IMPACT OF LOW-FREQUENCY HIGH-POWER ULTRASOUND ON SPOILAGE AND POTENTIALLY PATHOGENIC DAIRY MICROBES

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any other university for a degree.

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ABSTRACT

Thermal pasteurisation failures in the dairy industry have often been found to cause end-products of poor quality and short shelf-life. Therefore, alternative methods to eliminate microbial contaminants in raw milk are being studied. Ultrasonication is one such non-thermal technology that could offer the dairy industry an alternative to traditional pasteurisation.

The main objective of this dissertation was to evaluate the use of high-power low-frequency ultrasound (20 kHz, 750 W, 124 µm) applied in batch mode to eliminate a selection of spoilage and potentially pathogenic microbes, commonly associated with milk. These included Gram-positive and negative microbes, comprising of rods and cocci, an endospore-former, and a yeast (*Escherichia coli*, *Bacillus cereus*, *Chryseobacterium meningosepticum*, *Lactobacillus acidophilus*, *Lactococcus lactis*, *Listeria monocytogenes*, *Micrococcus luteus*, *Pseudomonas fluorescens* and *Saccharomyces cerevisiae*).

Three strains of *E. coli* (1×10^6 cfu.ml^{-1}) tested, viz. ATCC 11775, a wild strain from raw milk, and an O157:H7 strain from milk were sensitive to ultrasonication. Complete elimination of viable cells occurred within 10 min. Viable counts of *P. fluorescens* were reduced by 100% within 6 min of ultrasonication and *L. monocytogenes* was reduced by 99.0% within 10 min. *Lactococcus lactis* was reduced by 97.0% and *M. luteus*, *B. cereus* and *C. meningosepticum* by 88.0%, 87.0% and 85.0% respectively. *Lactobacillus acidophilus* showed the most resistance to ultrasound with only 78.0% of viable cells being eliminated. Under similar conditions, *S. cerevisiae* was reduced by 99.7%. Microbial cell morphology, size and Gram status did not necessarily influence the efficacy of ultrasonication. Sterile saline solution and UHT milk were used as the suspension media, and the reputed protective effect of milk fat was not observed under the parameters used in this study. A higher wave amplitude (100%; 124 µm) was found to be more efficient in eliminating microbes than a lower wave amplitude (50%; 62 µm). Pulsed-ultrasonication did not enhance the efficiency of ultrasonication indicating that standing waves were absent.

Limited success was achieved by ultrasonication itself, and the long batch treatment time (10 min or more) was found to be unrealistic for industrial implementation. Hence the simultaneous application of ultrasound and heat (thermo-ultrasonication) was examined.

Thermo-ultrasonication proved to be more effective than either an ultrasonic or heat treatment with all viable *M. luteus* cells being eliminated within 4 min (100% amplitude at 72℃). Similarly, to eliminate *E. coli* and *Lb. acidophilus* from milk, only 2 min and 4 min thermo-ultrasonication was required, respectively. *Bacillus cereus* endospores remained resistant and after a 10 min thermo-ultrasonic treatment only 78.04% were eliminated.

During this investigation both extensive surface (SEM) and internal (TEM) cell damage caused by ultrasonication were observed in *E. coli*, *Lb. acidophilus* and *S. cerevisiae*. Hence ultrasonication physically/mechanically damages these microbial cells causing cell death/injury.

Microbial proteins and DNA released from cells into the environment after an ultrasonic treatment was measured and an increase in released microbial proteins and DNA was found to be indicative of a decrease in the number of viable cells, providing that the initial cell concentration was high enough. It was, however, not possible to correlate the concentration of released microbial proteins and DNA with the exact number of viable cells eliminated, rendering it an ineffective quality indicator for the industry.

Ultrasonication had no statistically significant influence on the protein, fat and lactose content of both raw and pasteurised milk. The somatic cell count of raw and pasteurised milk was found to decrease after ultrasonication. Unlike with heating, activity of alkaline phosphatase and lactoperoxidase were not reduced by ultrasonication. Hence neither enzyme can be used to indicate a successful ultrasonic treatment of milk.

This study has demonstrated that ultrasonication offers a viable alternative to pasteurisation as it is effective in eliminating microbes, and does not alter native milk components. However, to attain a more effective killing, thermo-ultrasonication is recommended for the treatment of milk to be used for the production of different dairy products.

UITTREKSEL

Oneffektiewe hitte pasteurisasie in die suiwelindustrie word gereeld verbind met eindprodukte met 'n swak kwaliteit en kort rakleeflyd. Alternatiewe metodes om mikrobiese kontaminante te elimineer word dus ondersoek. Ultrasonikasie is so 'n nie-hitte tegnologie wat vir die suiwelindustrie 'n alternatief vir tradisionele pasteurisasie kan bied.

Die hoof doelwit van hierdie dissertasie was die evaluasie van die vermoë van hoë-krag lae-frekwensie ultraklank (20 kHz, 750 W, 124 µm) om a seleksie van bederf en potensiële patogeniese mikrobes, algemeen met melk geassosieer elimineer. Dit sluit Gram-positiewe en -negatiewe mikrobes, bestaande uit stafies en kokki, 'n endospoor vormer, en 'n gis (*Escherichia coli*, *Bacillus cereus*, *Chryseobacterium meningosepticum*, *Lactobacillus acidophilus*, *Lactococcus lactis*, *Listeria monocytogenes*, *Micrococcus luteus*, *Pseudomonas fluorescens* and *Saccharomyces cerevisiae*).

Drie *E. coli* (1 x 10^6 cfu.ml^{-1}) stamme is getoets, nl. ATCC 11775, 'n wilde stam wat uit melk geïsoleer is, en 'n O157:H7 stam uit melk, en daar is gevind dat al drie stamme sensitief is vir ultrasonikasie. Alle lewensvatbare selle is binne 10 min geëlimineer. Lewensvatbare tellings van *P. fluorescens* is met 100% verminder binne 6 min van ultrasonikasie en *L. monocytogenes* is met 99.0% verminder binne 10 min. *Lactococcus lactis* is met 97.0% verminder en *M. luteus*, *B. cereus* en *C. meningosepticum* met onderskeidelik 88.0%, 87.0% en 85.0%. *Lactobacillus acidophilus* was die meeste bestand teen ultraklank met net 78.0% van die lewensvatbare selle wat geëlimineer is. *Saccharomyces cerevisiae* is onder soortgelyke omstandighede met 99.7% verminder. Mikrobiese selmorfologie, grootte en Gram-status het nie noodwendig die effektiwiteit van ultrasonifikasie beïnvloed nie. Steriele fisiologiese sout oplossing en UHT melk is as suspensie media gebruik, en die sogenaamde beskermende effek van melkvet is nie, met die parameters soos in hierdie studie gebruik, waargeneem nie. Daar is gevind dat 'n hoër golf amplitude (100%; 124 µm) mikrobes meer effektief elimineer as 'n laer golf amplitude (50%; 62 µm). Die effektiwiteit van ultraklank is nie verhoog deur dit te puls nie wat aandui dat staande golwe nie gevorm het nie.

Beperkte sukses is met ultrasonikasie behaal, en daar is bevind dat die lang behandelings tye (10 min of meer) onrealisties is vir die suiwelindustrie. Die gelyktydige toepassing van ultraklank en hitte (termo-ultrasonikasie) is dus ondersoek.

Termo-ultrasonikasie was meer effektief as slegs 'n ultraklank of hitte behandeling en het alle lewensvatbare *M. luteus* selle binne 4 min (100% amplitude teen 72°C) geëlimineer. Slegs 2 min en 4 min termo-ultrasonikasie was nodig om onderskeidelik *E. coli* en *Lb. acidophilus* in melk te elimineer. *Bacillus cereus* endospore was steeds bestand, en na termo-ultrasonikasie van 10 min kon slegs 78.04% selle geëlimineer word.

Gedurende hierdie ondersoek is beide ekstensiewe oppervlak (SEM) en interne (TEM) selskade a.g.v. ultrasonikasie in *E. coli*, *Lb. acidophilus* en *S. cerevisiae* waargeneem. Dus beskadig ultrasonikasie die mikrobiese selle fisies/meganies wat lei tot sel doding/beskadiging.

Mikrobiese proteïene en DNS wat na 'n ultraklank behandeling uit die selle in die omgewing vrygestel word is gemeet en 'n verhoging in die vrygestelde mikrobiese proteïene en DNS was 'n aanduiding van 'n vermindering in die aantal lewensvatbare selle, mits die aanvanklike selkonsentrasie hoog genoeg was. Dit was egter nie moontlik om die konsentrasie vrygestelde mikrobiese proteïene en DNS met 'n presiese aantal lewensvatbare selle te korreleer nie, wat hierdie metode 'n oneffektiewe kwalteitsindikator vir die industrie maak.

Ultrasonikasie het geen statisties beduidende invloed op die proteien, vet of laktose inhoud van rou of gepasteuriseerde melk gehad nie. Die somatiese seltelling van rou en gepasteuriseerde melk het na ultrasonikasie afgeneem. Anders as met hitte, is die aktiwiteit van alkaliese fosfatase en laktoperoksidase nie deur ultrasonikasie verminder nie. Geen van die ensieme kan dus gebruik word om die sukses van 'n ultraklank behandeling van melk aan te dui nie.

Hierdie studie het gedemonstreer dat ultrasonikasie 'n geldige alternatief vir pasteurisasie is, aangesien dit mikrobes effektief kan elimineer, en ook nie die melk komponente verander nie. Om egter 'n meer effektiewe doding van selle te verseker word aangeraai dat termo-ultrasonikasie gebruik word vir die behandeling van melk wat gebruik kan word vir die produksie van 'n verskeidenheid suiwelprodukte.

CONTENTS

Language and style used in this dissertation are in accordance with the requirements of the *International Journal of Food Science and Technology*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

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If I have seen further, it is by standing on the shoulders of giants

Sir Isaac Newton

Explanation for nomenclature usage

To prevent confusion between older organism names and recent official genus and species nomenclature, the following species names were used:

Chryseobacterium meningosepticum	= *Elizabethkingia meningoseptica*
Lactococcus lactis	= *Lactococcus lactis* subsp. *lactis*
Streptococcus thermophilus	= *Streptococcus salivarius* subsp. *thermophilus*

CHAPTER 1

INTRODUCTION

Milk production for human consumption began more than 6 000 years ago (Anon., 2003), and milk may be considered nature's perfect food as it is such a rich source of proteins, fats, vitamins and minerals (Frõlich, 2002). Unfortunately, milk is also ideal for sustaining microbial life (Buffa *et al*., 2001) which could lead to spoilage of the milk and in extreme cases could be hazardous when ingested. Over the years many methods have thus been evaluated in order to extend the shelf-life of milk.

A heat treatment is most commonly applied to milk to eliminate spoilage and potentially pathogenic microbes (Buffa *et al*., 2001). The type of heat treatment may be classified as thermisation (63° - 65°C for 15 s) (Anon., 2003), low temperature long time (LTLT) pasteurisation (63°C for 30 min), high temperature short time (HTST) pasteurisation (72°C for 15 s) (Anon., 1997), or ultra high temperature (UHT) treatment (130 - 150°C for a few seconds) (Anon., 2003). The "Extended Shelf-life" process and "ultra-pasteurisation" (125° - 138°C for 2 - 4 s) are also being applied more frequently, although these products still need to be refrigerated after the heat treatment has been applied (Anon., 2003).

High temperature short time pasteurisation is the most commonly applied heat treatment for the extension of the shelf-life of milk. However, despite its popularity, there have been numerous reports of microbes isolated from pasteurised milk (Ternström *et al*., 1993; García-Armesto & Sutherland, 1997; Grant *et al*., 2001; Stopforth *et al*., 2003), which could lead to pasteurisation failures.

Alternative milk treatment methods have been investigated, each with its own advantages and disadvantages. One such an application is bactofugation which is the removal of microbes by using centrifugal forces (Kosikowski & Mistry, 1997). Although it works well for eliminating bacterial endospores, this method requires very high centrifugal forces (in excess of 10 000 x *g*) to be effective (Su & Ingham, 2000). The specialised and expensive equipment needed to produce such high centrifugal forces on industrial scale makes bactofugation a questionable alternative.

Another alternative method is the lactoperoxidase (LP) system, which is a naturally occurring antimicrobial system in milk (Wolfson & Sumner, 1994). Three components are needed to activate the LP system of which only lactoperoxidase is

found in sufficient concentration in raw milk. Thiocyanate and hydrogen peroxide need to be added to the raw milk (Reiter, 1985). Although the LP system is effective in eliminating some Gram-negative microbes (e.g. coliforms and pseudomonads) (Reiter *et al*., 1976), this system can only inhibit some of the Gram-positive microbes such as lactobacilli (Oram & Reiter, 1966). Furthermore, the food legislation of some countries, including South Africa (Anon., 1977), does not permit the addition of these components to raw milk, rendering this method of preservation illegal (Muir, 1996).

