrostatic pressure of the system and the energy transmitted to the medium by ultrasound. The data by Mañas et al. (2000a) demonstrate that the lethal efficacy of ultrasound is related to the energy transmitted to the medium. Figure 11.2 shows the relationship existing between the logarithm of D_{MS} values of *Listeria monocytogenes* and the energy transmitted to the medium by the ultrasonic waves at different amplitudes (62, 90 and 117 μm) and pressures (0, 100, 200 and 300 kPa). As shown in the figure, the decimal reduction time to the MS treatment is an exponential function of the energy transmitted to the medium by ultrasound and, therefore, similar to heat treatments, a Z_{MS} value can be defined. In the example $Z_{\mathrm{MS}} = 56.2$, which means that an increase in 56.2 W in the energy transferred to the medium by the ultrasound, the inactivation rate of *L. monocytogenes* will be

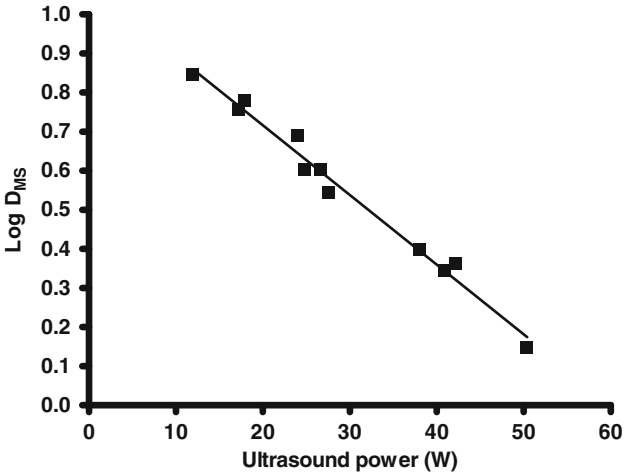

Fig. 11.2 Decimal reduction time curve of *L. monocytogenes* to manosonication

increased by 10 times. Later, we will discuss the effect of pressure and amplitude on the energy transferred to the medium by the ultrasound, as well as on the lethal efficacy.

2.2 Microbial MS/MTS Resistance

As mentioned in the corresponding sections, cavitation intensity is influenced by a multitude of physical parameters (wave frequency and amplitude, pressure, viscosity and temperature of the medium, etc.), but the geometry of the chamber and that of the sonication horn is also important. Since cavitation is limited to an area close to the transducer (the intensity of ultrasonic waves being inversely proportional to the square of the distance to the sonication tip), the effect of ultrasound depends on the shape and volume of the treatment chamber. Furthermore, cavitation should not take place too close to the horn's tip, as the bubbles generated could attenuate the effect by dispersing sonic waves (Berlan and Mason, 1992). The geometry and size of the horn also determine the energy transmitted to the medium (Berliner, 1984). The characteristics of the treatment medium can also affect the intrinsic resistance of the microorganism against MS/MTS. Because of all the above, it is very difficult to compare the values of resistance to ultrasound obtained by different investigators with different methodologies. In this chapter, we will use mainly those data obtained by our research group with the same equipment, previously described by Raso et al. (1998a). All data included in tables and figures in this section were obtained in a McIlvaine buffer of pH 7; therefore, the D_{MS} values in Table 11.1 are directly comparable.

Table 11.1 D_t(62°C) and D_{MS}(40°C, 200 kPa, 117 μm) values of different bacterial species

Bacterial species	$D_{62°C}$(min)	D_{MS}(min)
Bacterial spores		
B. subtilis	Insensitive	10
B. circulans	Insensitive	6.5
B. coagulans	Insensitive	9
Gram-positive vegetative cells		
L. monocytogenes	0.62	1.5
E. faecium	15.3	4.0
S. aureus	0.5	2.5
Gram-negative vegetative cells		
Y. enterocolitica	0.39	1.2
P. aeruginosa	0.18	0.92
E. coli	0.012	0.87
S. enteritidis	0.068	0.73
S. typhimurium	0.12	0.80
S. senftenberg	1.1	0.84
A. hydrophila	0.024	0.86

Table 11.1 shows the D_{MS} values of different bacterial spores and vegetative species, both gram positive and gram negative. Their resistance to heat is also included as a reference. The data in the table indicate that it is possible to inactivate bacterial spores at sublethal temperatures; therefore, MS treatments could be used as a food sterilization method, although the D_{MS} values for spores are relatively high in the experimental conditions tested. We should also highlight the small difference concerning resistance to ultrasound under pressure between the three species of spore formers investigated, as compared to their heat resistance. Whereas the D_{100} values of *Bacillus circulans* differ practically 10 times in regard to those of *Bacillus coagulans* ($D_{100} = 0.47$ and 5.0, respectively) (Mañas, 1999; Raso, 1995), the D_{MS} values barely differ by about 30%. Finally, it is worth highlighting that resistance against ultrasound is about 10 times higher in spores than in vegetative cells, a negligible difference in regard to the 10^7 times that their resistance to heat changes.

As far as vegetative cells are concerned, it can be concluded that gram-positive species are more resistant to manosonication, as well as against heat, than gram-negative species. As in the previous case, the differences between the D values of both groups are much lower against manosonication than against heat. Within the gram-positive group, whereas the heat resistance of *S. aureus* is lower than that of *L. monocytogenes*, the opposite occurs in regard to manosonication. This could indicate that spherical cellular shapes are more resistant to MS than bacillary cells, as previously suggested for ultrasound at ambient pressure (Alliger, 1975; Jacobs and Thornley, 1954). The gram-negative group is the most sensitive against heat and manosonication. However, as in the above-mentioned groups, differences in resistance to MS among genera and strains are lower than to heat. It is also worth mentioning that whereas the heat resistance of *Salmonella senftenberg* 775 W is

10–100 times higher than that of the rest of species, its resistance against MS is about in the middle of the range. A similar phenomenon has been observed when studying the resistance of *Salmonella* against irradiation (Álvarez et al., 2006b) and high pressure (Sherry et al., 2004).

The above results imply that the mechanisms of inactivation by heat and manosonication are different, although the target molecule might not be different. In any case this behaviour may have remarkable practical consequences. Current pasteurization treatments are calculated by establishing a determined survival risk for the most heat-resistant pathogenic species that can usually be expected to contaminate the food. Under these circumstances, if the raw material is contaminated by other pathogenic species that are less frequent but more heat resistant, the increased safety risk may be enormous. On the contrary, considering the scarce differences in resistance to MS, a change in the pathogenic species that usually contaminate the food would have a much smaller effect on the safety of the product pasteurized by MS. For example, according to the data included in Table 11.1, we can conclude that current pasteurization treatment of liquid whole eggs in the USA (3.5 min at 60°C, which would be equal to 1.81 min at 62°C) would allow the reduction of the population of our strain of *Salmonella typhimurium* by 15 logarithmic cycles, which exceeds by far the usually recommended 7–8 logarithmic cycles. However, if liquid whole egg is contaminated by the strain of *S. senftenberg* 775 W (data included in Table 11.1), the treatment would allow reduction of the microbial population by 1.64 cycles only. In other words, the safety risk would increase by $10^{13.36}$. On the contrary, an MS treatment designed to inactivate, as in the previous case, the population of *S. typhimurium* by 15 cycles (12 min, 40°C, 200 kPa, 117 μm) would also reduce the population of *S. senftenberg* by 14.28 cycles, i.e. the safety risk would not increase more than five times, although the serotype of the *Salmonella* contaminating the liquid egg would change. An additional advantage of MS in the above-mentioned example would be its effect on the spores of *B. circulans*, which is a usual spoilage agent of ultrapasteurized liquid whole egg. Whereas the above-mentioned treatment of heat pasteurization would not affect its viability at all, the corresponding MS treatment would allow a reduction of the population of *B. circulans* by 70 times, which would probably contribute to the extension of its shelf life. Obviously, there are not only advantages; whereas the heat treatment of the example would be 1.8 min at 62°C, the manosonication should be extended for 12 min. However, this MS treatment would still be more efficient to inactivate *S. senftenberg* and *B. circulans* than pasteurization at 62°C for 12 min.

