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Electron microscopic analysis of dairy microbes inactivated by ultrasound

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ABSTRACT

Ultrasonication is a non-thermal method of food preservation that has the advantage of inactivating microbes in food without causing the common side-effects associated with conventional heat treatments, such as nutrient and flavour loss. The aim of this study was to evaluate the use of ultrasound as an alternative to heat pasteurisation and to assess cell damage using transmission electron microscopy (TEM). Three spoilage microbes, previously isolated from pasteurised milk, were used as "test" microbes. Saline solution (SSS) and UHT milk were used as suspension media and were inoculated with exponential growth phase "test" microbes at a microbial concentration of 1×10^4 cfu ml^{-1}. The samples were subjected to power ultrasound (20 kHz, 750 W), at 100%/124 μm wave amplitude for different time intervals. Both *Escherichia coli* and *Saccharomyces cerevisiae* were reduced by >99% (for both suspension media) after ultrasonication and *Lactobacillus acidophilus* was reduced by 72% and 84% in SSS and milk, respectively.

Transmission electron microscope micrographs showed that ultrasonication inflicts extensive microbicidal/microbistatic external and internal damage on all three "test" microbes. In *E. coli*, sonication-induced emulsification caused the formation of unique minute lipopolysaccharide vesicles from the fragmenting cell envelope.

1. Introduction

Milk is generally given some form of a heat treatment to control microbial growth. Heat processing is not effective against all microbes [1–3] associated with milk and may trigger unwanted reactions such as loss of flavour, nutrients and vitamins [4,5]. To avoid the unwanted effects of heat, efforts are being made to find alternate methods of food preservation, based on new inactivation procedures [6]. Ultrasonication is a non-thermal method of food preservation that has the advantage of inactivating microorganisms in food without causing the common side-effects associated with conventional heat treatments.

The use of ultrasound to inactivate microbes was reported in the late 1920's [7], but its limited lethal effect on spoilage microbes prohibited it from being used as a sterilisation method. Improvements in ultrasound generation technology over the last decade have again stimulated interest in microbial inactivation by ultrasound [8].

Ultrasonic waves are generated by mechanical vibrations of frequencies between 20 kHz and 800 kHz [9]. When these waves propagate into liquid media, alternating compressions and rarefactions are produced. If the amplitude of the ultrasonic wave is high enough, cavitation, which is the making and breaking of microscopic bubbles, will occur [10]. When the bubbles reach a critical size, they collapse violently. This violent collapse is thought to generate mechanical forces resulting in the breaking and shearing of cell walls leading to cell death.

There are numerous ways of evaluating the lethality of thermal or non-thermal treatments on microbial populations present in food products. A suitable microbial plating medium is generally used for the enumeration of surviving microbes to ensure that correct counts of microbes are recorded [11]. Although enumeration of microbes before and after a treatment (e.g. ultrasonication) provides information on the effectiveness of the treatment on cell viability, no information is available on the type or extent of morphological or physical damage to the microbial cells. Visual information would provide insight into ultrasound-induced damage to the cell. Furthermore, visual information can also assist in characterising the type and magnitude of changes that occur to cell composition in response to the treatment, and it enhances the understanding of how and why a given treatment is effective against a particular microbe [12].

The aim of this study was to determine the effectiveness of power ultrasound (20 kHz, 124 μm) on the survival of *Escherichia coli*, *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* in sterile saline solution (SSS) and ultra high temperature (UHT) milk, and to

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gain visual information (TEM) on the extent of structural damage inflicted ultrasonically on the selected microbes.

2. Materials and methods

2.1. Bacterial cultures

The three "test" microbes used and their growth requirements, are listed in Table 1. Sterile growth medium (10 ml) was inoculated with a pure culture of the selected "test" microbe and incubated at the appropriate temperature (Table 1) for 24 h. Five millilitre of each 24 h culture was then used as inoculum (150 ml sterile growth medium) and incubated for a further 24 h prior to ultrasonication. Standard growth curves of each "test" microbe were used to determine the inoculum size of the samples to be ultrasonicated.