Microwave processing is not a new method for eliminating microbes with reports dating back to the 1940's (Flemming, 1944). In 1969, Hamid *et al.* proposed microwave processing as a viable alternative for pasteurising milk. The main advantage of using microwaves for processing milk is the faster start-up and shut-down times with consequent energy savings (Decareau, 1985). There are, however, several disadvantages when using the microwaves to extend the shelf-life of milk. These include: cost of equipment; low efficiency of conversion of electrical energy to microwave energy; and probably the most important for the consumer, organoleptic changes in the product (Vasavada & Cousin, 1993).

Ultraviolet (UV) irradiation of milk has also been investigated as a possible alternative to traditional heat treatments. Although UV is regularly used for disinfecting air (Bintsis *et al*., 2000), it is a known fact that microbes suspended in a liquid are more resistant to UV than microbes suspended in air (Koller, 1965). Some of the factors that have limited the application of UV in the dairy industry are the lack of penetration of UV in opaque liquids (Snowball & Hornsey, 1988; Lodi *et al*., 1996) and also the development of off-flavours in milk (Koller, 1965).

Microbial survival has been associated with all the above "alternative" options, including the different heat treatments. This has encouraged the search for new, non-thermal alternatives such as pulsed electric field inactivation, high pressure and ultrasonication (Piyasena *et al*., 2003). Although the elimination of microbes by ultrasonication was reported as early as the 1930's, the scant lethal effect of ultrasonication has limited its application as a "sterilisation" method. Improvements in ultrasound technology during the last decade have, however, renewed the interest of researchers in using ultrasonication to study the effective elimination of microbes from food products/liquids.

Renewed interest in ultrasonication led to several aspects that need to be answered before successful utilisation of this technique. The questions include: Which soundwave frequency is best for the elimination of microbes? What makes ultrasound

lethal to microbes and what happens to the microbes physically? Is it effective for the elimination of microbes? Is ultrasound effective against spoilage and potentially pathogenic microbes? Does ultrasonication affect the native milk components? Is a single treatment of ultrasound lethal enough, or will it be necessary to use ultrasound in combination with another method?

Ultrasound is defined as sound waves with a frequency of 20 kHz or more (Butz & Tauscher, 2002). High-power low frequency ultrasound, which is recommended for the elimination of microbes, refers to ultrasound at frequencies of between 20 and 100 kHz, also known as "power-ultrasound" (Mason & Lorimer, 2002). Although consensus has not been reached among researchers, the main killing mechanism of ultrasound is thought to be cavitation. During ultrasonication, longitudinal sound waves are formed in the liquid medium creating regions of alternating compressions and rarefactions (Sala *et al*., 1995). The constant change of pressure between the two regions causes cavitation to occur. Gas bubbles are formed in the liquid medium and grow in size after each rarefaction stage until a critical size is reached after which some of the cavitating bubbles violently collapse (Goldman & Lepschkin, 1952). As these bubbles collapse, shock waves occur creating micro-regions of very high temperature and pressure. Some researchers are of the opinion that it is rather these very high temperatures (5 500°C) and pressures (50 000 kPa) that are responsible for the elimination of microbes (Suslick, 1990). Other researchers argue that the life-time of these localized "hotspots" are too short to have any bactericidal effect and that it is rather the violent collapse of the cavitating bubbles and associated powerful eddies in the aqueous milieu that causes the microbial cell walls to rupture and shear, resulting in cell death (Alliger, 1975; Morton *et al*., 1982). It has also been suggested that as bubbles collapse, the formation of free radicals, such as various species of oxygen, as well as hydrogen peroxide could inactivate microbes (Piyasena *et al*., 2003).

There are reports of ultrasound being more effective against Gram-negative microbes than Gram-positives (Hülsen, 1999; Villamiel & de Jong, 2000), and also that smaller and cocci shaped microbes are more resistant to elimination by ultrasound (Alliger, 1975). Scherba *et al*. (1991) has, however, found that there is no correlation between the effectiveness of ultrasonication and the size, morphology or Gram status of microbes. Apart from the type and number of microbes to be treated, there are also other factors that are known to affect the efficacy of microbial elimination by ultrasound. These include: the amplitude of the ultrasonic waves; treatment time; treatment volume; treatment temperature; and the composition of the food (Hoover, 2000).

Although there is some information available on ultrasonication as applied as a microbicide in milk, it is evident that more research needs to be done on the effectiveness and impact of ultrasound in a dairy milieu before the dairy industry will consider investing in this new and powerful alternative to traditional thermal pasteurisation.

Before ultrasonication can be recommended as an alternative to thermal pasteurisation, it is essential to know if ultrasound can effectively eliminate microbes that are commonly associated with dairy products from milk. Furthermore, assuming that ultrasonication is effective, knowledge of any adverse effects of ultrasound on native milk components is imperative as this will influence the application of this method for the treatment of milk intended for cheese or yogurt production.

The main objective of this study was to evaluate the impact of high-power low-frequency ultrasound as an alternative to thermal pasteurisation in the dairy environment. This will be done by: investigating the effectiveness of ultrasound to eliminate *Escherichia coli* from milk; determining the impact of ultrasound on the survival of a selection of spoilage and potentially pathogenic microbes commonly associated with milk; determining if an ultrasonic treatment would lead to an increase in released microbial protein and DNA, which could possibly be used for the quantification of ultrasonically induced cell damage; and also to visualise (SEM and TEM) ultrasonically inflicted cell damage to gain some insight on the damage mechanism of ultrasound; determining if ultrasound has any detrimental impact on native milk components. Should ultrasound not be enough to ensure a microbiologically safe product, ultrasonication will be investigated as part of a hurdle treatment by assessing the microbicidal effects of a simultaneous application of ultrasound and heat (thermo-ultrasonication).

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CHAPTER 2

LITERATURE REVIEW

A. Background

Milk is an important part of the human diet and may even be considered to be "nature's perfect food", as it is an extremely rich source of nutrients (Frõlich, 2002). It is also an important source of vitamins and minerals, although, the two principle nutrients of milk are lipids and proteins (Frõlich, 2002). Unfortunately, it is also near perfect for sustaining microbial life (Buffa *et al*., 2001). Although milk should be practically free from microbes after milking of a clean, healthy cow, it is almost impossible to maintain this status. Microbes from the milking equipment, container, milker or air may contaminate the product (Fox & Cameron, 1982) and lead to spoilage. This limits the quality and shelf-life of fresh milk and it may also spread human pathogens (Cerf, 1986). Heat treatment is the oldest and most widely used technological process applied to extend the shelf-life of milk by eliminating spoilage and pathogenic microbes (Buffa *et al*., 2001).

The growth of pathogenic microbes is not a determinant of the shelf-life of fresh milk, however, the growth of spoilage microbes is. These microbes will degrade milk constituents by means of extracellular and intracellular enzyme activity. Four types of enzyme activity are encountered:

- lactose may be fermented to lactic acid during acidification of the product;
- rancidity may develop when lipids are hydrolysed by lipases - both microbial and the native milk enzymes;
- proteinase activity may result in breakdown of milk proteins and the development of intense bitter flavours; and
- phospholipases may attack the milk fat globule membrane which stabilises the native emulsion of milk fat, resulting in churning of the fat and this is generally referred to as 'bitty cream'.

The determinants of shelf-life of fresh dairy products are usually the spoilage microbes, which are capable of growth, even at refrigeration temperatures. Natural milk enzymes or non-microbially induced chemical reactions in milk seldom lead to spoilage

(Muir, 1996). In general, spoilage of fresh raw milk by lipolysis, do not occur until microbial counts in excess of 5 x 10^6 cfu.ml^{-1} (colony forming units per ml) are reached. The shelf-life may thus be regarded as the time taken for the microbial count to reach this threshold level (Muir *et al*., 1978).

The International Dairy Federation (IDF) recommends raw milk to be pasteurised by either using the batch, low temperature long time (63°C for 30 min) or the high temperature short time (72°C for 15 s) option (Cerf, 1986). The two most heat resistant milk pathogens, *Mycobacterium tuberculosis* and *Coxiella burnetii*, were used as reference strains to determine the minimum heat treatment to render milk free of pathogens. The problem with pasteurisation is that especially endospore-formers such as *Bacillus cereus* (Choma *et al*., 2000), and also some other spoilage microbes (e.g. micrococci, lactobacilli and some streptococci) (Burton, 1986) are able to survive minimum treatments. The ability of psychrotrophs to grow at refrigeration temperature is a further limiting factor, in terms of keeping quality, if they are not eliminated during pasteurisation (Cousin, 1982). Spoilage microbes such as *Pseudomonas* and also pathogens like *Listeria* do not normally survive pasteurisation and their presence is rather ascribed to post-pasteurisation contamination (Aaku *et al*., 2004; Kells & Gilmour, 2004).

Pasteurisation failures and the consumers' increasing demand for minimally processed, additive-free, shelf-stable products motivated the investigation of physical treatments as potential alternatives to traditional heat treatments. These alternatives include new ways of applying heat such as microwave heating, and also non-thermal methods such as sonication, pulsed electric fields and high pressure (Smelt, 1998). Non-thermal processes have the advantage of low processing temperatures, low energy utilisation and thus better retention of flavours, nutrients and a 'fresher' taste, while inactivating the spoilage and pathogenic microbes and enzymes (Vega-Mercado *et al*., 1995a).

B. Heat treatment technologies

The single most effective and straightforward way of reducing microbial numbers in milk is to apply a heat treatment (Muir, 1996). The heating time and temperature combination will determine the efficiency of the treatment. It was also found that higher processing temperatures require shorter times to kill microbes that may be present in

milk (Anon., 2003). There are three main categories of heat treatment of milk, namely thermisation, pasteurisation and ultra high temperature treatments.

Thermisation

The most moderate heat treatment with a significant practical application is "thermisation", a term used to describe a heating process that prevents raw milk spoilage during storage at refrigeration temperatures (Muir, 1996) by decreasing the number of psychrotrophic microbes (Shah, 1994). Although thermisation is effective for inhibiting spoilage it does, however, offer no guarantee of microbial safety. Thermisation does not stand on its own as a heat treatment in the same sense as pasteurisation, but must rather be seen as a means of extending the shelf-life of raw milk until it can undergo pasteurisation or a more severe heat treatment (Anon., 2003). As far as can be concluded from the literature, there is no legal definition for thermisation and also no consensus of the thermal specifications.

Shah (1994) reported thermisation to be a heat treatment of 74°C for 20 s. In contrast, it has been reported (Anon., 2003) that thermisation is a pre-heat treatment of milk to temperatures below pasteurisation, in the range of 63° - 65°C for 15 s. This time/temperature combination does not inactivate the phosphatase enzyme, but, aerobic endospore-forming microbes are prevented from multiplying by rapidly cooling the milk to 4°C or below. Thermisation also has a favourable effect on certain endospore-forming microbes by activating the endospores to revert back to vegetative cells, which may then be destroyed by the subsequent pasteurisation. According to the IDF (Cerf, 1986), thermisation involves heating to 63° - 65°C for 15 - 20 s, with the aim of improving the keeping quality of milk at the plant pending its final use. Researchers at the Hannah Research Institute (Muir, 1996) did extensive research on thermisation and found temperatures in excess of 65°C to be the most effective. It is now generally accepted that the term 'thermisation' applies to a heat treatment in the 65° - 70°C for 15 - 30 s range. After the heat treatment the thermised milk must be promptly cooled to below 6°C to be effective (Muir, 1996).

The organisms which grow in thermised milk during refrigerated storage are similar to those found in raw milk with pseudomonads being the predominant microbes found in thermised milk after prolonged storage (Muir, 1996). Pseudomonads are a rich source of extracellular enzymes and their presence and activity will limit the shelf-life of thermised milk. It has also been reported that *Streptococcus thermophilus* may reach high numbers in the regeneration section of plate heat exchangers over long,

continuous processing times (in excess of 5 h). These increases of *S. thermophilus* could lead to spoilage of the thermised milk (Muir, 1996). Thus, subsequent microbial growth in thermised milk is highly temperature dependent and, therefore, to ensure the efficiency of the treatment, it is vital that the temperature of the thermised milk be lowered as fast as practically possible to below 6°C and that the milk be stored at low temperatures (2° - 4°C) (Muir, 1996).