3 Effect of Physical Parameters on the MS/MTS Lethal Effect

The lethal efficacy of MS/MTS depends on the cavitation intensity, which is highly influenced by a multitude of physical parameters. Among these, ultrasonic wave amplitude, pressure of the medium and temperature are the most significant, from a practical point of view. Other parameters, including viscosity, are also very

important, but they cannot be easily modified in an industrial process. Therefore, their relevance, for our purposes, is lower. Further information concerning the influence of these variables on the energy transmitted to the medium by the ultrasound in our equipment can be found in Raso et al. (1999) and Condón et al. (2005).

3.1 Effect of the Amplitude of Ultrasonic Waves

The amplitude of ultrasonic waves influences cavitation intensity by determining the number of bubbles that implode by time unit (Suslick, 1988). Therefore, this parameter should be carefully controlled and maintained during sonication (Berliner, 1984). The data published by Raso et al. (1999) and Mañas et al. (2000a) demonstrate that, in our equipment, the energy transmitted to the medium by MS/MTS is an exponential function of the amplitude of the sonic waves. Furthermore, we have demonstrated that the lethal efficacy of MS at constant pressure and temperature is also an exponential function of the amplitude, i.e. the logarithm of the D_{MS} values decreases linearly with the ultrasonic wave amplitude, both in bacterial spores (Raso et al., 1998b) and vegetative cells of gram-positive species, e.g. *L. monocytogenes* (Pagán et al., 1999a) and *Enterococcus faecium* (Pagán et al., 1999c), and in gram-negative species, e.g. *Yersinia enterocolitica* (Raso et al., 1998a), *Aeromonas hydrophila* (Pagán et al., 1999c), *Salmonella enteritidis*, *S. typhimurium* and *S. senftenberg* (Mañas et al., 2000b).

Since it seemed that the effect of ultrasonic waves on D_{MS} values was the same for all vegetative cells investigated, we developed the following general equation:

$$\log D_{MS} = \log D_0 - 0.0091 \times (A - 62)$$

where D_{MS} is the decimal reduction time to MS, D_0 is the decimal reduction time to MS at an amplitude of 62 μm and A is the amplitude of ultrasonic waves.

The above equation fits all data obtained in our investigations (Álvarez, 2000; Mañas, 1999; Pagán, 1997) between 62 and 150 μm of amplitude, for the different species, with a correlation higher than 0.98. The good fit of the above equation has been well demonstrated by the works of Pagán et al. (1999c), Mañas et al. (2000b) and Condón et al. (2005). The equation parameters imply that the inactivation rate of vegetative cells by ultrasound will increase by 10 times when increasing the amplitude of ultrasonic waves by 110 μm. At present, there are no sufficient experimental data available that allow demonstration of the utility of the above equation to predict the inactivation of bacterial spores by MS.

The good fit of this unique equation to the experimental data obtained with different microbial species, whose resistance to manosonication varies about 10 times (Table 11.1), indicates that the effect of the amplitude is not related to a change in the intrinsic resistance of each species, but, most likely, to its physical effects. The existence of an exponential relationship between the energy transmitted to the medium by ultrasound and the amplitude of the ultrasonic waves (Mañas et al.,

2000b; Raso et al., 1999) seems to confirm this hypothesis. Bacterial inactivation by ultrasound seems to be due to very high pressure and temperature (Frizzell, 1988; Harvey and Loomis, 1929) and/or to the release of free radicals in the medium (Jacobs and Thornley, 1954; Riesz and Kondo, 1992) by transient cavitation. The higher inactivation rate at greater amplitudes could be due to an increase of the number of bubbles liable to implode per unit of time in a given volume and/or to an increase of the volume of liquid in which transient cavitation is liable to occur (Suslick, 1990).

3.2 Effect of Hydrostatic Pressure

When the hydrostatic pressure of a system increases, the intramolecular cohesion forces are strengthened, which reduces the tension of the vapour of the solvent and increases its viscosity. Both circumstances hinder the transient cavitation phenomenon. However, if the generator's power is sufficient to maintain the amplitude of the ultrasonic waves and cavitation occurs, its physicochemical and biological effects are higher (Berliner, 1984). We have demonstrated (Mañas et al., 2000a; Raso et al., 1999) that, in our equipment, the energy released to the medium by ultrasound increases exponentially with the hydrostatic pressure, until reaching a threshold value that depends on the temperature. Above this threshold, the effect of pressure progressively decreases, until it vanishes. A similar relationship has been found when correlating the D_{MS} values and the hydrostatic pressure of the medium (Fig. 11.3), both in bacterial spores (Raso et al. 1998b) and in vegetative cells (Mañas et al., 2000b; Pagán et al., 1999a; Raso et al. 1998a).

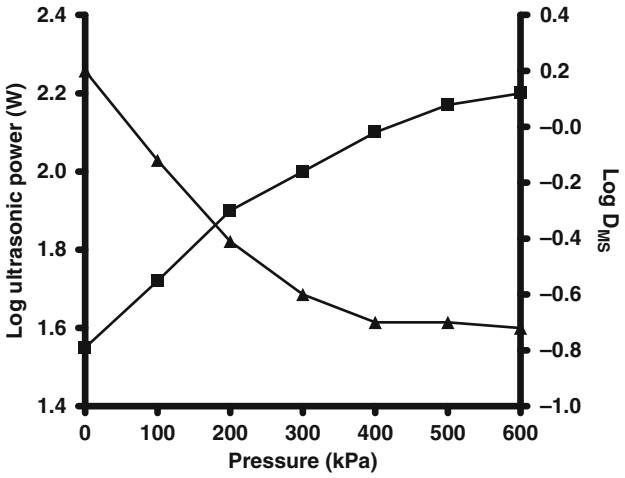

Fig. 11.3 Effect of pressure on ultrasonic output power (■) and on lethality of ultrasound (▲) (40°C, 20 kHz, 150 μm)

In 1999c Pagán et al. developed the following polynomial equation:

$$\log D_{MS} = \log D_0 - 0.0026 \times P + 2.2 \times 10^{-6} \times P^2$$

where D_{MS} is the decimal reduction time corresponding to an MS treatment at an amplitude of 117 μm and 40°C, D_0 is the D value corresponding to ultrasonic treatment of the same amplitude and temperature but at room pressure and P is the static relative pressure.