2.2. Ultrasonication

Two millilitre of the appropriate "test" microbe was centrifuged for 10 min at 6000*g* (Eppendorf 5415D centrifuge, Eppendorf, Hamburg, Germany). The pellet was suspended in SSS and the data from the standard curve were used to determine the desired cell concentration for inoculation of the suspension media. The suspension medium, either full cream (3.4% milk fat) UHT milk or SSS was inoculated with an aliquot of culture to yield an approximate inoculum level of 1×10^4 colony forming units per ml (cfu ml^{-1}) (initial concentration = N_0).

For ultrasonication, a 40 ml sample of the inoculated suspension media was measured into a sterile, jacketed glass sample holder connected to an ice-waterbath to maintain a temperature of between 24° and 26 °C. A 750 W, 20 kHz Vibra-Cell High Intensity Ultrasonic Processor VCX 750 (Sonics & Materials, Inc., Newtown, CT USA), fitted with an autoclavable 13 mm diameter probe fitted with a replaceable titanium tip was used for ultrasonication. With this unit, feedback from the probe was continuously evaluated, and the frequency and power were automatically adjusted to ensure optimum ultrasonic delivery. The Vibra-Cell is also able to monitor the energy (in Joules) and the temperature of the sample being processed. Samples were treated using five different time regimes: 2.5, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude.

Duplicate ultrasonic treatments were done and duplicate dilutions were made from each treated sample. The pour-plate technique and appropriate media (Table 1) were used for enumeration. Plates with between 30 and 300 colonies were selected for counting. UHT milk and SSS samples that had not been inoculated with any "test" microbes served as controls. In all cases, no microbial growth was observed after 48 h of incubation on the controls.

The efficacy of ultrasonic treatments in terms of eliminating microbes was measured by their decimal reduction time (*D*) which, for this study, was defined as the time (min) of a given treatment for the number of survivors to be reduced by one log cycle. *D*-values were calculated from the slope of the regression line plotted with the counts (cfu ml^{-1}) of the straight portion of the survival curve. In this study, the *D*-value at 20 kHz/750 W was abbreviated as D_{US}.

2.3. TEM sample preparation

The growth medium (150 ml) containing cells in the exponential phase were centrifuged at 5000*g* (Beckman Coulter TJ-25 Centrifuge, Beckman Coulter Inc., USA) for 10 min. The supernatant was discarded and the pellet washed twice by resuspension in SSS (0.85% m/v) followed by centrifugation. The final pellets were resuspended in 150 ml SSS. Samples were treated ultrasonically as described above.

2.4. Transmission electron microscopy

Eight millilitre of each of the ultrasonicated samples were centrifuged (5000*g*; 10 min) (Force 7 Microcentrifuge, Denver Instrument Company, USA) and the cells fixed by suspending the pellet in 2.5% glutaraldehyde (BDH) (in 0.1 M K_2HPO_4, KH_2PO_4 buffer; pH 7.0) (BDH) and stored at 4 °C. Cells that received no ultrasonic treatment served as the control. After aldehyde fixation, the samples were washed twice with phosphate buffered saline (PBS) (pH 7.4). Cells were post-fixed with 1% osmium tetroxide for 30 min. Cells were again washed with PBS and the pellet suspended in 10 μl distilled water. The cells were allowed to set in 20 μl 2% low melting agarose gel at 4 °C. The gel was cut into blocks and dehydrated for 10 min in ethanol in a series of solutions: 30%, 50%, 60%, 70%, 80%, 90%, 95% and 100%. The cells were then allowed to dehydrate in 100% acetone for a further 10 min. The dehydrated cells were infiltrated with increasing concentrations of agar low viscosity resin (Agar Scientific), a replacement for Spurr's resin, over 3 days [13]. The polymerisation of the resin to form specimen blocks was accomplished in an oven at 60 °C for 24 h. The specimen blocks were hand trimmed with a razor blade and sectioned with a glass knife using a Reichert Ultracut S Ultramicrotome (Leica). Microtome sections of 120 nm were placed on 200 mesh copper grids. The sections were stained with 2% uranyl acetate and lead citrate [14], and viewed with a Leo 912 transmission electron microscope operating at 120 kV.