Pasteurisation

Pasteurisation is the most commonly applied heat treatment of milk products (Muir, 1996). If pasteurisation is properly applied, it may reduce the health risks associated with raw milk by the almost complete elimination of pathogenic microbes, and it also inhibits spoiling by microbes. Pasteurisation processes have been given clear legal definitions because of its importance in guaranteeing the safety of milk and milk products (Muir, 1996). The IDF has given the following definition for pasteurisation (Cerf, 1986): "*Pasteurization - Is a process applied to a product with the aim of avoiding public health hazards arising from pathogenic microorganisms associated with milk by heat treatment which is consistent with minimal chemical, physical and organoleptic changes in the product*". The pasteurisation process may include two heat treatments:

Low temperature long time (LTLT)

According to the South African "milk law" (Anon., 1997): "*pasteurisation of milk shall be performed by heating every particle of the milk to a temperature of at least 63°C (not exceeding 65,5°C) and keeping it at that temperature for at least 30 minutes, which heating shall be followed by cooling within 30 minutes to a temperature lower than 5°C (this process is referred to as the "holder method" or the "batch method")*". The Scottish designation for LTLT pasteurisation recommends that milk must be held at not less than 62.8°C and not more than 65.6°C for at least 30 min, followed by immediate cooling to 6°C (Muir, 1996).

High temperature short time (HTST)

The SA "milk law" (Anon., 1997) further states that: "*pasteurisation of milk shall be performed by heating every particle of the milk to a temperature of at least 72°C and keeping it at that temperature for at least 15 seconds, which heating shall be followed immediately by cooling to a temperature lower than 5°C (this process is referred to as the "high-temperature short-time method")*". The Scottish designation for HTST

pasteurisation recommends that milk must be held at not less than 71.7°C and not more than 78.1°C for at least 15 s, followed by immediate cooling to 6°C (Muir, 1996).

General comments on pasteurisation

Pasteurisation not only kills most common pathogens in milk but also eliminates the Gram-negative psychrotrophs which are the most common cause of spoilage in raw and thermised milk (Muir, 1996). One major shortcoming of the pasteurisation process is the failure to eliminate the thermoduric microbes which may grow at refrigeration temperatures. These microbes are mostly bacilli and include strains of *Bacillus cereus*, *B. circulans* and *B. mycoides* which are all capable of rapid germination and growth in refrigerated dairy products (Muir, 1996). *Bacillus cereus* may be considered to be a pathogen as the endospores and toxins survive pasteurisation and may cause serious food related illnesses (Granum & Lund, 1997).

It is generally accepted that although pasteurisation eliminates all but the endospore-forming organisms from the psychrotrophic population of milk, the process does not destroy their extracellular degradative enzymes. Roughly a third of the phospholipase and two thirds of the proteinase and lipase enzymes survive HTST pasteurisation (Patel & Blankenagel, 1972). Therefore, if the psychrotrophic count exceeds 5×10^6 cfu.ml^{-1} in raw milk before pasteurisation, there is a definite chance that shelf-life will be shortened (Patel & Blankenagel, 1972).

Fundamentally the time/temperature combination of the pasteurisation process reaches an equilibrium between sterility and shelf-life on the one hand, and nutrition and palatability on the other. Pasteurisation is effective because the heating applied causes individual microbial cells to die randomly. Two effects are at work: firstly, the longer the milk is held at an elevated temperature, the more organisms die, however, this may be detrimental to nutrition and taste; and secondly, the rate of death increases with increases in temperature. Brief heating, as applied during HTST pasteurisation, reduces microbial populations to acceptable levels without excessive destruction of the delicate substances that are valuable to milk nutrition and flavour (Harrison, 2002).

There is no significant difference between the nutritional quality of raw and pasteurised milk. Even though most of the pathogenic microbes are destroyed during pasteurisation, the total number of microbes is only reduced, and only certain enzymes are inactivated. This results in pasteurised milk having only a limited keeping capacity. One advantage of pasteurisation is that it converts hydroxy acids into lactones, resulting in a product with improved sensory characteristics. Pasteurisation has very little effect

on the nutritional quality of vitamins, although storage could lead to loss of some vitamins. The fat-soluble vitamins, A, D and E, and some of the vitamins in the B-complex (nicotinic acid, pantothenic acid, biotin and riboflavin) are relatively heat stable and generally only minor losses have been reported when milk is pasteurised (Frõlich, 2002). Heat treatments have not proved to have a negative influence on the availability of minerals (Weeks, 1985).

General pasteurisation equipment

LTLT – Although LTLT or "batch" pasteurisation has to a large extent been replaced by HTST pasteurisation, it is still widely practised in some parts of the world, and especially in laboratories, due to its simplicity and simple equipment requirements (Potter & Hotchkiss, 1995).

In this process, milk is heated and held in one vessel usually made of stainless steel fitted with a paddle for agitation of the milk. The holder vessel is insulated with some form of material, for example cork, to maintain the milk temperature. There are three methods used for batch pasteurisation: hot water is circulated in the jacket surrounding the holding vessel to heat the milk; or low-pressure steam is used directly for heating; or a combination of water and steam may be used. Once the desired temperature (63°C) has been reached, the milk is held for 30 min before being cooled to the required temperature (Harvey & Hill, 1967).

HTST – The two principles of heating applied by the dairy industry for this process are direct and indirect heating. Direct heating is efficient for rapid heating and is normally used to sterilise milk by steam injection, or infusion of milk into a steam-filled vessel. Heat is transferred by convection or conduction from the heating medium to the milk. Direct heating as a means of pasteurisation is forbidden by law in some countries on the grounds that foreign matter may be introduced into the product (Anon., 2003).

Indirect heat transfer is the most popular method used for pasteurisation of milk. In this method, there is always a partition of some sort between the product and the heating/cooling medium (Anon., 2003).

There are three classes of heat exchangers generally used for HTST pasteurisation: plate heat exchangers (PHE); tubular heat exchangers (THE); and scraped-surface heat exchangers (SSHE). The latter is used for viscous, sticky and lumpy products such as jams, chocolate, peanut butter etc. and will, therefore, not be discussed (Burton, 1988; Anon., 2003).

Plate heat exchangers are the most popular type of heat exchangers used by the dairy industry. Plate heat exchangers consist of a set of thin rectangular corrugated metal plates fitted into a frame. These metal plates act as heat-transfer surfaces and when they are fitted together, the corrugations on consecutive plates interlock to form narrow flow channels, in which hot and cold streams flow alternatively. The distance between the plates is determined by the thickness of the gasket surrounding each plate. The gasket seals the fluids in the PHE to prevent external leakage and internal mixing. Several flow patterns are possible, depending on the gaskets' configuration (Ribeiro & Caño Andrade, 2002; Anon., 2003).

Tubular heat exchangers are used for both pasteurisation and UHT treatment of dairy products. The THE may be operated for longer periods between cleaning cycles than the PHE during UHT treatment. The THE does however, need a higher flow velocity than the PHE to create efficient heat transfer. There are two fundamentally different types of THE: multi/mono tube and concentric tube (Anon., 2003).

In multitube THE, the product flows through a group of parallel tubes and the service medium between and around the tubes. The monotube is a version with only one inner tube and will permit particles with a diameter of up to 50 mm to pass through. Multi/mono tubes are suited for processes operating at very high pressures and high temperatures (Burton, 1988; Anon., 2003). In contrast, concentric tube THE's give efficient heating or cooling as there is heating/cooling media on both sides of the annular product channel. This type of THE is well suited for high viscous fluids with strong non-Newtonian behaviour (Anon., 2003).

Fouling can be a serious problem encountered during pasteurisation. During the heating process, protein, mineral and fat deposits (also known as milk stone) may accumulate on the contact surfaces of the equipment (Burton, 1988). This reduces efficiency by lowering heat transfer, and may also be a possible source of contamination. To overcome this problem, extensive and regular cleaning is required, especially when PHE are used. This process may be time consuming as the PHE have to be taken apart and the plates cleaned individually (Anon., 2003).

Monitoring pasteurisation

The IDF (Cerf, 1986) defined pasteurised milk as follows: "*Milk which has been subjected to pasteurization; which if retailed as such has been cooled without delay and has then been packaged with minimum delay under conditions which minimize contamination. The product must give a negative phosphatase test immediately after*

heat treatment". Thus, alkaline phosphatase is used as a statutory index of effective pasteurisation (Muir, 1996). These enzymes are believed to have a thermal resistance greater than that of the most heat-resistant non-endospore-forming pathogens commonly found in milk (Murthy *et al*., 1993). In contrast, thermisation does not inactivate the phosphatase enzyme (Anon., 2003). A negative test for alkaline phosphatase indicates proper pasteurisation. The test is negative if, after pasteurisation, milk is incubated with a colorimetric reagent susceptible to phosphatase action and a residual level of less than 10 µg of ρ-nitrophenol per ml of milk is found (Muir, 1996).

Heat treatments of above 100°C

There are several options available when a heat treatment of above 100°C is required or considered essential. "Extended Shelf-life" (ESL) is a process frequently applied in Canada and the United States of America (USA). Although there is no single definition for ESL, a typical temperature/time program is 125° - 130°C for 2 - 4 s (Anon., 2003). This type of heat treatment is sometimes also referred to as "ultra-pasteurisation" (UP) which is considered to be a heat treatment of 125° - 138°C for 2 - 4 s, with cooling to below 7°C. Both the ESL and UP heat treatments require the final product to be refrigerated (Anon., 2003).

According to the SA "milk law" (Anon., 1997): "*'UHT' or 'ultra high temperature treatment' means the process whereby milk or a dairy product is subjected to heat treatment above 100°C and aseptically packaged so that the end product, after incubation for not less than 14 days at a temperature of 30°C ± 1°C, is free from spoilage by micro-organisms*".

General comments on heat treatments of above 100°C

The shelf-life of pasteurised milk is normally measured in days, depending on the storage temperature and the number of microbes remaining (Harrison, 2002). In order to extend the storage time to several months, all microbes and enzymes need to be inactivated. This may be achieved by using UHT treatments (Frõlich, 2002). It has been suggested (Anon., 2003) that the UHT process ensures food safety via commercial sterility and a long shelf-life at ambient temperatures. Souring (or acidification), due to microbial metabolic processes, may be greatly slowed by processing at much higher temperatures, with a trade-off in reduced nutrition and palatability, however, even this above-boiling temperature (>100°C) does not kill all

microbes and souring will still occur after a few months (Harrison, 2002). Griffiths *et al*. (1986) suggested that a heat treatment of 115℃ for 5 s is the most promising for the control of endospore-forming microbes.

The UHT process is, however, not always recommended as a replacement for pasteurisation, since it adversely affects amino acid bioavailability and, consequently, nutritional value (Efigênia *et al*., 1997). Frõlich (2002) also reported that UHT treatments may result in considerable losses of some selected nutrients. Heating at higher temperatures produces higher concentrations of lactones which lead to improved sensory characteristics, but also leads to the formation of aldehydes and methyl ketones which may have an adverse effect on the flavour of the final product. During prolonged heat treatments at high temperatures and/or storage, oxidation may cause loss of the fat-soluble vitamins. Ultra high temperature treatment of milk may also lead to a significant decrease of some of the vitamins (Frõlich, 2002), however, more rapid heating and cooling has less impact on chemical changes that might occur including taste, colour and nutritional value. It was also found that the higher processing temperatures required shorter times to kill the microbes present in the milk (Anon., 2003).

Extended heat treatments have been studied as a means of controlling the growth of psychrotrophic endospore-forming microbes, as other heating methods have limited efficiency in controlling this specific problem. Simply increasing the temperature of pasteurisation is ineffective because the higher temperatures often result in activation of otherwise dormant endospores (Brown *et al*., 1980).

Double HTST heat treatments have also been studied. The application principle of such combined heat treatments is that the first heat treatment activates the microbial endospores which, after a suitable incubation period, germinate. The vegetative cells are then readily destroyed by the second HTST pasteurisation. In contrast, the application of double pasteurisation, coupled with a range of intermediate incubation conditions, has not been successful to control spoilage (Brown *et al*., 1979). The lack of success of double heat treatment may be explained by two factors. Firstly, it has been found to be impossible to achieve complete germination of all endospores by a single heat treatment. Secondly, even when maximum germination has been achieved some new sporulation may occur in the period of incubation allowed for the activated endospores to convert into true vegetative cells. Thus, double pasteurisation does not hold a considerable advantage over single HTST pasteurisation in terms of the

elimination of endospores, and may be deemed unnecessary and cost-ineffective/uneconomical.

There are two ways of producing long-life milk for ambient storage, either by sterilisation or UHT treatment. Milk is given an in-container heat treatment at 115° - 120°C for 20 - 30 min to produce sterilised milk. The bottles (either glass or plastic) may be sterilised in bulk in an autoclave, or may be sterilised continuously in horizontal or vertical hydrostatic towers. Ultra high temperature milk requires a heat treatment of 135° - 140°C for a few seconds, followed by aseptic filling, normally in carton boxes (Anon., 2003).