This general equation allows the adjustment of all D_{MS} values of vegetative cells obtained in our equipment, at sublethal temperatures (up to 40°C) and at different pressures (up to 400 kPa) (Mañas et al., 2000b; Pagán et al., 1999a; Raso et al., 1998a), with a correlation that is always higher than 0.98. The good fit of the experimental data to this general equation is supported by the works of Mañas et al. (2000b) and Condón et al. (2005). According to this equation, an increase of the pressure of the system from 0 to 400 kPa of relative pressure reduces the D_{MS} values by approximately five times. At present, there are not enough available data allowing demonstrating the adequacy of this equation to predict the effects of the pressure of the system on the lethal efficacy of manosonication on bacterial spores. The only available data (Raso et al., 1998b) seem to indicate that the percentage of survivors of a spore population of *Bacillus subtilis* subjected to MS treatment (250 kHz, 117 μm, 70°C) during a fixed time exponentially decrease with pressure up to 500 kPa. Above this threshold value, the lethal efficacy of the process decreases. The difference between the threshold values above which the increase of pressure barely affects the efficacy of MS on vegetative cells (300–400 kPa) and spores (500 kPa) is most probably due to the different treatment temperatures used (40 and 70°C, respectively). Raso et al. (1999) demonstrated that if the temperature of the manosonication medium is increased, the pressure of the system must be increased as well in order to achieve a constant power output. They also proved that the curve correlating both variables shows a profile similar to the water vapour pressure curve.

As with the amplitude, a good fit of a unique equation to the experimental data obtained at different pressures with different species showing a different resistance to MS would indicate that the differences in cell structure do not affect the effect of pressure, which will most likely be related to changes in cavitation intensity. Increased pressure will affect the occurrence of cavitation, but if cavitation takes place, the intensity of the implosion will be increased (Whillock and Harvey, 1997) and, therefore, its lethal effects. When a particular pressure threshold is reached, the ultrasonic field will be incapable of overcoming the combined forces of overpressure and the cohesive force of the liquid molecules, and the number of bubbles undergoing cavitation will decrease (Suslick, 1988), as well as the microbial inactivating effects of the treatment. This is a significant aspect that should be taken into account when trying to establish the optimum pressure of treatment in an eventual industrial process, where an increased pressure not always will lead to increased lethal efficacy.

3.3 Effect of Temperature

The earliest bibliographic references about the effect of temperature on the bactericidal efficacy of ultrasound date back to the 1970s. At that time it was demonstrated that prior ultrasonic treatment sensitizes bacterial spores to subsequent heat treatment (Burgos et al., 1972). Some years later, it could be proven that the application of ultrasound at high temperatures synergistically increased the efficacy of the combination on spores (García, 1985). In the 1980s, Ordóñez et al. (1986) proposed the name "thermoultrasonication" to designate this combined process. Thermoultrasonication has proven to be efficient in inactivation of vegetative cells of different species (Earnshaw et al., 1995; Hurst et al., 1995; Ordóñez et al., 1984) and, in some temperature ranges, on bacterial spores (García et al., 1989). However, in the latter, the sensitizing effect of heat decreased with temperature, until it practically vanished at temperatures close to the boiling point (García, 1985).

Changes in the lethal efficacy of ultrasound at different temperatures may be attributed to direct and indirect mechanisms. As discussed below, the increased temperature of the medium may determine some structural changes in the envelopes of vegetative cells, which would lose a part of their mechanical resistance. Nevertheless temperature can also act by modifying cavitation intensity, although this effect can be misleading. Bubbles form and grow more quickly as the temperature of the liquid becomes higher, because vapour pressure increases and tensile strength decreases (Suslick, 1988). However, the violence of these collapses is lower, because high vapour tension inside the bubble acts as a cushion (Alliger, 1975). The final biological effect of the combination of heat and ultrasound will depend on the balance between these opposite phenomena, whose relative significance will also depend on the range of temperatures under study. Since the slope of the vapour tension line progressively increases with temperature, the undesirable effects on cavitation intensity will increase as well, especially at temperatures higher than 70–80°C (Raso et al., 1999). This physical phenomenon can easily explain the loss of efficacy of thermoultrasonication on bacterial spores, as described by García (1985).

Sala et al. (1992) developed an instrument that allowed application of ultrasonic treatments at different pressures and temperatures. We demonstrated that it was possible to maintain the bactericidal efficacy of ultrasound even at temperatures higher than 100°C, just by increasing the hydrostatic pressure of the system. We named this new combined process "manothermosonication". From a theoretical point of view, if the cavitation intensity depended only on changes of the vapour tension of the solvent, such intensity could be maintained by keeping constant the difference between the hydrostatic pressure of the system and the vapour pressure of the solvent at treatment temperature. Actually, there are small deviations (Raso, 1995), as heating produces other changes, including decreased viscosity, which also influences cavitation.

On the other hand, in order to study the influence of temperature on the microbial lethal efficacy of ultrasound at constant cavitation intensities, the hydrostatic pressure of the system should be constantly corrected. However, in practice, values sufficiently close to constant pressure can be obtained, provided that the differential

pressure is very high with regard to changes of vapour pressure of the solvent within the range of temperatures under study. These conditions occur between room temperature and 70°C, a range where the slope of the vapour tension line is very low. Raso et al. (1999) demonstrated that at 200 kPa a change of temperature from 20 to 70°C barely modifies the energy transmitted to the medium by ultrasound by 10%.

Figure 11.4 shows the relationship existing between the $D_{MS/MTS}$ values (20 kHz, 117 μm, 200 kPa) and the treatment temperature for two species (i.e. *L. monocytogenes* and *A. hydrophila*) with high and low resistance to manosonication. The corresponding decimal reduction time curves (DRTC) against heat, as well as a theoretical curve obtained through a predictive model developed by our group (which will be discussed below), are also included for reference purposes. As shown in the figure, resistance to ultrasonic treatments under pressure is kept practically independent from temperature until reaching a threshold value, above which the $D_{MS/MTS}$ values decrease rapidly until becoming equal to D_t values. This behaviour implies that inactivation by ultrasonic waves under pressure and inactivation by heat are two independent processes. At low treatment temperatures (sublethal temperatures), the lethal effect of the treatment would only be due to the action of ultrasonic waves under pressure. When lethal temperatures are reached, the independent inactivating effects of ultrasonic waves under pressure and heat would be added. Thus, the total lethal effect achieved would be equal to the addition of the lethal effects produced by each technology, i.e. an additive effect. From this point, whereas the lethal efficacy of ultrasonic waves under pressure would remain constant when increasing the temperature, the lethal effects of heat would increase exponentially, and the contribution of the ultrasonic waves under pressure to the total lethal effect would obviously decrease, until it would practically disappear and the $D_{MS/MTS}$ values

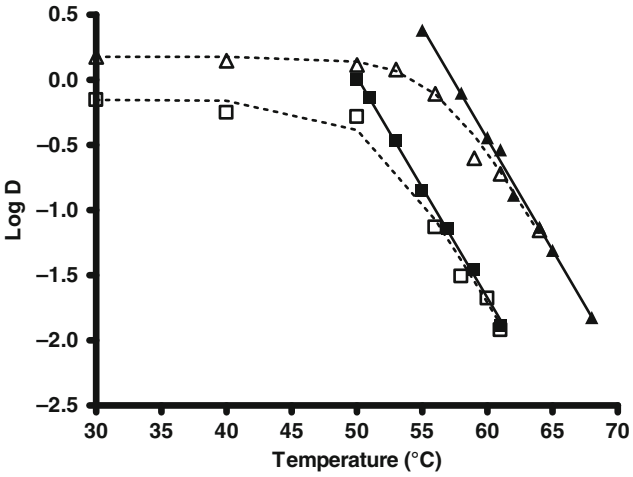

Fig. 11.4 Theoretical (*dotted line*) and experimental (*symbols*) D_{MS} values (200 kPa, 117 μm) of *L. monocytogenes* (▲) and *A. hydrophila* (■). *Straight lines* represent the corresponding decimal reduction time curves to heat treatments

would equal the D_t values. The above results encouraged us to distinguish two processes, which we called manosonication (MS) and manothermosonication (MTS). Manosonication is the process in which heat has no lethal effect and depends on cavitation intensity only. Manothermosonication is a similar process, in which the lethal effects of ultrasound under pressure and those of heat would be added. Obviously, the temperature at which the MS process becomes MTS will depend on the microbial species under study and, more specifically, on its resistance to heat. The data in Fig. 11.4 imply that MS would become MTS at 50 and 55°C approximately, for *A. hydrophila* and *L. monocytogenes*, respectively.