Table 1
Growth media, incubation times and temperatures used for cultivation of the different "test" microbes

Microbe	USFSCC[a]	Medium	Incubation		Gram	Morphology
			Time (h)	Temperature (°C)		
Escherichia coli	11775	NB[b]/PCA[c]	24	37°	–	Rod
Lactobacillus acidophilus	1348	MRS[d]	24	35°	+	Rod (chains)
Saccharomyces cerevisiae	462	YDP[e]/MEA[f]	24	25°	NA	Yeast

NA = not applicable.
[a] USFSCC = University of Stellenbosch, Food Science Culture Collection.
[b] NB = Nutrient broth (Biolab).
[c] PCA = Plate count agar (Biolab).
[d] MRS = de Man, Rogosa & Sharpe broth (Biolab).
[e] YDP = Yeast dextrose peptone broth (Biolab).
[f] MEA = Malt extract agar (Biolab).

3. Results and discussion

The three "test" microbes used in this study were chosen for different reasons. *E. coli* was included as it is considered to be an indicator of faecal contamination by the dairy industry [15]. Furthermore, this small Gram-negative rod grows rapidly and well on simple, non-specific growth media and is easy to detect. *L. acidophilus* was included as it is a Gram-positive rod which produces lactic acid [16], and may therefore, be considered a potentially spoilage microbe of fresh milk. *S. cerevisiae* was included as it is a eukaryote, and has been isolated from fresh milk.

3.1. Survival determinations

Elimination of all viable *E. coli* cells were achieved after 10.0 min of ultrasonication for both batch inoculum concentrations (1×10^4 cfu ml^{-1} or 1×10^5 cfu ml^{-1} in both SSS and milk). The

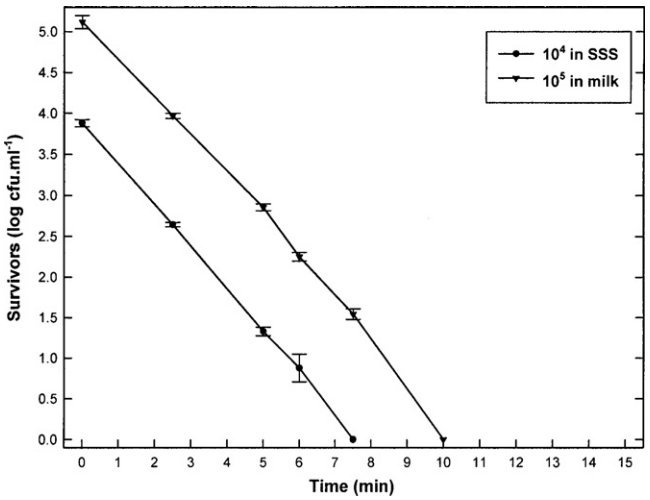

Fig. 1. Regression (95% confidence level) of the data showing the impact of ultrasonication at 20 kHz on *Escherichia coli* in either SSS or UHT milk (Each data point represents quadruple values. The standard deviation was used as the error-bar).

1×10^4 cfu ml^{-1} inoculum in SSS was reduced by $\log_{10}$ 3.88 after the 10.0 min treatment (Fig. 1). Similar results were obtained for the other initial concentration (N_0) where 1×10^5 cfu ml^{-1} in milk was reduced by $\log_{10}$ 4.42 reduction. The D_{US}-values for *E. coli* were 2.0 min in both SSS and milk (Table 2).

Salleh–Mack and Roberts [17] reported a $\log_{10}$ 5.54 reduction for *E. coli* in distilled water after ultrasonication for 10 min at a non-lethal temperature below 30 °C. The higher inoculum used, higher frequency (24 kHz), larger probe (22 mm diameter), larger sample volume and different suspension medium accounts for the different logarithmic reduction reported in their study [17] when compared with the results obtained from this investigation. D'Amico et al. [18] reported a $\log_{10}$ 4.63 reduction in viable *E. coli* cells after 6 min ultrasonication at 20 °C. However, they [18] used an acidic suspension medium, apple cider, and used a sample volume of only 15 ml. These differences may account for the different reduction values reported [18] when compared with the values reported here.