There are two ways of giving milk an UHT treatment, either by direct or by indirect heating. During direct heating the product comes in direct contact with the heating medium, followed by flash cooling in a vacuum. Steam can be injected into the milk (steam injection), or the milk can be introduced into a steam-filled vessel (steam infusion) (Anon., 2003).

Indirect heating is more cost-effective than direct heating. There are two main methods used for the UHT treatment of milk: plate heat exchangers and tubular heat exchangers (Burton, 1988; Anon., 2003). The main principles have already been discussed.

C. Alternative treatments technologies

Membrane treatment

Membrane processes have been employed by the dairy industry for many years (Rosenberg, 1995). Membrane separation is a logical choice for the fractionation of milk as many milk components may be separated on the basis of size (Brans *et al*., 2004). The four main membrane processes employed by the dairy industry are microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) (Rosenberg, 1995). The illustration in Fig. 1 shows the differences between these processes in terms of size and milk components that may be separated. The dairy industry uses MF for different purposes: preservation of the functional properties of milk proteins; fractionating caseins and whey proteins (Rosenberg, 1995); and the elimination or reduction of microbes and endospores from milk (Eckner & Zottola, 1991; McSweeney *et al*., 1991; Rosenberg, 1995; Guerra *et al*., 1997; Saboya & Maubois, 2000). When dealing with membrane processing in the dairy industry, MF is generally

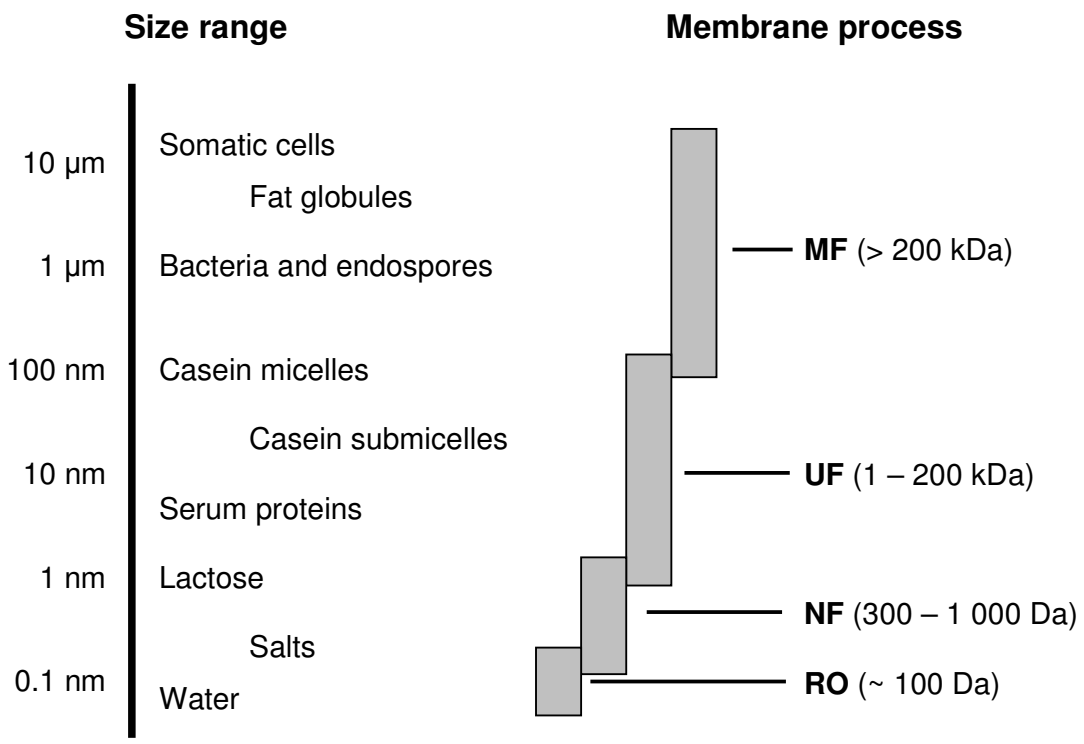

Figure 1 Different membrane processes and size indication of the milk components that may be separated. MF – microfiltration; UF – ultrafiltration; NF – nanofiltration; RO – reverse osmosis (combination of data taken from Rosenberg, 1995 and Brans *et al*., 2004).

used for the elimination of microbes, therefore, only MF will be discussed in this dissertation.

Microfiltration (MF) is a pressure-driven membrane process that uses membranes with a pore size of 0.2 - 2 µm (Rosenberg, 1995). Raw milk that has been subjected to MF is usually divided into two fractions: the fluid retained by the membrane (the retentate) which consists of mainly fat globules, microbes and endospores; and the liquid passing through the membrane (the permeate or microfiltrate) (Saboya & Maubois, 2000). Theoretically, this permeate may be considered to be fat-free and bacteria-free milk (Maubois, 1991).

A major disadvantage of MF is the build-up of foulants on the membrane surface and also inside the pores. This leads to significant reductions in the efficiency of the process (Sheldon *et al*., 1991). The foulants include fat globules, proteins, microbes and endospores (Malmberg & Holm, 1988). Fouling of the system necessitates frequent membrane cleaning which may be time consuming, costly and it also shortens the 'lifetime' of the filters (Nyström, 1989).

Rosenberg (1995) reported that although MF is efficient in removing microbes and endospores, the MF process cannot be used to guarantee the removal of all pathogenic microbes present in milk, and thus cannot be considered an alternative to pasteurisation.

Bactofugation

Bactofugation is mainly used for removing microbes present in milk by a centrifugal force based on the difference between the density of the microbial cell and that of the milk serum (Kosikowski & Mistry, 1997). In the USA, pasteurisation is legally required to be part of a hurdle technology where bactofugation is used for the treatment of fresh milk (Kosikowski & Mistry, 1997). During bactofugation the force steers the microbes to the outer wall of the centrifuge where they exit through several ports, while the milk leaves at points nearer the centrifuge centre. This method is preferred to traditional pasteurisation especially when the typical flavour of raw milk is required for "ripened" cheese. During bactofugation, milk is subjected to centrifugal forces of approximately 9 000 x *g* to remove microbes and endospores (Kosikowski & Mistry, 1997; Sarkar, 1999). The resulting pellet may be heat treated and added back to the milk to avoid yield losses associated with bactofugation (Kosikowski & Mistry, 1997).

Ingham *et al*. (1998) showed that *Clostridium* endospores may be present in raw milk, and subsequently survive pasteurisation. Members of the genus *Clostridium* may

metabolise lactic acid to produce butyric and acetic acid, carbon dioxide, and hydrogen (Cato *et al*., 1986; Su & Ingham, 2000). Importantly, it has been shown (Klijn *et al*., 1995) that *C. tyrobutyricum* may cause late blowing in cheese.

Bactofugation has been well studied as a means of removing endospores from milk (Su & Ingham, 2000; Harrison, 2002). Su & Ingham (2000) reported that the higher the centrifugal force, the greater the degree of endospore removal. They reported a 66.2% reduction in *C. tyrobutyricum* endospores after centrifugation at 3 000 x *g* for 30 s compared to a 98.6% reduction for a 30 s treatment at 12 000 x *g*.

Lactoperoxide

The lactoperoxidase (LP) system is a naturally occurring antimicrobial system found in raw milk (Kamau *et al*., 1990; Wolfson & Sumner, 1994). To activate the LP system three components are required: LP, thiocyanate (SCN^-) and hydrogen peroxide (H_2O_2). Lactoperoxidase is normally found in sufficient concentrations in raw milk, however, thiocyanate and hydrogen peroxide need to be added (Reiter, 1985). The enzyme lactoperoxidase catalyses the oxidation of thiocyanate by H_2O_2, resulting in intermediate products with antimicrobial properties that may cause the inhibition of different spoilage and pathogenic organisms, thus enhancing the microbiological quality of the raw milk (Björck, 1978; Wolfson & Sumner, 1993). Apart from the importance of preserving the quality of raw milk, the LP system may also be used to extend the shelf-life of pasteurised milk (Barrett *et al*., 1999).

The antimicrobial activity of the LP system against psychrotrophic (Björck, 1978) and mesophilic (Zajac *et al*., 1983) microbes has been investigated. In these studies it was found that the LP system exerts both bacteriostatic and bactericidal activities against Gram-negative catalase positive microbes such as coliforms, pseudomonads and salmonellae depending on the pH, temperature, incubation time and cell density (Björck *et al*., 1975; Reiter *et al*., 1976). Gram-positive, catalase negative microbes (e.g. lactobacilli and streptococci) are, however, usually only inhibited by the LP system (Oram & Reiter, 1966). Zajac *et al*. (1981) also reported that the LP system was ineffective against the endospores of *Bacillus cereus*. This is in accordance with the hypothesis that the site of action of the LP system is on the bacterial plasma membrane and thus does not impact the endospores.

The timespan of the antimicrobial effect achieved by the LP system is inversely related to the storage temperature of the milk. When milk is stored at 15°C the antimicrobial effect lasts for 24 - 26 h, whereas it only lasts for 7 - 8 h when milk is

stored at 30℃ (IDF, 1988). Therefore, the LP system, when allowed (Kamau *et al*., 1990; 1991) is usually used in conjunction with a heat treatment. Muir (1996) reported that legislation in the United Kingdom does permit the use of the LP system for preservation of raw milk.

Microwave

Microwave pasteurisation of milk is by no means a new treatment. In 1969, Hamid *et al*. reported the use of a microwave system for pasteurising milk. Since then several researchers have done work on the microbiological impact of this system (Knutson *et al*., 1988; Thompson & Thompson, 1990; Heddleson & Doores, 1994). Microwaves are generated by a magnetron and are then absorbed by the food being treated; the dipole molecules in the food align with the microwave field which cause friction among the molecules resulting in heating of the product (Knutson *et al*., 1987).

There is some controversy as to the exact microbial killing mechanism of microwaves. Flemming (1944) treated an unknown concentration of *Escherichia coli* cells with electromagnetic fields at various frequencies (11 - 350 MHz) and found that the system had a lethal effect, with no significant rise in temperature, on the microbes. In another study, Brown & Morrison (1954) tried to duplicate Flemming's studies without success. After extensive research, they concluded that any microbial reduction was rather brought about by thermal effects and not the microwaves as such. This was later confirmed by other researchers (Goldblith & Wang, 1967; Lechowich *et al*., 1969; Hamrick & Butler, 1973). Similarly, Vela & Wu (1979) exposed lyophilised microbial cultures to microwaves, with no detrimental effect. They theorised that without free water, the cells could not absorb enough energy to generate heat to inactivate the microbes. Decareau (1985) reviewed a considerable amount of literature and concluded that the majority of research supports the argument that heat, and not microwave radiation alone, kills the microbes.

Merin & Rosenthal (1984) compared raw milk heated for 30 min in a microwave oven (2 450 MHz, 700W) to raw milk heated for 30 min in a waterbath at 63℃. The chemical composition of the two batches of milk was similar, both tested negative for phosphatase, and no coliforms could be detected. Plate counts done according to standard methods (Merin & Rosenthal, 1984) revealed a decrease in numbers by a six log value. A five log reduction was observed for psychrotrophs. Knutson *et al*. (1988) found that a microwave process that simulated high temperature short time (HTST) pasteurisation (71.1℃ for 15 s) did not inactivate all cells of *Salmonella typhimurium*,

Pseudomonas fluorescens or *E. coli* at an inoculation concentration of 10^3 - 10^4 cfu.ml^{-1}. Similarly, they found that the simulated LTLT process (62.8°C for 30 min) did not eliminate *Streptococcus faecalis* (1 x 10^6 cfu.ml^{-1} inoculum) to the same level as conventional batch pasteurisation of milk. They suggested that uneven heating in microwave ovens might be the reason. Chiu *et al*. (1984) also studied the extension of the shelf-life of pasteurised milk by microwave heating. They found that microwave heating of eight day old milk to 60°C reduced the psychrotrophic microbial count (1.8 x 10^6 cfu.ml^{-1}) to zero, thus extending the shelf-life of milk.

It has in the past been claimed that the rapid temperature rise in microwave heating results in less destruction of nutrients compared with conventional processes (Datta & Hu, 1992). There is, however, some disagreement between researchers on the effect of microwave heating on vitamins. Sieber *et al*. (1996) reported no loss of vitamins A, E, B_1, B_2, and B_6 in milk, with Sigman-Grant *et al*. (1992) reaching the same conclusion for vitamins B_2 and C in infant formula milk. Medrano *et al*. (1994), however, reported a significant loss of vitamins A and B_2 in milk. Vidal-Valverde & Redondo (1993) reported a significant loss of vitamin B_1 and found a thiamine loss of >50% in full cream milk and 65% in skimmed milk after microwave treatment at 80°C for 4 min.