In order to check the above hypothesis indirectly, we developed a predictive model that allowed us to compare experimental values and theoretical values estimated by assuming that microbial death by ultrasound under pressure and by heat is caused through different and independent mechanisms of action, and that, in both cases, the inactivation follows an exponential kinetics. Under these conditions, the course of microbial inactivation by heat, MS and MTS would meet the following equations:

$$\begin{aligned} \log N_t &= \log N_0 - (1/D_t) \times t \quad \text{for heat} \\ \log N_t &= \log N_0 - (1/D_{\mathrm{MS}}) \times t \quad \text{for manosonication} \\ \log N_t &= \log N_0 - (1/D_{\mathrm{MTS}}) \times t \quad \text{for manothermosonication} \end{aligned}$$

where N_t is the number of cells surviving after a treatment time "t", N_0 is the number of living cells in the time t=0 and D_t, D_{MS} and D_{MTS} are the corresponding decimal reduction times.

If heat and ultrasound under pressure act in an independent manner, the inactivation rate by MTS can be estimated from the individual inactivation rates by MS and heat. This will allow deduction of the relationship existing between the decimal reduction times against each technology through the following equation:

$$D_{\mathrm{MTS}} = (D_{\mathrm{MS}} \times D_t)/(D_{\mathrm{MS}} + D_t)$$

The dotted lines in Fig. 11.4 represent the estimations of $D_{\mathrm{MS/MTS}}$ values for *A. hydrophila* and *L. monocytogenes* at different temperatures, as obtained with the above equation. The good fit of the experimental data to theoretical estimations seems to confirm the starting hypothesis. This equation allowed us to estimate the D_{MTS} values at different temperatures of most vegetative cells investigated, both gram positive (*L. monocytogenes,* Pagán et al., 1999a) and gram negative (*Y. enterocolitica*, Raso et al., 1998a; *A. hydrophila*, Pagán et al., 1999c; *S. enteritidis, S. typhimurium* and *S. senftenberg*, Mañas et al., 2000b). These results indicate that microbial inactivation by ultrasonic waves under pressure is independent of temperature when the cavitation intensity is kept constant. However, in the past few years, we have found some cases in which the lethal effect of MTS is synergistic, i.e. the total lethal effect of the treatment is higher than the mere addition of the lethal effect of ultrasound under pressure plus heat. This topic will be further discussed in Section 5.

3.4 Interactions

As mentioned in the above sections, the rate of microbial inactivation by ultrasonic waves exponentially increases with hydrostatic pressure, but only up to a definite threshold value, above which it may even decrease. Moreover, in narrow temperature ranges and below 70°C, the lethal effect of ultrasound barely depends on temperature when the pressure is kept above 200 kPa. Investigations supporting these conclusions were designed, keeping constant two of the three parameters and

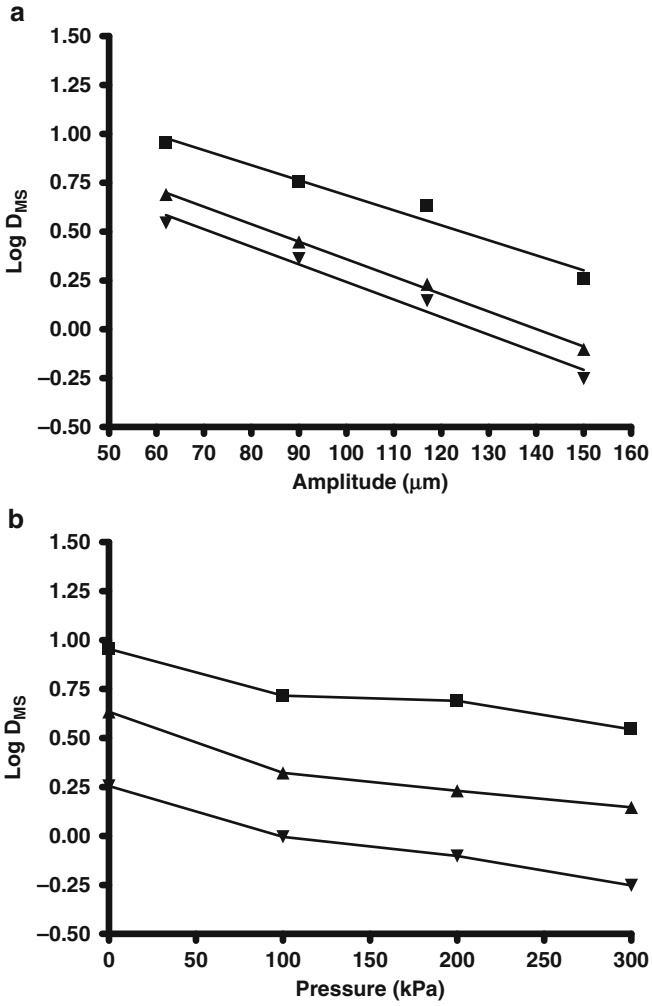

Fig. 11.5 (**a**) Effect of amplitude on D_{MS} values of *L. monocytogenes*: 0 kPa (●), 200 kPa (▲) and 300 kPa (■). (**b**) Effect of pressure on D_{MS} values of *L. monocytogenes*: 62 μm (●), 117 μm (▲) and 150 μm (■)

modifying only the third one, which raises a doubt about their eventual interactions. This is an interesting aspect about which, as far as we know, there are no data available except those by Pagán (1997).

Figure 11.5a,b shows the relationship between amplitude of ultrasonic waves and D_{MS} values for *L. monocytogenes* at various treatment pressures and the relationship between D_{MS} values of the same species and hydrostatic pressure at various amplitudes, respectively. These results indicate that the effect of amplitude is independent of pressure and vice versa. Raso et al. (1999) demonstrated that the increase of energy transmitted to the medium by increased amplitude was independent of the pressure of the system at constant temperature, which seems to indicate that the lethal efficacy of MS depends only on cavitation intensity, no matter if the changes of intensity occur by modifying the pressure, the amplitude or both variables.

4 Environmental Factors Affecting Bacterial MS/MTS Resistance

It is a well-known fact that microbial resistance against any inactivation agent has a genetic component, but it is also influenced by a multitude of environmental factors such as growth conditions and treatment media. Actually, in regard to some technologies such as heat, these environmental factors may even mask those differences that are genetically defined (Pagán et al., 1999c, d; Stumbo, 1965; Tomlins and Ordal, 1976). Therefore, it is important to determine the effect of these environmental factors on the resistance against the inactivating agent under study for every species of reference, since the species limiting the treatment may change in each specific situation.