It has also been reported [19] that *E. coli* was reduced by 99.17% in saline after 10 min when treated with 700 kHz, but surprisingly, the researchers reported no inactivation of *E. coli* in milk. The type of milk, which could influence the efficacy of ultrasonication, was not mentioned by the researchers [19]. The results obtained in this study compares well with the findings of Utsunomiya et al. [19] in terms of the reduction (cfu ml^{-1}) achieved after a 10 min treatment in saline. However, Utsunomiya et al. [19] did not achieve an *E. coli* reduction of >99%, as was achieved in this study (Table 2). Important factors not referred to by Utsunomiya et al. [19] and which could influence this result would be wave amplitude obtained at 700 kHz and the inoculum concentration. Contrary to the findings of Utsunomiya et al. [19], the suspension media used in this study (SSS and milk) had no effect on the efficiency of ultrasonication to eliminate *E. coli*. It has also been suggested [20] that the fat globules present in milk might have a protective effect against microbes undergoing ultrasonication, rendering the process inefficient. This was not observed when using the parameters applied during this study.

Table 2
Summary of the D_{US}-values, and $\log_{10}$ reductions obtained for the different "test" microbes evaluated over a 10 min treatment time

Microbe	Treatment time (min)	Inoculum (cfu ml^{-1})	SSS		Milk	
			D_{US} (min)	$\log_{10}$ red	D_{US} (min)	$\log_{10}$ red
Escherichia coli	10	1×10^4	2.0	3.88	2.0	4.42
Lactobacillus acidophilus	10	1×10^4	nc	0.55	nc	0.82
Saccharomyces cerevisiae	10	1×10^4	2.8	3.62	4.3	2.10

nc = not calculated (D_{US}-value could not be calculated as a single log reduction was not reached).

After ultrasonicating *L. acidophilus* for 10.0 min, a $\log_{10}$ 0.55 reduction of the initial 1×10^4 cfu ml^{-1} in SSS was achieved. In milk, $\log_{10}$ 0.82 of the initial 1×10^4 cfu ml^{-1} was eliminated. These results precluded the calculation of D_{US}-values for *L. acidophilus* (Table 2). Although similar results were achieved for *L. acidophilus* treated in SSS and milk, as was the case for *E. coli*, *L. acidophilus* was found to be more resistant to the destructive effect of ultrasound. It is thus clear that every microbe has a unique response to ultrasonication.

Ultrasonication of the *S. cerevisiae* strain for 10 min in SSS resulted in a $\log_{10}$ 3.62 reduction of the initial 1×10^4 cfu ml^{-1} inoculum, and in milk a $\log_{10}$ 2.10 reduction of the initial 1×10^4 cfu ml^{-1} was achieved. The D_{US} in SSS was calculated to be 2.8 min, and in milk 4.3 min (for this specific microbe the D_{US} was taken as the average of two values) (Table 2). It is evident that the nature of the suspension medium had an influence on the D_{US}-values obtained when *S. cerevisiae* was treated.

Elsewhere, *D*-values in water for *S. cerevisiae* of 1.6 min (100 W) and 2.4 min (180 W) (initial concentration of approximately 1×10^8 cfu ml^{-1} in sterile water) have been reported [21]. One difference between this study and their study [21] was that, although they used a frequency of 20 kHz, they combined ultrasound with a heat treatment (55 °C) and this would account for differences in *D*-values obtained. In addition, the inoculum was composed of rehydrated freeze-dried *S. cerevisiae* which would influence the *D*-values. Other researchers [22,23] also used ultrasound in a hurdle system and applied three different temperature combinations (35°, 45° and 55 °C). Reported *D*-values were 21.4 min (35 °C), 14.1 min (45 °C) and 1.3 min (55 °C) at 90% wave amplitude. The differences in process parameters used by the different authors make valid comparisons of the results obtained difficult.