It has been proposed that microwave treatment be considered an alternative method for heating milk and milk products (Gallmann & Eberhard, 1993; Valero *et al*., 2000). The technical advantages of microwave processing include the speed of operation, energy savings, precise process control and faster start-up and shut-down times (Decareau, 1985). Heat exchangers foul and this reduces the heat transfer and causes flavour changes in pasteurised milk while with the microwave process, milk heats directly. Fouling may, therefore, be avoided by elimination of the steep temperature gradient of conventional pasteurisation (Kudra *et al*., 1991; Aktas & Özilgen, 1992). Although there are advantages for using microwaves in milk processing, several disadvantages, especially the cost of equipment and operation, low efficiency of conversion of electrical energy to microwave energy and uneven product heating and organoleptic changes in products, have prevented widespread adoption of this technology (Vasavada & Cousin, 1993). Heddleson & Doores (1994), however, reported that uneven heat distribution could be avoided by the use of a continuous system. Similarly, Aktas & Özilgen (1992) reported satisfactory levels of microbial destruction by microwave treatment of milk in a continuous system.

Ultraviolet irradiation

Ultraviolet (UV) irradiation may be used for the sterilisation of surfaces, air and liquids (Bintsis *et al*., 2000). The UV spectrum may be subdivided into three categories: long-wave (UVA) with wavelengths of 320 - 400 nm; medium-wave (UVB) with wavelengths of 280 - 320 nm; and short-wave (UVC) with wavelengths of 200 - 280 nm (Giese, 1964). Most microorganisms, including bacteria, viruses, fungi, yeasts and algae are killed by UV irradiation at wavelengths between 250 - 260 nm (UVC), with the maximum bactericidal effect being at 254 nm. The microbial DNA is directly altered by UVC, which leads to a large decrease in the microbial population (Giese, 1992). It may, however, happen that microbes that were presumably killed by UV irradiation may be revived when exposed to visible wavelength light and will, therefore, contribute to the spoilage of the product. This phenomenon is called photoreactivation (Cords *et al*., 2001).

The advantages of UVC irradiation are that the process is not pH or temperature dependant (Cords *et al*., 2001), and cause no change in either colour, flavour or odour when used for the disinfection of water (Snowball & Hornsey, 1988). There are, however, several disadvantages when using UV, especially in the dairy industry: the development of off-flavours in milk (Koller, 1965); lack of penetration in opaque liquids (Snowball & Hornsey, 1988; Lodi *et al*., 1996); the presence of fat molecules which may lead to rancidity; variable antimicrobial efficiency and long exposure to UV irradiation may cause damage to the eyes and skin (Cords *et al*., 2001).

Temperatures of between 5° and 37°C have little influence on the antimicrobial action of UV irradiation; however, moisture may drastically decrease its efficiency. Microbes that are suspended in a liquid such as milk or water are much more resistant than those suspended in air, even after making allowance for absorption of UV by the medium (Koller, 1965).

In 2002, Smith *et al*. proposed the use of pulsed UV laser light as an effective means for the cold pasteurisation of milk. They did, however, only use a 1 ml sample which might be too small for any conclusive results. They suggested that their process was capable of controlling endospore-forming microbes, without conducting any appropriate tests. Furthermore, they reported a 21 d shelf-life of the treated product after no growth was detected on trypticase soy broth plates (inoculated with only 50 µl milk) (Smith *et al*., 2002). From the above assumptions made by them, one may question the validity of the results and recommendations.

D. Microbial survival

According to the IDF standards, milk is pasteurised to render it free from pathogenic microbes, which may pose as a health risk to the consumer (Cerf, 1986). However, the use of industrial pasteurisation processes cannot guarantee milk to be free of pathogenic microbes, either because they are present in too large numbers in the raw milk, or it might have entered the milk as post-pasteurisation contamination (Grant *et al*., 1996). Thermoduric microbes also lead to pasteurisation failures (Schröder, 1984; Muir, 1990).

Bacillus, *Aerococcus*, *Staphylococcus*, *Flavobacterium*, *Enterobacter*, *Pseudomonas*, *Micrococcus*, *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Microbacterium*, *Propionibacterium*, *Leuconostoc*, *Streptococcus*, *Proteus* and coliforms have all been isolated from raw milk (ICMSF, 1980; Frank *et al*., 1993; Jay, 1996). In addition, members of *Bacillus cereus*, *Pseudomonas*, *Enterobacteriaceae*, *Aeromonas* (Ternström *et al*., 1993), *Citrobacter freundii* (Lindberg *et al*., 1998), *Enterobacter sakazakii* (Skladal *et al*., 1993), *Listeria monocytogenes* (Doyle *et al*., 1987) and *Mycobacterium paratuberculosis* (Grant *et al*., 2001) have been isolated from pasteurised milk.

Salmerón *et al*. (2002) reported the presence of *Lactobacillus*, *Lactococcus* and *Pseudomonas* in HTST pasteurised milk. Ternström *et al*. (1993) indicated that at least half of the consumer milk on sale in Sweden and Norway have in the past been spoilt by Gram-negative psychrotrophs, especially members of the genus *Pseudomonas*. The ability of these microbes to grow at refrigeration temperatures holds serious consequences in terms of shelf-life.

Bacillus cereus is a common contaminant of raw (Lin *et al*., 1998) and pasteurised milk (Griffiths & Phillips, 1990; Larsen & Jørgensen, 1997), and has been reported to be the leading cause of food poisoning in several countries (Beattie & Williams, 1999). The endospores of *B. cereus* have a decimal reduction time of 2.2 - 5.4 min at 100°C (Choma *et al*., 2000) and will thus be able to survive pasteurisation. Dufrenne *et al*. (1994) reported the average generation time of a psychrotrophic *B. cereus* strain to be 8.2 h at 7°C. Some of the psychrotrophic strains are also known to grow in food at temperatures as low as 4° - 6°C (Andersen Borge *et al*., 2001).

The presence of *B. cereus* can lead to spoilage of pasteurised milk by the production of protease enzymes which lead to coagulation of the milk and a bitter

tasting product. Furthermore, lipolysis may also be found, which will result in fat aggregation of the cream (Andersson *et al*., 1995; Frank, 2001). *Bacillus cereus* has also been classified as a pathogen producing at least three diarrhoeal toxins (enterotoxins) (Granum, 2001) and an emetic (vomit-inducing) toxin (Kramer & Gilbert, 1989; Andersson *et al*., 1995). Andersson *et al*. (1995) reported that *B. cereus* levels of between 10^3 and 10^4 cfu.ml^{-1} have been implicated in food poisoning outbreaks.

Listeria monocytogenes is a pathogenic microbe responsible for human listeriosis (Loncarevic *et al*., 1997), and has been described as the most important pathogen isolated from milk (Lovett *et al*., 1987). It is commonly found in raw milk (Harvey & Gilmour, 1992), and has the ability to grow well at refrigeration temperatures (Rosenow & Marth, 1987). Although it is generally believed that *L. monocytogenes* does not survive pasteurisation (Piyasena *et al*., 1998) there are several studies reporting its heat resistance and subsequent survival of pasteurisation (Fenlon *et al*., 1996; Senczek *et al*., 2000). This is mainly ascribed to the protective nature of leukocytes present in milk and to the survival advantage of the pathogen (Doyle *et al*., 1987; Lovett *et al*., 1987). A further serious health safety aspect is that biofilm cells of *Listeria* are reported to be more resistant to disinfectants than planktonic cells (Chae & Schraft 2000).

It is somewhat alarming that pathogens, such as *B. cereus*, *L. monocytogenes*, *Escherichia coli* O157:H7, *Salmonella typhimurium* and *Campylobacter jejuni* can readily form biofilms (Somers *et al*., 1994; Joseph *et al*., 2001; Stopforth *et al*., 2003). Food spoilage microbes such as *Pseudomonas aeruginosa*, *P. fragi*, *Micrococcus* spp. and *B. subtilis* have also been associated with biofilms (Abrishami *et al*., 1994). Biofilms on heat exchangers may seriously decrease the efficiency of heat transfer. Carpentier & Cerf (1993) reported that microbes within biofilms are more resistant to heat, which may lead to pasteurisation failures (Schmid *et al*., 2004), and subsequently lower the shelf-life of the product and cause the spreading of diseases (Zottola, 1994; Mittelman, 1998).

E. New technologies

Pulsed electric field processing

Pulsed electric field (PEF) processing is one of the promising new technologies for the production of fluid milk (Jeyamkondan *et al*., 1999). This form of processing works by direct inactivation of microbes (Gould, 1996). The product to be processed is placed between two electrodes and the high-voltage pulses (typically 20 - 80 kV.cm^{-1}) are

applied to the product. The pulses are very short (µs), thus minimum heat is generated and the process remains non-thermal.

The PEF process is based on the fact that food normally contains ions, and these will cause a current to flow through the product which then causes microbial inactivation by dielectrical breakdown and electroporation of the cell membrane (Zhang *et al*., 1995a; Pothakamury *et al*., 1997). When an external electric field is applied to a cell, a transmembrane potential is induced across the cell membrane. Free charges build up on both sides of the membrane and attract each other, which compresses the cell membrane. When the generated transmembrane potential is greater than the cell's natural potential (1 V), the cell membrane looses stability and dielectric breakdown of the membrane occurs (Castro *et al*., 1993). This causes pore formation in the cell membrane with subsequent membrane permeability changes. There are several theories that explain pore formation but it is still unclear whether it occurs in the lipid or the protein matrices (Barbosa-Cánovas *et al*., 1999). The inability of a cellular membrane to function properly and regulate electron transport that controls small molecule movement in and out of the cells would, in time, result in microbial inactivation (Hamilton & Sale, 1967). Pothakamury *et al*. (1997) observed that the mechanism of inactivation by PEF differed from the mechanism for thermal inactivation which, at a temperature of 66°C for 10 min, damaged the cell organelles but no cell walls were ruptured as was the case for PEF treatments.

The effectiveness of PEF on the inactivation of microbes depends on several processing parameters: electric field strength; treatment time; treatment temperature; pulse length; pulse frequency; flow rate of the liquid product; pulse shape; and the number of pulses per chamber (Barbosa-Cánovas *et al*., 1999).

Pothakamury *et al*. (1996) reported that when *E. coli* cells are in the growth phase they are more sensitive to the effect of PEF than cells in the lag or stationary phases. Zhang *et al*. (1995b) observed that the initial concentration (1.15×10^3 - 7.14×10^8 cfu.ml^{-1}) of *E. coli* in simulated milk ultrafiltrate (SMUF) did not influence the rate of inactivation. Yeasts were found to be more susceptible to PEF than bacteria (Wouters & Smelt, 1997), while Gram-positive microbes are more resistant than Gram-negative microbes (Evrendilek *et al*., 1999; Rodrigo *et al*., 2001). Grahl & Märkl (1996) reported that endospore inactivation was not significant as no lethal effects were observed with *Clostridium tyrobutyricum*, *Bacillus cereus* and *B. nivea* endospores after a PEF treatment.

Grahl & Märkl (1996) noticed a decrease in efficiency of PEF when skimmed milk was used as substrate compared to when a buffer solution was used under the same processing parameters. They concluded that the presence of the fat particles of milk seemed to protect microbes against PEF. However, Reina *et al*. (1998) while evaluating the effect of the fat content of skimmed, 2% fat and full cream milk, reported no differences in the microbial elimination results.

No dangerous chemical reactions have been observed during PEF treatments, and the process also leads to minimal loss of fat and water soluble vitamins (Calderón-Miranda *et al*., 1999; Bendicho *et al*., 2002). Similarly, no significant flavour changes have been reported (Grahl & Märkl, 1996). Undesirable enzymes required more severe PEF treatments than microbes to obtain significant inactivation (Ho *et al*., 1997).

There are disagreements in the literature as to the levels of enzyme inactivation that may be obtained during PEF treatments, and especially the process parameters must be taken into consideration when comparing results between different authors. It has been shown that the degree of reduction of enzyme activity depends on the intensity of the electric field, the number of pulses applied, and also the characteristics of the particular enzyme (Vega-Mercado *et al*., 1995b; Grahl & Märkl, 1996; Bendicho *et al*., 2002). The media containing the enzyme, the treatment temperature and the enzyme concentration also influences the degree of enzyme inactivation (Castro *et al*., 2001). Castro *et al*. (2001) reported a 65% decrease in alkaline phosphatase activity in SMUF and 59% in 2% and full cream milk. In contrast, Grahl & Märkl (1996), Ho *et al*. (1997) and Van Loey *et al*. (2002) did not find any significant enzyme inactivation in either milk or aqueous solution. The differences in inactivation results might be due to differences in electrical parameters. Vega-Mercado *et al*. (2001) reported an 80% reduction in microbial protease (from *P. fluorescens*) activity when the enzyme was suspended in tryptic soy broth enriched with yeast extract. A 62% inactivation of commercial lipase from *P. fluorescens* suspended in SMUF was reported after a batch treatment, however, with a continuous process only a 13% inactivation was reached (Bendicho *et al*., 2002).