The factors that may modify the microbial resistance vary greatly, and these factors have been traditionally classified, according to the moment in which they act, as either previous or simultaneous to treatment. Among the former, temperature of growth and stress factors are the most relevant; among the latter, pH, water activity and chemical composition of the medium are the most investigated. Sometimes, a third group of factors, which act "after the treatment", is also included, but these are actually related to the phenomena of cell damage and recovery, and they will be discussed in Section 5.

Table 11.2 summarizes the data currently available on this topic, under conditions of reference. These data were obtained by different researchers in our group (Álvarez, 2000; Álvarez et al., 2003b, 2006a; Mañas, 1999; Mañas et al., 2000a; Pagán, 1997; Pagán et al., 1999a, c; Raso, 1995) over the course of 15 years, but using the same equipment and with a similar methodology, which allows their direct comparison.

4.1 Factors Prior to Treatment

It is well known that changes in growth temperature induce some changes in cell envelopes, which can largely modify their resistance to different lethal agents. This

Table 11.2 Effect of some environmental factors on the D_{MS} (40°C, 200 kPa, 117 μm) values of different bacterial species

	Growth temperature (°C)			Treatment medium pH		Water activity			Medium Composition			Heat shock	
	37	20	4	7.0	4.0	>0.99	0.96	0.93	Buffer	Milk	Egg	Non-HS	HS
L. monocytogenes	1.5	–	1.5	1.5	0.96	1.6	–	3.1	1.5	1.8	–	1.7	1.6
Y. enterocolitica	0.31	0.59	0.81	0.89	1	–	–	–	0.81	0.82	–	–	–
A. hydrophila	0.69	0.23	–	–	–	–	–	–	–	–	–	–	–
S. typhimurium	0.80	0.81	–	–	–	–	–	–	0.80	–	0.84	–	–
S. enteritidis	–	–	–	0.73	0.56	0.89	1.3	–	0.73	–	0.76	–	–
S. senftenberg	–	–	–	0.84	0.96	1.7	2.1	1.8	0.84	–	1.4	0.85	0.78

is especially significant when the inactivating agent specifically acts on these structures, as in the case of MS (see Section 5). Amazingly, growth temperature barely affects the MS resistance of any of the species investigated, as compared with changes in their resistance to heat. For example, an increase of growth temperature from 4 to 37°C in *L. monocytogenes* increases its heat resistance by 2.1 times (Pagán et al. 1999a), but it does not affect its resistance to MS.

From a scientific point of view, the influence of this factor on the MS resistance of *Y. enterocolitica* is particularly interesting. Pagán et al. (1999d) demonstrated that the heat resistance of this microorganism was independent of culture temperature between 4 and 20°C, but in cells grown at 37°C, the D_{62} values increased by a factor of 4. These results may be explained by the increase in the ratio of saturated to unsaturated fatty acids of the cell membrane. Tsuchiya et al. (1987) found that an increase of the culture temperature from 5 to 25°C barely changed the composition of the cell membrane of *Y. enterocolitica*, but a further raise to 37°C increased that ratio drastically. On the contrary, as results in Table 11.2 show, the MS resistance of *Y. enterocolitica* exponentially decreases with increased growth temperature, which would indicate that the mechanisms through which ultrasound and heat treatments affect the cell membrane are different and that the composition in fatty acids does not affect its MS resistance.

Heat shocks develop a degree of protection against subsequent heat treatments in microorganisms (Lindquist, 1986; Mackey and Derrick 1986). Heat shocks trigger a physiological response that leads to the synthesis of a specific set of proteins known as heat shock proteins (HSPs) (Lindquist, 1986; Schlesinger 1986). The action mechanism of HSPs is not fully understood. Parsell and Lindquist (1993) suggested that, among others, the role of HSPs could be to prevent the accumulation of aberrant proteins generated by stress and also to protect the original structure and metabolic activity of other important proteins by avoiding their aggregation and by restoring heat damage. Pagán et al. (1999b) carefully studied the effect of time and temperature of heat shocks on the heat resistance of the strain *of L. monocytogenes* included in Table 11.2. Their work indicated that the probability of survival to heat of a population of this strain increased by thousands of times after being subjected to a sublethal heat shock. On the other hand, the heat shocks changed the profile of the survival curves, which showed prolonged shoulders in the case of heat-shocked cells. Furthermore, their results proved that the higher heat resistance of the shocked cells was partly due to a higher thermal stability of their cell structures and partly due to a higher capacity of damage repair. As shown in Table 11.2, sublethal heat shocks do not protect *L. monocytogenes* and *S. senftenberg* against manosonication. These results imply that heat shocks do not stabilize the cell structures against ultrasound. Moreover, they indicate that either sublethal damages previous to the inactivation by MS do not occur or the shocks do not increase the capacity of repairing the damages caused by ultrasound.

As mentioned above, the lethal effect of manothermosonication was additive on most species investigated. However, the only work existing on this topic (Pagán et al., 1999b) implies that when MTS (62°C, 200 kPa, 117 μm) is applied to heat-shocked cells (180 min at 45°C) of *L. monocytogenes*, the combination shows a

synergistic effect, as it reduces the population 10 times more than expected after 1.5 min treatment. This synergistic effect on heat-shocked cells when heat and ultrasonic waves under pressure (MS) act simultaneously (MTS) could be due to a lower resistance to MS of the cells damaged by heat during the MTS treatment. In order to check this hypothesis, the authors compared the lethal efficacy of MTS and that of a treatment in which the heat treatment (1.5 min at 62°C) and the MS treatment (1.5 min, 200 kPa, 117 μm) were applied successively and not simultaneously. The authors demonstrated that the previous heat treatment makes the cells more sensitive against manosonication, but its lethal effect does not equal that of the corresponding MTS. They concluded that, most likely, ultrasound interferes with the phenomena of recovery of the damages inflicted by heat, and that heat alters the physical characteristics of the cell envelopes by sensitizing them against ultrasound.

4.2 Factors Simultaneous to Treatment

The pH is one environmental factor with high influence on microbial resistance to different technologies, such as heat (Jay, 1992; Pagán, 1997; Tomlins and Ordal, 1976), high pressure (Alpas et al., 2000; Koseki and Yamamoto, 2006; Mackey et al., 1995; Stewart et al., 1997; Wouters et al., 1998) and pulsed electric fields (Álvarez et al., 2000, 2002; Aronsson and Rönner, 2001; Aronsson et al., 2004; García et al., 2003, 2005; Geveke and Kozempel, 2003). Acidification, besides its remarkable depressing effect on resistance, is easily modifiable in foods. Therefore, it is frequently applied in the food industry. Contrary to most other technologies, the pH of the treatment medium barely affects microbial resistance to MS. As Table 11.2 shows, a reduction of pH from 7.0 to 4.0, which reduces the D_t values of *L. monocytogenes* by four times (Condón et al., 2005), only reduces its D_{MS} value by 1.6 times. The effect on other studied species is even lower. At present, the mechanism of sensitization to heating in media of low pH is not known with accuracy. It has been suggested that acidification of the medium could facilitate the denaturation of cellular proteins by physical agents (Bender and Marquis, 1985) and/or produce lixiviation of divalent cations from cell envelopes, thereby reducing their resistance (Alderton et al., 1964; Ando and Tsuzuki, 1983). The lethal effect of MTS on *L. monocytogenes* cells treated in acid media has, as in neutral media, an additive effect (Pagán, 1997).