It has been reported that smaller sized bacteria are more resistant to the effect of ultrasound [20,24], and that rods are more easily eliminated than cocci. Gram-positive bacteria have also been reported to be more resistant [24–26]. Although the specific strain of *S. cerevisiae* (5–10 μm in diameter) used in this study was found to be more sensitive than *L. acidophilus* (0.6 μm × 2.0–5.0 μm), the smaller Gram-negative rod, *E. coli* (0.5 μm × 1.5 μm), was most susceptible to ultrasound-induced death under the conditions applied in this study. Others have reported that there is no difference in ultrasound resistance between Gram-positive and negative bacteria [27]. Similarly, Cameron [28] working under standardised experimental conditions (e.g. growth phase, suspension volume, wave amplitude, etc.), reported no correlation between the Gram-status, shape or size of microbes and resistance to ultrasonication at non-lethal temperatures. The microbes tested included: *E. coli*; *L. acidophilus*; *Lactococcus lactis* subsp. *lactis*; *Bacillus cereus*; *Listeria monocytogenes*; *Pseudomonas fluorescens*; *Elizabethkingia meningoseptica* and; *S. cerevisiae*. Results from this extensive study showed that there was no direct correlation between cell size or Gram characteristic of the three microbes tested, and response to ultrasound. Although Gram-positive bacteria have a thicker and a more tightly adherent peptidoglycan layer than Gram-negative bacteria, it was suggested that it is not the cell wall thickness that protects against ultrasound [26]. Instead, it was proposed that the "target" of ultrasonic damage might rather be the cytoplasmic membrane, which normally consists of a lipoprotein bilayer.

It was reported [21] that the cavitation field in a microbial suspension generated by ultrasonic waves does not deactivate, break up or kill the cells when using a concentrated freeze-dried inoculum (2×10^{10} cfu ml^{-1}), but rather that cavitation damages the cell wall and possibly the cytoplasmic membrane, thus affecting the cellular permeability. The cytosol and intracellular contents would then leak out of the damaged cytoplasmic membrane and cell wall and they thus ascribed this "leakage" as the cause of bacterial inactivation and ultimately elimination. The violent collapse of cavitating bubbles induces the formation of shock waves and reactive free radicals. However, it seems likely that it is the shock waves, rather than the radicals that lead to microbial cell death [29,30]. To verify results reported by [27] and [21], ultrasonically induced microbial cell damage was visually examined with TEM.

3.2. Transmission electron microscopy

Transmission electron microscopy is generally used to investigate the internal structure of microbial cells. Thin (<120 nm) sections were made from cells embedded in resin to observe any possible microstructural changes resulting from ultrasonication. Untreated and treated *E. coli*, *L. acidophilus* and *S. cerevisiae* cells were examined using TEM. Both external and internal cell damage caused by ultrasonication was visible in the micrographs.

After 2 min ultrasonication *E. coli* cells were reduced by 1 log (Table 2). Transmission electron micrographs of *E. coli* after this treatment showed that when compared to untreated controls (Fig. 2a), most of the cells had been extensively damaged, both internally and externally (Fig. 2b and c). This damage was not uniform. Intact, as well as damaged cells were seen (Fig. 2d and b). Untreated controls had intact smooth cell boundaries which surrounded regions of cytoplasm packed with ribosomes, as well as the less dense regions of the prokaryotic nucleoid. After ultrasonication, many fragments of cells remained which were devoid of all cell content (Fig. 2b). Ultrasound thus caused lethal damage to cell boundaries. A striking feature of *E. coli* cells after ultrasonication was the presence of minute vesicles ($\leqslant$20 nm) (Fig. 2b). As vesicles do not occur in prokaryotic cells, and were not seen in the untreated cells, these vesicles must have formed as a result of ultrasonication. The vesicles were stable, remaining intact throughout the preparation for TEM. Vesicles were seen within and external to the cells (Fig. 2b and c). It is suggested that these vesicles are bounded by lipopolysaccharide membranes and are formed from the outer and inner cell membranes of the Gram-negative *E. coli*. At times, the dissociation of the three layers of the Gram-negative cell boundary and associated formation of the vesicles was visualised (Fig. 2c). The folding inward of the Gram-negative envelope to form vesicles was also evident (Fig. 2b). The