Although PEF can hold its own as a non-thermal method for the pasteurisation of milk, it has been reported that a simultaneous mild heat treatment (<50°C) enhances the efficiency of PEF (Sensoy *et al*., 1997; Reina *et al*., 1998). Sepulveda *et al*. (2005) found that the shelf-life of HTST pasteurised milk could be extended to 60 d at a storage temperature of 4°C when pasteurisation of the milk was immediately followed by a PEF

treatment at 35 kV.cm^{-1}. When HTST pasteurised milk was treated with PEF eight days after pasteurisation, a 78 d shelf-life was reached with storage at 4°C.

High pressure processing

High pressure (HP) of up to 1 000 MPa may be considered a non-thermal method of food preservation with the advantage of inactivating microbes in the food without adversely affecting the nutritional and sensory qualities or the colour of the product (Farkas & Hoover, 2000; Furukawa *et al*., 2001). Although the mechanism of inactivation of microbes by HP is not clearly understood, it is thought to induce changes in the morphology, biochemical reactions, genetic mechanisms and cell membrane and cell walls of microbes (Hoover *et al*., 1989). Smelt *et al*. (1994) showed changes in biochemical reactions when an increase in extracellular ATP was found in pressure-treated cells. Benito *et al*. (1999) confirmed this increased uptake of propidium iodide and ethidium bromide.

There are a number of factors influencing the resistance of microbes to high pressure treatments and include: treatment temperature; magnitude; treatment time; growth stage of microbes; and the composition of the suspension medium. The presence of lipids, carbohydrates, proteins and a low water activity influences the sensitivity of microbes to the lethal effect of high pressure (Isaacs & Chilton, 1995).

Mackey *et al*. (1995) found that microbes in the exponential phase are more sensitive to pressure. They reported a 7 log reduction for *Listeria monocytogenes* in the exponential phase after a 10 min treatment at 400 MPa, but only a 1.3 log reduction when viable cells in the stationary phase had undergone the same treatment. Gram-positive microbes are more resistant to high pressure than Gram-negative microbes (Patterson *et al*., 1995; Smelt, 1998). Yeasts and moulds are inactivated by pressures between 200 and 300 MPa. At 25°C, Gram-negative microbes require a pressure treatment between 300 - 400 MPa for inactivation, while Gram-positive microbes need at least 500 - 600 MPa for 10 min to achieve inactivation.

It is a well known fact that endospores may survive pressure treatments in excess of 1 000 MPa (Smelt, 1998). However, Hayakawa *et al*. (1994) reported that endospores may be destroyed by a combination of high temperatures (80°C) and high pressure (600 MPa), but the combination was only effective when the pressure treatment was applied in short pulses. Pressures between 50 and 300 MPa leads to the initiation of germination of endospores which may then be inactivated by further pressure or heat treatments.

Several research groups have reported that a combination of high pressure with heat increases any subsequent microbial inactivation (Earnshaw *et al*., 1995; Patterson & Kilpatrick, 1998). The use of such hurdle combinations has the advantage of reducing energy costs and also the production of safer and more palatable products (Black *et al*., 2005).

As with most non-thermal pasteurisation alternatives, a method to determine an adequate treatment is a serious problem where HP treatments are concerned. Alkaline phosphatase is not sensitive to HP, and can therefore not be used to indicate efficient 'pasteurisation' (Mussa & Ramaswamy, 1997). Up to now, HP has found little application in the dairy industry. This is mainly due to the initial capital expenditure (Smelt, 1998) and also the non-availability of large-scale commercial equipment (Mertens & Deplace, 1993).

F. High-power low-frequency ultrasound

There is a continuous industrial interest in developing alternative food preservation methods which may be used to replace the severe heat-based methods that are commonly used. Recent advances in the search for such non-thermal processing methods led researchers to investigate the application of ultrasound. The "killing" potential of ultrasound was realised when sonar was being investigated for use in anti-submarine warfare, and it was noticed that the sound waves caused fish to die (Earnshaw *et al*., 1995; Earnshaw, 1998).

Ultrasound can be defined as the energy generated by sound waves of 20 kHz to 800 kHz (Hoover, 1997). Large-scale applications involve high energy, low-frequency waves (between 20 and 100 kHz) and are often referred to as "power ultrasound". There is also an increasing interest in higher frequencies, however, these are normally well below the MHz range where it is difficult to achieve cavitation (Mason & Lorimer, 2002). Most macroscopic applications of ultrasound depend on compound acoustic phenomena occurring in matter, which, in turn, are caused by primary vibratory inputs. Thus, as acoustic pressure causes cavitation and microstreaming in liquids, vibratory stress causes heating and fatiguing in solids, and ultrasonic acceleration causes surface instability at the liquid-liquid and liquid-gas interfaces (Shoh, 1988).

The physics of ultrasound is complex and despite information available, many aspects of the mechanism remain obscure (Suslick, 1988). There are numerous theories as to the precise mechanism of microbial inactivation by ultrasound, with

cavitation being the most popular theory. An ultrasonic wave travelling through a liquid consists of alternate compressions and rarefactions. If the amplitude of the ultrasonic wave is high enough, cavitation, which is the making and breaking of microscopic bubbles, will occur (Goldman & Lepschkin, 1952). The cavitation bubbles grow by a process known as rectified diffusion. This is where small amounts of vapour from the medium enter the bubble during its expansion phase that is not fully expelled during compression. The bubbles grow over the period of a few cycles to an equilibrium size for the particular frequency applied. It is the fate of these bubbles when they intensely collapse in succeeding compression cycles that generates the energy for chemical and mechanical effects (Neppiras, 1984; Henglein, 1987; Suslick, 1990). There are two types of cavitation, stable and transient cavitation, and each exhibits different kinds of bubble behaviour in response to an acoustic field (Frizzell, 1988).

During stable cavitation, bubbles oscillate in response to the ultrasonic pressure field. The bubble radius varies about an equilibrium value, and the bubble exists for a number of acoustic cycles without collapsing or otherwise leaving the field. Acoustic streaming and high shear stresses may be associated with such stable cavitation activity (Ter Haar, 1988). During oscillation, stable cavitation can, under given circumstances, change to transient cavitation, and vice versa (Scherba *et al.*, 1991). It has been reported that the killing of cells relies on transient cavitation (Leighton, 1995).

Transient cavitation occurs during the compression phase in media that experience a tension stress during a portion of the rarefaction phase of the acoustic disturbance (Frizzell, 1988). The bubble oscillates in an unstable manner about the equilibrium radius, grows several times its equilibrium size, and collapses violently (Ter Haar, 1988). Each bubble collapse acts as a localised 'hotspot' generating temperatures of above 5 000°C and pressures of roughly 500 MPa, depending on the liquid medium being treated (Suslick, 1988; 1989; 1990). These high pressures and temperatures, as well as high pressure shockwaves that radiate from the location of the bubble are capable of causing mechanical damage to the surrounding material (Frizzell, 1988). Butz & Tauscher (2002) proposed that the mechanism of microbial killing is mainly due to the thinning of cell membranes, localised heating and the production of free radicals. The high temperatures may cause bond dissociations in molecules, thus producing free radicals that can react with biomolecular species in much the same way as those produced by ionising radiations (Frizzell, 1988). There is also evidence that sonication of cells in a suspension may lead to cell lysis, and subsequently, complete cell destruction. Cavitation has been shown to be the major cause of this effect

(Kaufman *et al*., 1977; Morton *et al*., 1982). Where cell lysis occurs, it is an immediate consequence of ultrasonic exposure, and eukaryotic cells in mitosis may be more susceptible than those in other stages of the cell cycle (Clarke & Hill, 1970).

The inhomogeneous cyclic field around stably oscillating bubbles can cause microcurrents by creating a steady flow of the liquid medium surrounding the bubbles. This phenomenon is known as microstreaming (Frizzell, 1988). The effect of microstreaming near bubbles can be one of mixing, however, if the streaming velocities are great enough, it may be sufficient to damage cells (Hughes & Nyborg, 1962; Frizzell, 1988).

Scherba *et al*. (1991) reported that is has been mathematically demonstrated that practically the whole of the lethal effect of sonication is due to the pressure changes responsible for the disruption of cellular structures. They proposed that the target of ultrasonic damage may be the cytoplasmic membrane, which consists of a lipoprotein bilayer, since the structure of the peptidoglycan layer did not appear to be a factor. Davies (1959) suggested that mechanical disruption is the most probable mechanism of action of ultrasound. It has been observed that microbes can survive high pressures; however, they are incapable of withstanding the quick alternating pressures produced during cavitation.

Critical processing factors

When ultrasound is applied in the food industry as a "pasteurising" or "sterilising" technology the critical processing factors will probably be the amplitude of the ultrasonic waves, contact time with the microbes, the type and number of microbes present, and the volume and temperature of the liquid to be processed (Hoover, 2000).

It is generally assumed that the larger the microbial cells are in size, the more sensitive to the effects of ultrasound they will be. It has been reported that coccoids show more resistance when compared to rods (Jacobs & Thornley, 1954). Gram-positive microbes have also been found to be more resistant to sonication, as opposed to Gram-negatives. Similarly, aerobic microbes are more resistant than anaerobes (Ahmed & Russell, 1975). The age of the cells is another important factor affecting sensitivity. Kinsloe *et al*. (1954) reported that young (4 h) *Saccharomyces cerevisiae* cells were more sensitive than older cells (24 h). Sanz *et al*. (1985) reported that endospores are much more resistant than vegetative cells, and even questioned the ability of ultrasound to disrupt endospores.

Jacobs & Thornley (1954) reported, after studying different microbes, that resistance to ultrasound was always greater in food than in nutritive broth. They also found that *E. coli* was more resistant when treated in milk containing more fat. Lee *et al*. (1989) reported that when treating *Salmonella* in either peptone water or chocolate milk, the chocolate milk had a definite protective effect. When *Salmonella eastbourne* was treated in peptone water (0.1%), a *D*-value of 3.8 min was achieved for a 6.3×10^6 cfu.ml^{-1} inoculum, and the population was reduced to 2.0×10^4 cfu.ml^{-1} after a 10 min treatment. When *S. eastbourne* was inoculated in milk chocolate, a 0.78 log reduction (74%) was achieved after 30 min of sonication.

Parameters influencing cavitation

The cavitational threshold of a medium can be defined as the minimum oscillation of pressure that is required to produce cavitation. There are a number of factors determining the cavitational threshold of a medium including: dissolved gas; hydrostatic pressure; specific heat of the gas in the bubble; specific heat of the liquid; and also the tensile strength (Atchley & Crump, 1988). It has also been reported that there is an inverse relationship between the cavitational threshold of a medium and the reciprocal of temperature. As temperature increases, the cavitational threshold decreases and becomes zero at the boiling point of the liquid (Atchley & Crump, 1988). Liquids should therefore be processed at the lowest possible temperature.

Berlan & Manson (1992) reported that the cavitational threshold will increase as the concentration of solids in a liquid medium decrease, or when the ion concentration increases. However, it will ultimately depend on the frequency of the sonic waves. The frequency of sonication in the usable range is an important parameter as it determines the maximum bubble size before implosion (Suslick, 1989). The lower the frequency, the bigger the critical size of the bubbles, and therefore, the intensity of collapse will be bigger. At very high frequencies (1 MHz) cavitation is more difficult, and above 2.5 MHz cavitation does not occur (Alliger, 1975). As the frequency of sound waves increases, the amplitude decreases, resulting in a decrease in size of the cavitating bubbles that may form. This leads to less violent collapses once the equilibrium sizes of the bubbles are reached (Alliger, 1975; Berliner, 1984).

The lethal effects of ultrasound also depend on the intensity of the implosions. Energy liberated by cavitation depends on the ratio of the radius of a bubble at maximum size to the initial ratio (Berliner, 1984). Intensity is a measure of the energy available per unit volume of sample, and it is directly related to amplitude. It is normally

assumed that "more electrical power is better", however, this is often not the case. As stated above, it is the intensity of cavitation that breaks cells, shears DNA strands and mixes immiscible liquids, not the total power applied to the system. Intensity is directly related to the amplitude of the radiating face of the tip or horn, thus, it is the amplitude that must be provided, maintained and monitored (Berliner, 1984).