According to published data, water activity is the parameter most influential on microbial resistance to different physical agents of inactivation. For example, it has been demonstrated that the reduction of water activity of the treatment medium may increase bacterial resistance to heat by hundreds of times (Kwast and Verrips, 1982; Sumner et al., 1991). There are only two works (Álvarez et al., 2003b, 2006a) dealing in detail with the influence of water activity on the resistance *of S. enteritidis* and *S. senftenberg* to MS/MTS and only one brief reference to its effect on *L. monocytogenes* (Pagán et al., 1999a). A reduction of water activity from 0.99 to 0.96 increases the D_t values of *S. enteritidis* by 30 times, but it barely increases its D_{MS} value twofold (Álvarez et al., 2003b). Similarly, the reduction of water activity from

0.99 to 0.93, which increases the D_t values of *S. senftenberg* by four times, does not increase its resistance to MS (Álvarez et al., 2006a). The addition of 57% (w/v) of sucrose to the treatment medium increases the D_{61} value from 0.22 to 5.7 min and the D_{MS} from 1.5 to 3.1 min. These results indicate that the hypothetical advantages of a pasteurization treatment by MS in regard to heat pasteurization will be higher in foods with low water activity.

Contrary to what happens in media with water activity close to 1, at low water activities the lethal efficacy of MTS is the result of a synergistic effect. Figure 11.6 shows that the influence of treatment temperature on the $D_{MS/MTS}$ values of *S. enteritidis* treated in media of water activity = 0.96. The figure includes, as a reference, a theoretical curve calculated by assuming that the total lethal effect is the addition of the independent effects of ultrasound under pressure and heat. As the figure demonstrates, the D_{MS} values are remarkably lower than expected between 50 and 65°C. For example, at 50°C, the lethal efficacy of manosonication is four times higher than expected. Álvarez et al. (2006a) demonstrated that the lower the water activity of the treatment medium, the higher the synergistic effect. They developed a tertiary mathematical model able to predict the survival of *S. senftenberg* to MTS in media of different water activity.

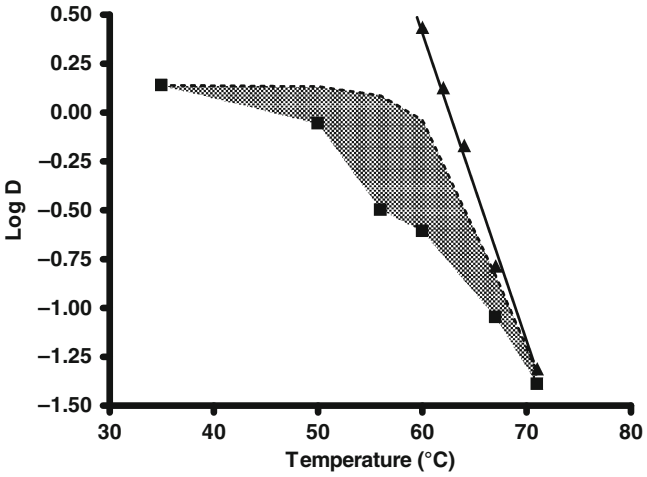

Fig. 11.6 Resistance of *S. enteritidis* to ultrasound (117 μm) under pressure (175 kPa) at different temperatures in medium with $a_w = 0.96$. Decimal reduction time values to ultrasonic waves under pressure (■) and to heat (▲) are shown. The *dotted line* represents the theoretical DRTC to ultrasonic waves under pressure calculated with the equation $D_{MTS} = (D_t \times D_{MS})/(D_t + D_{MS})$

It has been demonstrated that increased heat resistance in media of low water activity is partly due to a stabilization of the cell structures against heat and partly due to a higher capacity of repairing the damages inflicted by heat (Álvarez et al., 2003d). Microbial inactivation by MS at low water activities is, as in high water

activities, an "all or nothing" event, which could partly explain the small influence of this factor on resistance to MS.

It is well known that bacterial thermal tolerance changes with heating media (Doyle and Mazzotta, 2000; Mañas et al., 2001; Murphy et al., 2000; Tomlins and Ordal, 1976). It has been suggested (Hansen and Riemann, 1963; Mañas et al., 2001) that these changes in resistance could be due to pH and/or to water activity differences. However, some authors (Condón and Sala, 1992; Mañas et al., 2001) have demonstrated that microorganisms can show different heat resistance in several media with the same pH. It has also been demonstrated that microorganisms suspended in media of the same water activity, through the addition of different solutes, can show different thermal sensitivities (Baird-Parker et al., 1970; Corry, 1974; Mañas et al., 2001). Therefore, it could be concluded that chemical components, regardless of pH and water activity, could protect bacterial cells against heat treatments. Similar results have been obtained when comparing resistance to high hydrostatic pressure (Hauben et al., 1998; Patterson et al., 1995; Simpson and Gilmour, 1997) and to pulsed electric fields (Grahl and Märkl, 1996; Hülsheger et al., 1981) both in laboratory media and food. Table 11.2 shows that MS resistance barely changes in laboratory media and liquid foods, such as milk or liquid whole egg. At present, all of the mechanisms involved in the increased heat tolerance are not accurately known. Mañas et al. (2001) demonstrated that, at least in milk and liquid whole egg, the entire heat protective effect of foods on microbial resistance was not the result of the addition of the protective effect of each component, but the result of a synergistic effect of the interaction of some of the components. We demonstrated that low-molecular weight milk components protected *S. senftenberg* envelopes against heat by a mechanism involving divalent cations (Mañas et al., 2001). According to the data included in Table 11.2, this increased thermostability of cell envelopes is not accompanied by an increased mechanical resistance to shock waves generated by cavitation.

4.3 Summary

From the results discussed in the above sections it can be deduced that, contrary to what happens with other preservation technologies, environmental factors have very little effect on MS resistance. Figure 11.7 summarizes and exemplifies the effect of each factor investigated on the resistance of *L. monocytogenes* to heat and to MS. As shown in the figure, whereas the D_t values may vary about 100 times depending on the growth conditions and the kind of treatment medium, the D_{MS} values change barely by twofold.

The above observations imply significant practical conclusions. As compared to heat, MS will probably not be a choice technology when intending to sanitize acid foods, such as juices. On the other hand, whereas acidification is applied as a hurdle in a combined process based on a heat treatment, it will be of little interest for those based on the application of ultrasound under pressure. Manothermosonication will be especially useful when cells are subjected to different stresses during industrial

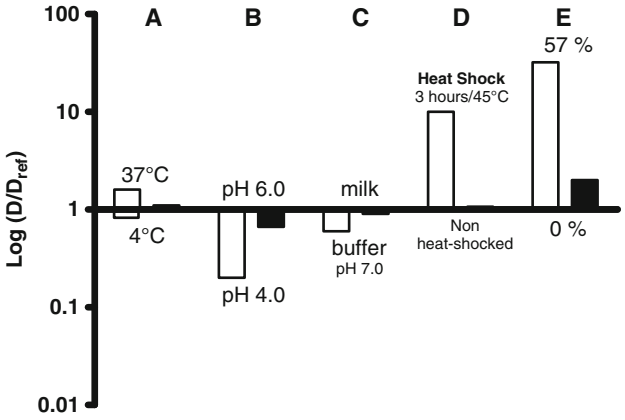

Fig. 11.7 Effect of different environmental factors on heat (*white bars*) and MS (*black bars*) resistance of *L. monocytogenes*. Effect of growth temperature (**a**), pH (**b**), medium composition (**c**), previous heat shock (**d**) and sucrose concentration (**e**). Values represented were calculated by dividing the *D* values obtained for each condition by the D_{ref} values of each technology (D_{ref} values are the D_t and D_{MS} values obtained for cells grown at 20°C and treated skim milk)

processing, allowing them to develop resistance against subsequent technological treatment, as in the case of heat shocks. MS/MTS is also a process especially interesting when intending to pasteurize foods with low water activity, in which the heat resistance of the microorganisms may increase by hundreds of times. In these foods, the bactericidal efficacy of ultrasound under pressure is practically the same as in media of high water activity and, furthermore, they exert a synergistic lethal effect when heat is applied simultaneously.