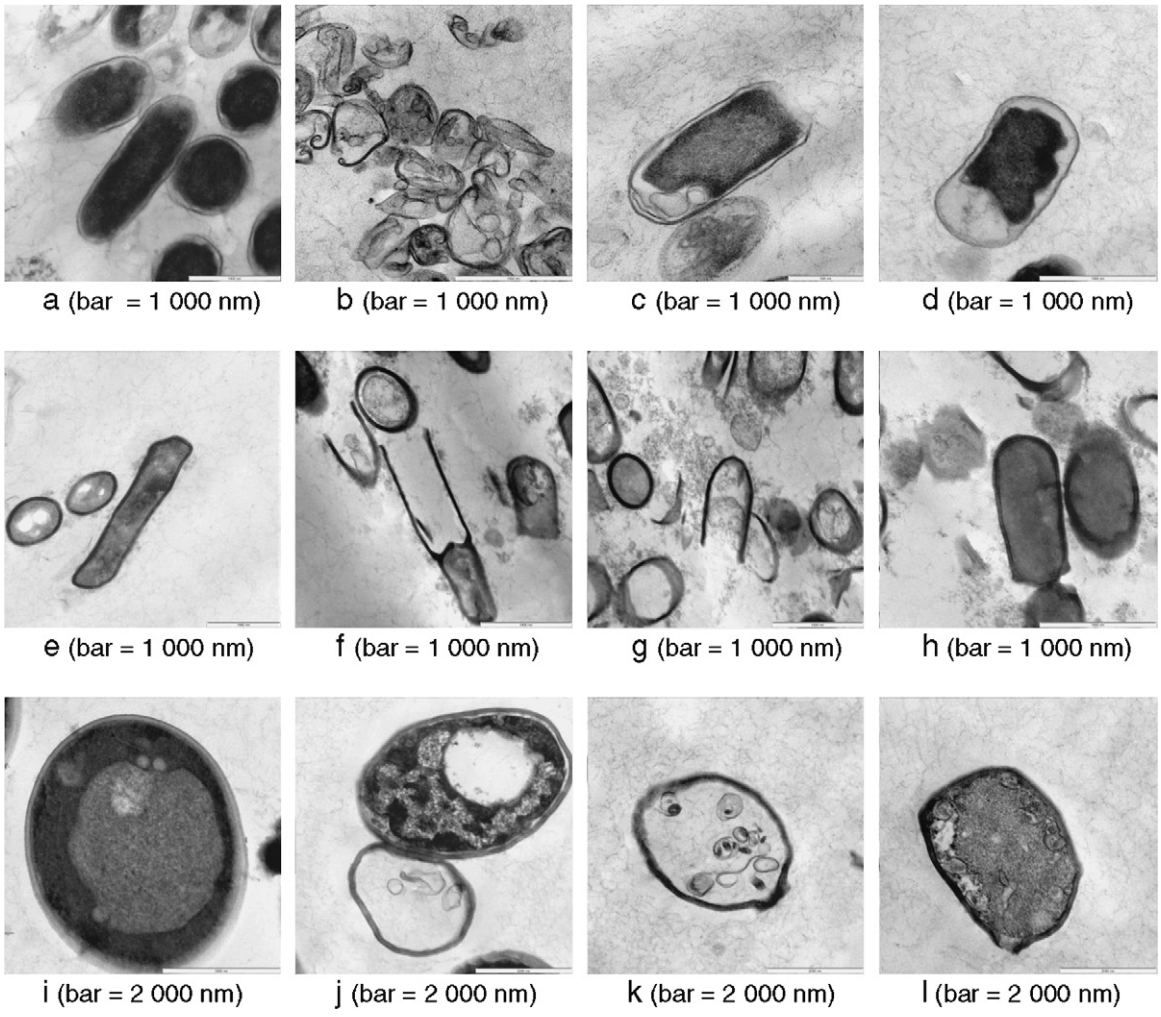

Fig. 2. Transmission electron micrographs of *E. coli* [untreated (a) and ultrasonicated (b–d)], *L. acidophilus* [untreated (e) and ultrasonicated (f–h)] and *S. cerevisiae* [untreated (i) and ultrasonicated (j–l)] cells.

formation of the vesicles was probably driven by an emulsification process of membrane lipids and air bubbles associated with cavitation. As far as we are aware, this is the first reporting of such liposome-like vesicles.