Another limiting factor is the temperature of the liquid. Cavitation nuclei and bubbles form and grow quicker at higher liquid temperatures, because the vapour pressure increases and the tensile strength decreases. However, although more bubbles are formed, the violence of collapse is smaller because the high vapour tension acts as a cushion (Alliger, 1975; Berliner, 1984). For this reason cell disruption processes should be carried out at the lowest possible temperature. Low temperature sonication, does however, require more power to overcome the lower vapour pressure, and results in higher intensity shock front propagation (Berliner, 1984). Higher pressures, viscosity, surface tension or concentrations require more power to produce cavitation. Power can be defined as the energy required to drive the radiating surface of a given horn per unit time (Berliner, 1984).

It is evident that there are many different parameters influencing the effect of cavitation, and most need to be optimised in order to maximise cavitation for food processing (Berlan & Mason, 1992).

Power ultrasound equipment

When an alternating voltage is applied to a piezoelectric crystal, the crystal changes shape in relation to the electric field (Alliger, 1975). The crystal acts as an ultrasonic transducer converting electrical energy into mechanical or acoustic energy (Mason, 1998). This is known as the piezoelectric effect. These continuous changes in shape or length are the pulsations which travel through the liquid (Alliger, 1975).

There are two main types of ultrasonic transducers, magnetostrictive transducers and piezoelectric transducers. Piezoelectric transducers are utilised most frequently for the generation of ultrasound, and use ceramics containing piezoelectric materials such as barium titanate or lead meraniobate. Lead zirconate titanate (PZT) is the most commonly used ceramic. It has a polarisation temperature of 350°C (Curie temperature) and can operate over the whole ultrasonic range (Shoh, 1988). Shoh (1988) reported the electromechanical conversion efficiency to be 98%. However, the PZT crystals have unique geometrics for different frequencies that need to be taken in

consideration when manufacturing a crystal for processing at any specified frequency (Mason, 1998).

Irrespective of the transducer used, the equipment needed to deliver the ultrasonic energy to the liquid system under investigation can be separated into three essential parts:

- a generator to convert electricity (220V ac) into high frequency voltage to drive the transducer;
- a transducer which converts the high frequency voltage into mechanical (ultrasonic) vibrations. The power available through a given transducer changes inversely with the square of the frequency, therefore, for higher power applications, equipment operating at lower frequencies is preferred; and
- a delivery system which matches the vibration to the liquid. Typically, in higher power systems the acoustic vibration is amplified and conducted into the liquid by the attachment of a 'horn' to the transducer. After prolonged high power use the tip of the horn may become eroded, thereby altering the overall length of the horn. Therefore, horns are normally fitted with a replaceable tip (Mason, 1998). Titanium alloy is the best horn material, combining outstanding acoustical properties with lightness, strength, abrasion resistance, and a chemical inertness better than that of stainless steel (Berliner, 1984).

G. Remarks on problems and applications

There are numerous options for the treatment of milk with the aim of rendering it free of spoilage and potentially pathogenic microbes. These options vary in terms of initial capital investment, operating costs, efficiency, treatment time, etc. One such technique that clearly stands out from the rest is sonication as the nature of this application makes ultrasound a very attractive option for the dairy industry. When ultrasound is compared to other treatment methods, there is not an abundance of data available on the lethality of ultrasound. However, promising results from the older literature, albeit quite dated, merits further research into the application of ultrasound as a pasteurisation alternative.

Ultrasound as a processing technique could possibly be installed in-line as part of an existing milk processing system. Equipment presently available to the dairy industry can be considered to be light and portable, it is easy to clean with fouling of pipes being eliminated, electrical efficiency is better than when compared to traditional heating processes, and no additional equipment, such as boilers, is needed.

The main crux of sonication as a means of lowering microbial levels is the kinetics of the process. In this case *D*-values are essential when comparing results from different researchers. The lack of *D*-values in research papers from the literature makes comparisons and conclusions between results from the literature difficult or even impossible. Furthermore, there is also difficulty and some confusion involved when evaluating published results as researchers use different types of sonication apparatus. To add to these comparison problems, different power levels are used by different researchers and this result in different cavitation intensities, subsequently influencing the efficiency/lethality of the technique.

Ultrasound has enormous potential as a sterilisation technique. However, the need exists for data obtained under standardised and reproducible conditions before the dairy industry will fully recognise the immense potential of this economical and eco-friendly alternative to traditional thermal pasteurisation.

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CHAPTER 3

IMPACT OF LOW-FREQUENCY HIGH-POWER ULTRASONICATION ON THE SURVIVAL OF *ESCHERICHIA COLI*

Abstract

Numerous reports in the literature suggest pasteurisation failures in the dairy industry as a possible cause for an end product with a poor quality. The aim of this study was to evaluate the use of ultrasonication as an alternative to heat pasteurisation. Three *Escherichia coli* strains, a reference strain (ATCC 11775), a wild strain isolated from "raw" milk, and an O157:H7 strain, were used as "test" microbes. The suspension media included a saline solution and UHT milk inoculated with the "test" microbes in the exponential growth phase to give a final microbial concentration of either 1 x 10^4 or 1 x 10^6 cfu.ml^{-1}. The samples were then subjected to ultrasound for different time intervals using low-frequency high-power ultrasound (20 kHz, 750 W). Viable cell counts of *E. coli* were reduced by 100% after 10.0 min for a 1 x 10^4 cfu.ml^{-1} inoculum. Ultrasonication with low cell inoculations (2 x 10^3 cfu.ml^{-1}) was very effective with a 100% elimination of *E. coli* cells within 5.0 min. It was also found that pulse-ultrasonication did not enhance the killing effect of ultrasound. A higher wave amplitude (100%) was found to be more efficient when compared with a lower wave amplitude (50%). No difference in sensitivity to the effect of ultrasound was detected between the different *E. coli* strains tested. The composition of the suspension media (saline solution or milk) did not influence the success of ultrasonication.

Introduction

Traditional thermal pasteurisation and sterilisation processes are the most common methods used by the food and dairy industry for the inactivation of microorganisms (Ciccolini *et al*., 1997; Piyasena *et al*., 2003). Although *E. coli* is reported to be destroyed by pasteurisation (Muir, 1996; Holsinger *et al*., 1997), there are reports on its ability (including the pathogenic strain O157:H7) to form biofilms within pasteurisation equipment, leading to pasteurisation failures (Dewanti & Wong, 1995; Stopforth *et al*., 2003).

Increases in temperature can irreversibly modify milk components, for example the degradation of vitamins, precipitation of Ca^{2+} and the denaturation of serum proteins. Heating may also disrupt the physiochemical equilibrium of milk components (Eckner & Zottola, 1991; Simon & Hansen, 2001). Consumer demands for food products that are fresher, more natural, healthier (Mertens & Knorr, 1992), with a better nutritional content and higher overall quality, are increasing (Piyasena *et al*., 2003). Thus, new preservation techniques including ultrasonication, which can eliminate microbial activity at lower or even without heat addition, are being developed (Qin *et al*., 1996; Piyasena *et al*., 2003).

Ultrasonication may be considered an alternative to the heat treatment of milk as it is less energy-intensive and therefore more cost-efficient and environmentally friendly (Piyasena *et al*., 2003). High-power ultrasound is known to damage or disrupt biological cell walls which will result in the destruction of living cells. Unfortunately, very high intensities are required if ultrasound alone is to be used for complete sterilisation (Mason *et al*., 1996). The bactericidal effect of ultrasound is generally attributed to intracellular cavitation (Hughes & Nyborg, 1962). It has also been suggested that micro-mechanical shocks are created by the making and breaking of microscopic bubbles generated by fluctuating pressures during the ultrasonication process. These shocks disrupt cellular structural and functional components and result in cell lysis (Hoover, 2000; Butz & Tauscher, 2002). According to Ciccolini *et al*. (1997), the effects of cavitation on microbial suspensions include: dispersion of microbial clumps; cell wall puncturing; modification of cellular activity; and increased sensitivity to heat. However, it must always be remembered that the effectiveness of ultrasonication is known to be influenced by the microbial strain tested, the suspending medium and the size of the cell (Wase & Patel, 1985; García *et al*., 1989; Lee *et al*., 1989).

Other advantageous effects of ultrasonic waves in milk include: fat may be homogenised; gases are removed (Burger & Winder, 1954); and the antioxidant activity enhanced (Taylor & Richardson, 1980). Villamiel & de Jong (2000) reported that continuous-flow ultrasonic treatment could be a promising technique for milk processing.

The aim of this study was to investigate the effectiveness of high-power, low-frequency ultrasound (20 kHz, 750 W) to eliminate *Escherichia coli* from milk.

Materials and methods

Bacterial cultures

The following *E. coli* strains where evaluated in this study: a standard reference strain ATCC 11775 obtained from the University of Stellenbosch, Department of Food Science Culture Collection (USFSCC); a strain isolated from "raw" milk using VRB agar (Merck); and an O157:H7 strain obtained from Prof. T.J. Britz, Department of Food Science, University of Stellenbosch. Strain purity was regularly checked by microscopy and Gram stains, and the identity confirmed using the API 20E system (bioMérieux sa, Marcy-l'Etiole, France).

A broth subculture of the appropriate *E. coli* strain was prepared by inoculating 10 ml sterile nutrient broth (Merck) with a "test" microbe, and incubating for 24 h at 37°C. A 100 ml sterile container, containing 90 ml broth was inoculated with 5 ml of the 24 h culture and incubated for a further 24 h prior to the ultrasonic treatments.

Standard growth curves

The optical density (OD) of each strain was determined spectrophotometrically at 500 nm (Spectronic 20 Genesys, Spectronic Instruments, Cape Town). A dilution series was made in 0.85% (m/v) sterile saline solution (SSS). Growth curves were done in triplicate.

Ultrasonication

Two ml of the appropriate *E. coli* culture was centrifuged for 10 min at 6 000 x *g* (Eppendorf 5415D). The pellet was suspended in SSS and the data from the standard curve was used to determine the desired cell concentration for inoculation of the suspension media. The suspension media, either full cream (3.4% milk fat) UHT (ultra high temperature) milk or SSS, were inoculated with an aliquot of culture to yield an approximate inoculum level of either 1×10^4 or 1×10^6 colony forming units per ml ($cfu.ml^{-1}$) (initial concentration = N_0).

For ultrasonication, a 40 ml sample of the inoculated suspension media was pipetted 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. In most studies *E. coli* ATCC 11775 was used. Samples were treated using five different time regimes:

1. 2.5, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude;
2. 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 min at 100% wave amplitude;
3. 2.5, 5.0, 10.0 and 15.0 min at either 50% or 100% wave amplitude;
4. 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude on low microbial levels;
5. 2.5, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude with on/off pulsing combinations of 5 s/5 s, 10 s/5 s or 10 s/10 s;
6. 2.5, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude on all three different *E. coli* strains.

All ultrasonic treatments were done in duplicate. Duplicate dilutions were made from each treated sample; the pour-plate technique and plate count agar (PCA) (Merck) were used for enumeration. Plates with between 30 and 300 colonies were selected for counting (Anon., 1997). UHT milk and SSS samples that had not been inoculated with a "test" microbe, served as controls. The controls showed no microbial growth after 24 h of incubation.

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}.

Results and discussion

The dairy industry generally consider the presence of *E. coli* in dairy products as an indication of faecal and post-pasteurisation contamination (Holsinger *et al*., 1997). The SA "milk law" states that when the VRB MUG agar method is used, no *E. coli* may be present in 1.0 ml of milk (Anon., 1997). Gram-negative microbes have been reported to be very sensitive to ultrasonication (Ahmed & Russell, 1975); however, small microbes tend to be more resistant despite their Gram-status (Jacobs & Thornley, 1954).

Although the use of ultrasound as a "sterilisation" technique, with reports dating back to before 1954, is not new, recent advances in acoustic technology has enabled researchers to construct equipment that is able to deliver more power than a decade ago. This increase in available power ultimately results in better cavitation, increasing the lethality of this technique.

The standard growth curve of *E. coli* ATCC 11775 (Fig. 1) was used as a reference to determine the cell inoculation size, after the appropriate dilutions (exponential phase), for the ultrasonication studies.