5 MS/MTS Bacterial Inactivation Mechanisms

The study of the mechanisms of action of ultrasonic waves, as well as any other agents intended for use for microbial inactivation in foods, is essential. An adequate design of new food preservation processes, especially in the case of combined processes, has to be based on the biological mode of action of the various hurdles used.

The lethal effect of low-power ultrasound is small, and most authors agree that the microbial lethal effect produced by high-power ultrasound is related to the cavitation phenomenon (Davies, 1959; Kinsloe et al., 1954; Pagán, 1997; Raso et al., 1998a), more specifically to its mechanical and chemical effects. When bubbles collapse under an ultrasonic field, high temperatures and pressures are generated at the implosion point. Therefore, heat, pressure shock waves or both could be responsible for the lethal effect of ultrasound. On the other hand, these extreme conditions lead to the dissociation of water into hydroxyl radicals and hydrogen atoms

(Suslick, 1990). These reactive radicals could also be involved in the inactivation of microorganisms through oxidative damage (Shin et al., 1994); however, this last effect seems to be negligible compared with the mechanical damage. Current available data may indirectly lead to a sound hypothesis about the mode of action of ultrasound and will be reviewed next.

The role of reactive radicals on the lethal effect of ultrasonic waves under pressure has been studied by the addition of the free radical scavenger cysteamine. Results have shown that the lethality of the treatment on bacterial spores (Raso, 1995) and vegetative cells (Allison et al., 1996; Pagán, 1997; Raso et al., 1998a) was the same when cysteamine was added to the treatment media. Moreover, oxidative agents generally provoke injuries in cell envelopes before inactivation begins. These injuries are commonly detected by the addition of sodium chloride to the recovery media, which constitutes the most frequently used technique to prevent bacterial damage recovery. MS survival curves of various vegetative cells were the same when cells were recovered in media with and without sodium chloride (Mañas, 1999; Pagán, 1997; Pagán et al., 1999a), which indicates that the mechanisms of inactivation by ultrasound and oxidative compounds are different. Therefore, any important contribution of the sonolysis to the inactivating effect of ultrasound on microorganisms has been discarded.

When a bubble generated by ultrasound implodes, heat is generated in the liquid around the cavity. The quantity of heated liquid is very small and heat dissipates quickly, but the temperature of this region remains very high (5,000°C) for a very short time, just a few microseconds (Flint and Suslick, 1991; Suslick, 1988). The occurrence of these hot spots could explain, following the Arrhenius equation, the exponential rate of death by ultrasound through a mechanism involving thermal inactivation. As has been discussed above, decimal reduction time values to MS/MTS treatments fit the theoretical curve calculated by assuming that heat and ultrasonic microbial inactivation are independent processes, which clearly points to different inactivation mechanisms for each agent. Furthermore, whereas bacterial heat resistance varies widely with the strain and the experimental conditions, resistance to MS is close in all vegetative cells studied under various environmental factors. Finally, the percentage of damaged cells of a bacterial population treated by heat increase throughout the treatment time, but damaged cells have not been detected after MS treatments (Pagán, 1997; Raso, 1995). This would also demonstrate different mechanisms of inactivation for heat and ultrasonic treatments. Therefore, all of these factors indicate that, in most occasions, heat would not contribute to the lethal effect of ultrasound.

Most authors have suggested that the mechanical effects of ultrasonic waves are probably the reason for its inactivating effect, in such a way that pressure waves passing through the liquid media would provoke the mechanical disruption of cell envelopes (Davies, 1959; Kinsloe et al., 1954; Lee et al., 2009; Pagán, 1997; Raso et al., 1998a). There are, however, only a few data supporting this idea. Raso et al. (1998a) studied the integrity of cells of *Y. enterocolitica* through a microscopic approach following various heat, MS and MTS treatments with equivalent lethality levels (more than 99% of bacterial inactivation). The

percentage of inactivated cells were calculated by the difference between plate counts before and after each treatment, and the percentage of disrupted cells were estimated through phase contrast microscopy. We observed that no disrupted cells were obtained after heat treatment. On the contrary, after an MS treatment of the same lethality, no undisrupted cells could be observed. After the corresponding MTS treatment, approximately 80% of cells remained undisrupted. Pagán (1997) found similar results with *L. monocytogenes*. These results confirmed that MS inactivates microorganisms through cell envelope breakdown.

In fact, the mechanical disruption of cell envelopes as the key event leading to cell death by ultrasound explains most of the physiological observations described. This hypothesis would also explain that the effect of pressure and amplitude of ultrasonic waves is the same in all of the bacterial species investigated, the additive lethal effect of manothermosonication treatments (inactivating effect of heat added to that of ultrasonic waves under pressure) and the profile of the corresponding DRTC. On the other hand, the absence of damaged vegetative cells indicates that mechanical cell disruption by ultrasound is an "all or nothing" event. This mode of action will complicate the inclusion of ultrasound in combined processes with a synergistic effect, which would only be expected if the additional hurdle added to the ultrasonic treatment modifies the mechanical resistance of the cell envelopes. Finally, it is also noticeable that from data obtained it would be concluded the possible differences in mechanical resilience among envelopes of the various vegetative cells would have a minor effect on bacterial inactivation by ultrasound.

Occasionally, as discussed above, a synergistic effect of ultrasound plus heat has been observed. However, this observation is not in disagreement with the general hypothesis. The synergistic lethal effect of manothermosonication has been reported on bacterial spores (Raso et al., 1994, 1998b), heat-shocked cells of *L. monocytogenes* (Pagán et al., 1999b), particularly heat-resistant vegetative cells (Pagán et al., 1999c), and several strains of *Salmonella* treated in low water activity media (Álvarez, 2000). Bacterial spores have a very complex structure and mechanically resistant envelopes, which isolate the protoplast from the environment. It has been reported that ultrasonic treatments are able to disrupt the spore exosporium (Berger and Marr, 1960). It has also been observed that ultrasound provokes the release of dipicolinic acid and low-molecular weight polypeptides from the cortex of some bacterial spores (Palacios et al., 1991). Furthermore, Raso et al. (1998b) found that manosonication treatment sensitized the spores of *B. subtilis* to lysozyme action. Some strains of spore formers are known to have naturally leaky coats and are therefore lysozyme sensitive, but, in most cases, lysozyme is only capable of hydrolyzing the peptidoglycan of the spore cortex if the overlying coat is first made leaky. These results suggest that the mechanism of action of ultrasonic waves on bacterial spores is also based on the mechanical disruption of the most external envelopes. The external damage would lead to the rehydration of the protoplast, which would result in a loss of heat tolerance (Sala et al., 1995). This would explain the observed synergistic effect of manothermosonication on bacterial spores. Ultrasonic waves under pressure would act, in this case, by sensitizing bacterial spores to heat.