Viable untreated *L. acidophilus* cells with the distinct Gram-positive cell wall are shown in Fig. 2e, and ultrasonicated cells are shown in Fig. 2f, g and h. An ultrasonic treatment of 5 min proved to be damaging to viable *L. acidophilus* cells. As was the case for *E. coli*, this damage was variable. Intact cells were seen (Fig. 2h), as well as cells devoid of content, where the cell terminus had been "sheared off" due to cavitation (Fig. 2f and g). At times, a single filament of cells showed uneven/dissimilar damage (Fig. 2f). Vesicles, as reported for *E. coli* were present in the *Lactobacillus* cells but in far lower numbers. However, the number of vesicles was less than those observed for *E. coli*. This is probably due to the fact that the Gram-positive cell is bounded by a thick peptidoglycan layer. There is no outer lipopolysaccharide membrane. The sole membrane is that internal to the wall and enclosing the cell contents. It is possible that the cell membrane was the source of the vesicles associated with *Lactobacillus*.

S. cerevisiae was subjected to 2 min ultrasonication after which, cell damage was clearly visible. Untreated oval-shaped cells showed an intact boundary, and the protoplasm contained the nucleus and organelles typical of a eukaryotic cell (Fig. 2i). After ultrasonication, cells showed variable signs of damage. Externally, cell walls appeared uneven, and many cells were devoid of content (Fig. 2j). Other cells retained organelles, some of which appeared damaged (Fig. 2k). This observation was probably a result of intracellular cavitation, first suggested by Hughes and Nyborg in 1962 [31]. In 1975, using light microscopy, Alliger [24] showed that disruption of subcellular particles in *S. cerevisiae* occurred rapidly and prior to disruption of cell walls when undergoing ultrasonication. More recently, others using TEM [22] have also reported on early internal damage to cell organelles in *S. cerevisiae*, whilst the outer cell wall remained intact. In this study, cells with punctured walls were also visualised, and this was ascribed to external mechanical forces associated with cavitation (Fig. 2l). No vesicles, as reported for the prokaryotes, were seen.

Although the damage to cells was variable, TEM studies indicate that *E. coli*, *L. acidophilus* and *S. cerevisiae* cells were severely damaged by ultrasound both internally and externally. It is thus evident that ultrasonication kills microbial cells by damaging the cell boundaries and the microstructures of the cells. Cell fragmentation was clearly evident, but based on the extent of internal cell damage induced by ultrasound, it is possible that fragmentation need not be the sole cause of cell death in cells subjected to cavitation [21].

4. Conclusions

Our studies have shown that when subjected to ultrasound in a milk milieu, the three microbes tested showed different inactivation rates. Furthermore, in the case of *S. cerevisiae*, the liquid in which the "test" microbes were suspended also influenced death rates.

The visual information obtained in this study showed that ultrasound structurally damages microbial cells. By using TEM, the destructive effect of ultrasound on both the cell wall and within the cell was found to be extensive. Thus the TEM data provide insight on the type and magnitude of damage due to the treatment. The data collected from the TEM micrographs provide evidence of the destructive effect of cavitation on microbial cells. The cavitational forces, induced by ultrasonication, caused irreparable damage to the outer cell wall and inner cell membrane of the tested microbes, however, in the case of the yeast, *S. cerevisiae*, internal damage to the cell organelles was also observed. It is possible that damage to only internal cell organelles may not be lethal in all cases, i.e., microbistatic.

Based on the preliminary results obtained in this study, is can be concluded that ultrasound holds promise as an alternative to traditional thermal pasteurisation. The use of ultrasound would be more environmentally friendly than heat treatments. Pilot scale studies are, however, required before any meaningful suggestions can be made to the dairy industry. The influence of sub-lethal cell injury also needs to be assessed in future studies.

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