Pagán et al. (1999a) reported the synergistic effect of manothermosonication on heat-shocked cells of *L. monocytogenes*, but not on native non-shocked cells (Pagán et al., 1999b). The authors reported that if a heat treatment was applied prior to an MS treatment, cells were slightly sensitized to ultrasound under pressure. However, this sensitizing effect did not explain the entire lethal effect of MTS. They concluded that, contrary to what was observed with bacterial spores, heat sensitized the cells to ultrasonic waves under pressure. Several authors have reported the occurrence of various changes induced by heat in cell envelopes (Helander et al., 1997; Stevens et al., 1992). Some of these changes modify cell membrane rigidity. It has been observed that heat may cause the release of some divalent cations from the cell envelope and melt cell membrane fatty acids (Helander et al., 1997; Stevens et al., 1992). Therefore, some temperature-induced changes occurring in the cell envelopes could weaken them against mechanical stress. The disruption of envelopes seems to be the mechanism of action of ultrasonic waves, even when a synergistic effect is observed. The synergistic effect reported in the highly heat-resistant *Streptococcus faecium* (Pagán et al., 1999c) and in *Salmonella* serotypes treated at low water activity (Álvarez, 2000) could be explained in a similar way. When heat-sensitive bacteria are treated by manothermosonication, the lethal effect of heat begins at low temperatures and the weakening effect of heat on the mechanical resistance of cell envelopes cannot be observed. As a consequence, an additive effect is expected. On the contrary, when heat-resistant bacteria or cells whose thermal resistance is increased by the particular characteristics of the treatment medium are treated by MTS, the lethal effect of heat only appears at higher temperatures. These higher temperatures may exert changes in cell envelopes and a synergistic effect should be expected. Specific investigations have not been carried out in this matter and new data would be very useful.

6 Control of MS/MTS Industrial Processes

Since it is impossible to ensure a "zero" risk when implementing an industrial process, it is convenient (if not necessary) to establish a kinetic model of inactivation. This model will allow selection of treatment conditions to achieve a definite objective, i.e. performance criteria. It is also important to determine those physical parameters on which the final efficacy depends, as well as to establish the relevant equations allowing to a forecast of the consequences from an error in the treatment conditions, in regard to the stability and safety of the manufactured product. Concerning the above questions, the MS/MTS process shows some advantages when compared to other technologies for the preservation and hygienization of foods.

For example, kinetics of inactivation by high hydrostatic pressure and pulsed electric fields does not follow an exponential pattern, which makes calculation and adjustment of industrial treatments very difficult. The survival curves obtained with these technologies often show tailing phenomena, whose importance, i.e. level of

surviving population, also varies with the treatment intensity. Therefore, it is difficult to even find primary models able to predict the lethal efficacy of a treatment at constant intensity of different durations. The current trend is to use models based on a Weibull-like distribution on the basis of their robustness and simplicity (Álvarez et al., 2003a, d; Chen and Hoover, 2003; Fernández et al., 2002; Heinz and Knorr, 1996; Mafart et al., 2002). However, at present, consensus has not been reached in regard to the convenience of their use, and there have been only two or three sporadic trials to develop secondary and tertiary models (Álvarez et al., 2007; Chen and Hoover, 2004; Gómez et al., 2005a, b), which are in fact the models that can really interest the industrial sector. As mentioned above, inactivation kinetics by MS/MTS fits an exponential primary model, very simple and robust, which has been widely used by the canning industry to adjust the intensity of heat treatment. Furthermore, as indicated in Section 2.1, it is possible to easily develop secondary models that are also based on first-order kinetics. In summary, simple, robust tertiary models, similar to those developed for the adjustment of heat treatments and of direct application to the industrial sector, can be developed.

On the other hand, since the efficacy of manosonication depends both on amplitude and pressure, it could be expected that the simultaneous control of both variables within an industrial process would be difficult, as well as quantification of the effect of any possible processing mistake. However, as has been described in previous sections, the effects of amplitude and pressure are independent of each other. Furthermore, the biological effects of each of them are directly related to the energy transmitted to the medium by ultrasound under any experimental conditions. Therefore, the energy transferred to the treatment medium is an appropriate parameter to measure in order to control the process. Mañas et al. (2000a) developed a very simple linear equation that allowed calculation of the lethal efficacy of MS treatments from the measurement of energy transferred to the medium. Following the same procedure, it would be very easy to develop some specific equations for particular industrial equipment, which would allow us to quantify the efficacy of the process by measuring just one parameter: the energy transferred. This simplification, for instance, could not be used to control pulsed electric fields processes, as the application of a given quantity of energy has different biological effects depending on the strength of the electric field applied (Álvarez et al., 2003c).

An additional aspect of relevance for the industry is the possibility of forecasting the consequences from an eventual processing error. In this regard, MS also shows some particular advantages. For example, in a heat treatment, the factor to be controlled is temperature, and there is the theoretical possibility of predicting the consequences from an error in treatment temperature through the use of z values. However, these calculations are very risky, as each microbial species has a different z value, and the raw materials are usually contaminated with heterogeneous bacterial flora. On the contrary, as we have discussed before, the effects of pressure and amplitude of the ultrasonic waves are the same for all species investigated. Therefore, the consequences of any eventual processing error could be easily estimated in a reliable manner.

Lastly, besides the actual practical utility of this technology for food processing, the MS/MTS processes could already be transferred to the industrial sector with the mathematical tools necessary for optimization of treatments and control of the process.

7 Concluding Remarks

As a consequence of consumer's increasing requirements, the food industry has been challenged to improve the safety, stability and convenience of foods and a great effort has been made to develop more appropriate methods of preservation/hygienization. At present, it can be foreseen that none of the new technologies under study will replace traditional preserving procedures for a wide range of products. However, each one shows some particular advantages that may make it the choice technology for given products or processes.

The major advantage of MS/MTS in regard to other new preservation technologies is their capacity to inactivate bacterial spores at sublethal temperatures, although the treatment times for the sterilization of foods with this technology would probably be long. Concerning the inactivation of vegetative cells, the major advantages are based on the exponential course of inactivation and on the fact that microbial resistance barely changes with species and the environmental factors. It seems that there are no mechanisms of development of cross resistance and occurrence of cell damage/recovery either, which represents an additional safety guarantee.

As far as the development of combined processes is concerned, current knowledge indicates that, with the exception of the combination with heat, the application of traditional hurdles will barely allow development of any processes of synergistic lethal effects, unless the hurdle is selected to lower the mechanical resistance of cell envelopes. In this regard, it is worth highlighting that bacterial spores represent an exception, since MS treatment sensitizes these spores against lysozyme. This is a most interesting aspect that should be further investigated.

The combination of ultrasound under pressure and heat (manothermosonication) could be especially advantageous. Although an additive effect has been generally observed, on some occasions when heat treatments are less effective, a synergistic effect can be achieved. Moreover, the application of manothermosonication would be highly energetically profitable, since almost all of the ultrasonic energy applied to the medium is finally converted into heat, which could be reused in the process. An additional advantage is that various different individual operations, such as mixture, emulsification, degasification, could be carried out in just one step.

The major limitation of manothermosonication could be its effects on the physicochemical and nutritional properties of foods. Unfortunately, there are not enough data available in this regard, and general conclusions cannot be drawn. Appropriate specific equipment to apply this technology to foods at a pilot-plant

level has not been developed either. Since knowledge about the biological effects of manothermosonication is enough and food characteristics may vary widely, probably the next step is to apply this technology to some particular product whose preservation/pasteurization is especially difficult at present. In this way, an overall assessment of its effects on the quality, in its different aspects, will be possible.

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