Study 1 - Ultrasonication for 2.5, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude

The actual counts (cfu.ml^{-1}) are given in Fig. 2 and the recalculated data are given in the form of log graphs (Fig. 3). In this study (Fig. 2), a 99.99 - 100% elimination of *E. coli* was achieved after 10.0 min of ultrasonication for all five batch inoculum concentrations (1 x 10^4 cfu.ml^{-1} or 1 x 10^6 cfu.ml^{-1}, in both the SSS and milk). The 1 x 10^4 cfu.ml^{-1} inoculum in SSS was reduced to zero cfu.ml^{-1} after the 10.0 min treatment (Fig. 2); this is equivalent to a 3.88 log reduction (Fig. 3). Similar results were obtained for the other initial concentrations (N_o) where 1 x 10^6 cfu.ml^{-1} in SSS was reduced to 142 (a 3.93 log reduction), 2 x 10^4 cfu.ml^{-1} in milk was reduced to zero (a 4.32 log reduction), 1 x 10^5 cfu.ml^{-1} in milk was reduced to zero (a 4.42 log reduction) and 1 x 10^6 cfu.ml^{-1} in milk was reduced to zero (a 5.34 log reduction) (Figs. 2 and 3). The D_{US} values for *E. coli* were, depending on the starting concentration, 2.0 min (1 x 10^4 cfu.ml^{-1}) and 2.7 min (1 x 10^6 cfu.ml^{-1}) in SSS and 2.3 min (2 x 10^4 cfu.ml^{-1}), 2.0 min (1 x 10^5 cfu.ml^{-1}) and 1.9 min (1 x 10^6 cfu.ml^{-1}) in milk. The steeper slope of the regression line for the 1 x 10^6 cfu.ml^{-1} inoculum in milk (Fig. 3), and the subsequent lower D_{US} value of 1.9 min, is ascribed to a different, newer set of apparatus used for this specific set of data. This is an important technical aspect that will be addressed in a later study.

In 1979, Utsunomiya & Kosaka reported a 0.83% survival of *E. coli* (99.17% reduction) in saline after 10 min when treated at 700 kHz, but surprisingly, they reported no inactivation of *E. coli* in milk. In their article Utsunomiya & Kosaka (1979) did not mention which type of milk was used, which would also influence the efficacy of ultrasonication. The results obtained in this study compares well with the findings of Utsunomiya & Kosaka (1979) in terms of the % reduction achieved after a 10 min treatment in saline. However, the initial inoculation concentration was not mentioned, and a very high initial concentration or unavailability of enough power at a frequency of

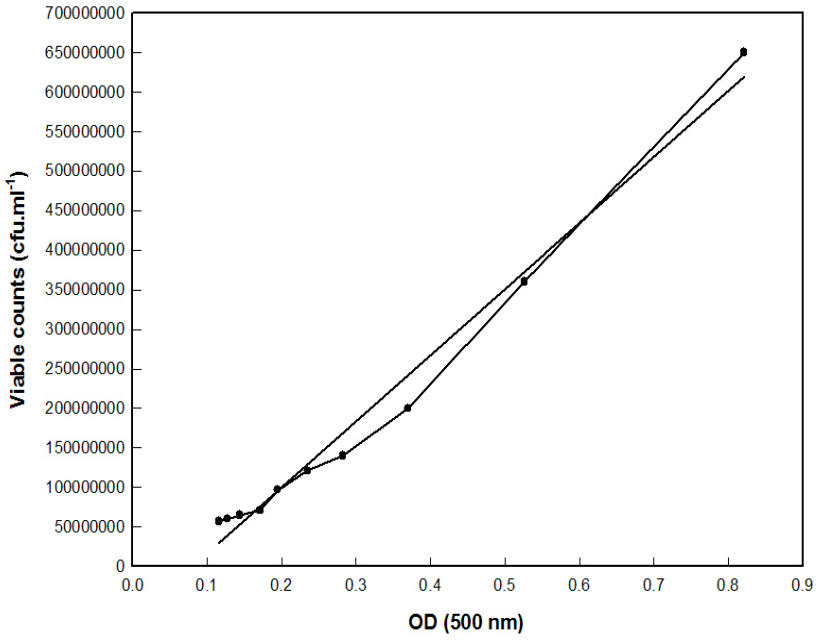

Figure 1 Standard growth curve of 24 h pure cultures of *Escherichia coli* ATCC 11775 (Data points represents averages of three repeats).

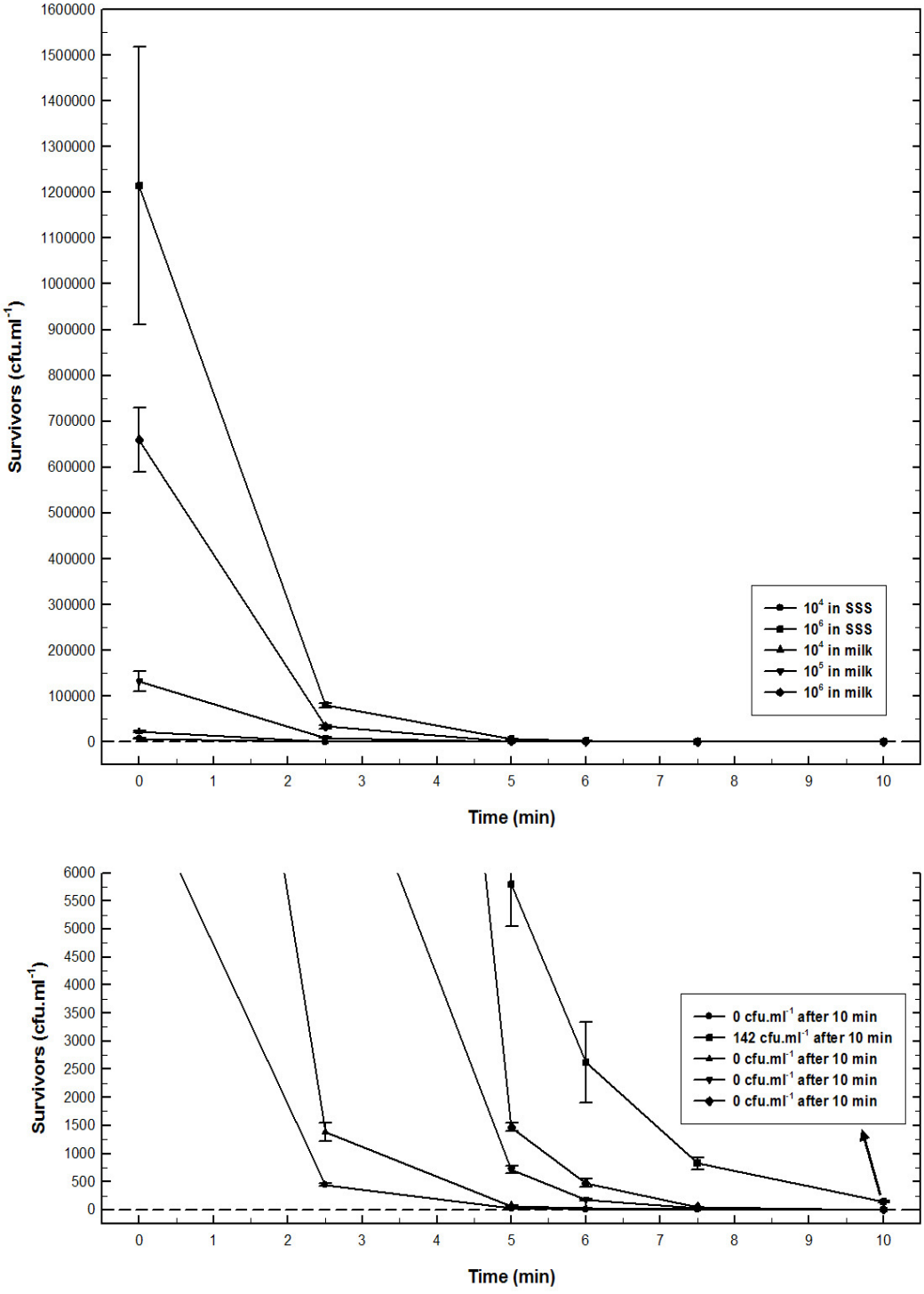

Figure 2 Impact of ultrasonication at 20 kHz on *Escherichia coli* ATCC 11775 at different starting concentrations in either SSS or UHT milk. The lower graph represents an expanded Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

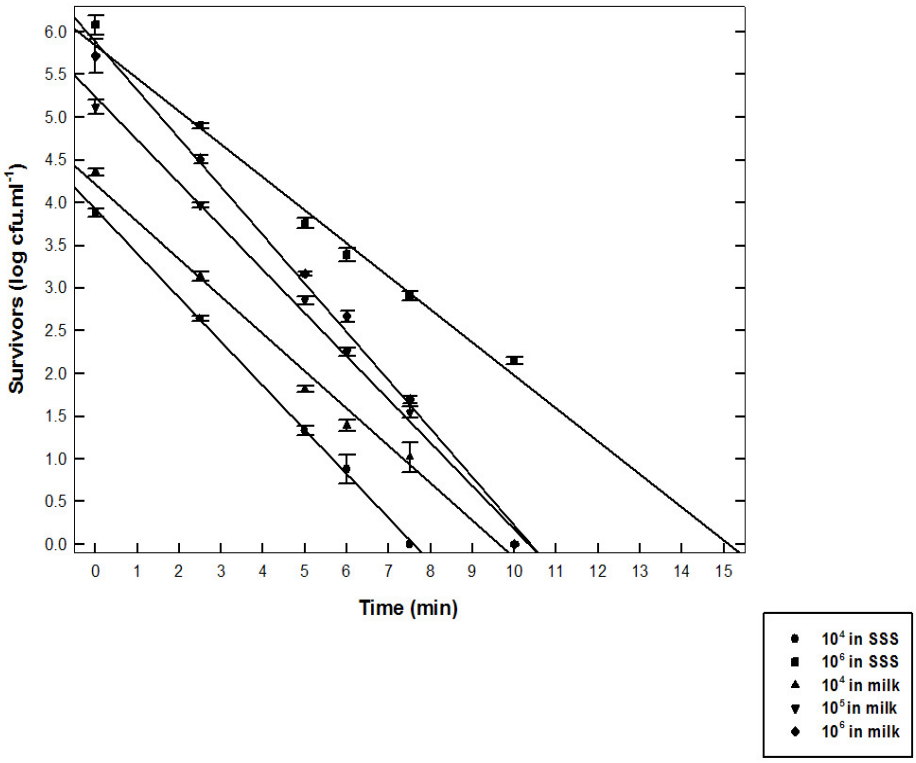

Figure 3 Regression (95% confidence level) of the data showing the impact of ultrasonication at 20 kHz on *Escherichia coli* ATCC 11775 at different starting concentrations in either SSS or UHT milk (Each data point represents quadruple values. The standard deviation was used as the error-bar).

700 kHz might be the reason why 100% elimination was not achieved. Contrary to the findings of Utsunomiya & Kosaka (1979), the suspension media had no effect on the efficiency of ultrasonication during this study. Jacobs & Thornley (1954) suggested that the fat globules present in milk might have a protective effect, rendering ultrasonication inefficient. This proclaimed protective effect of milk was not observed under the parameters used during this study.

Study 2 - Ultrasonication for 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 min at 100% wave amplitude

In Study 1, it was found that the first 2.5 min of ultrasonication often resulted in the highest elimination of viable cells (Fig. 2). To confirm this observation, the first 2.5 min of the treatment was split into 30 s sections to determine at which time the bacteria were the most sensitive to the effect of ultrasound. The actual counts ($cfu.ml^{-1}$) are given in Fig. 4 and the recalculated data are given in the form of log graphs (Fig. 5).

The results from this study confirmed the findings of Study 1, where 93.23% of viable *E. coli* cells were inactivated during the first 2.5 min, compared to 90.64% for this study when 1 x 10^4 $cfu.ml^{-1}$ was sonicated in SSS and 95.85% when 1 x 10^5 $cfu.ml^{-1}$ was sonicated in milk (Fig. 5). Thus, ultrasonication of 1 x 10^4 (SSS) and 1 x 10^5 (milk) viable *E. coli* cells for 3.0 min resulted in 1.15 and 1.75 log reductions, respectively (Fig. 5). The D_{US} was calculated to be 2.6 min (1 x 10^4 $cfu.ml^{-1}$ in SSS) and 1.6 min (1 x 10^5 $cfu.ml^{-1}$ in milk).

Study 3 - Ultrasonication for 2.5, 5.0, 10.0 and 15.0 min at either 50% or 100% wave amplitude

The impact of wave amplitude was investigated with an extended maximum treatment time of 15.0 min. The actual counts ($cfu.ml^{-1}$) are given in Fig. 6 and the recalculated data are given in the form of log graphs (Fig. 7).

In this study, ultrasonication of *E. coli* for 10.0 min at 100% wave amplitude in SSS resulted in a 4.02 log reduction (100%) for a 1 x 10^4 $cfu.ml^{-1}$ inoculum. When 50% wave amplitude was used, a 99.29% or 2.15 log reduction of initial viable counts was observed for a 1 x 10^4 $cfu.ml^{-1}$ inoculum after a 15.0 min ultrasonic treatment (Fig. 7). The D_{US} at 100% wave amplitude was calculated to be 2.6 min, and at 50% wave amplitude the D_{US} for *E. coli* was 7.0 min. The longer D_{US} for the sample treated at 50%

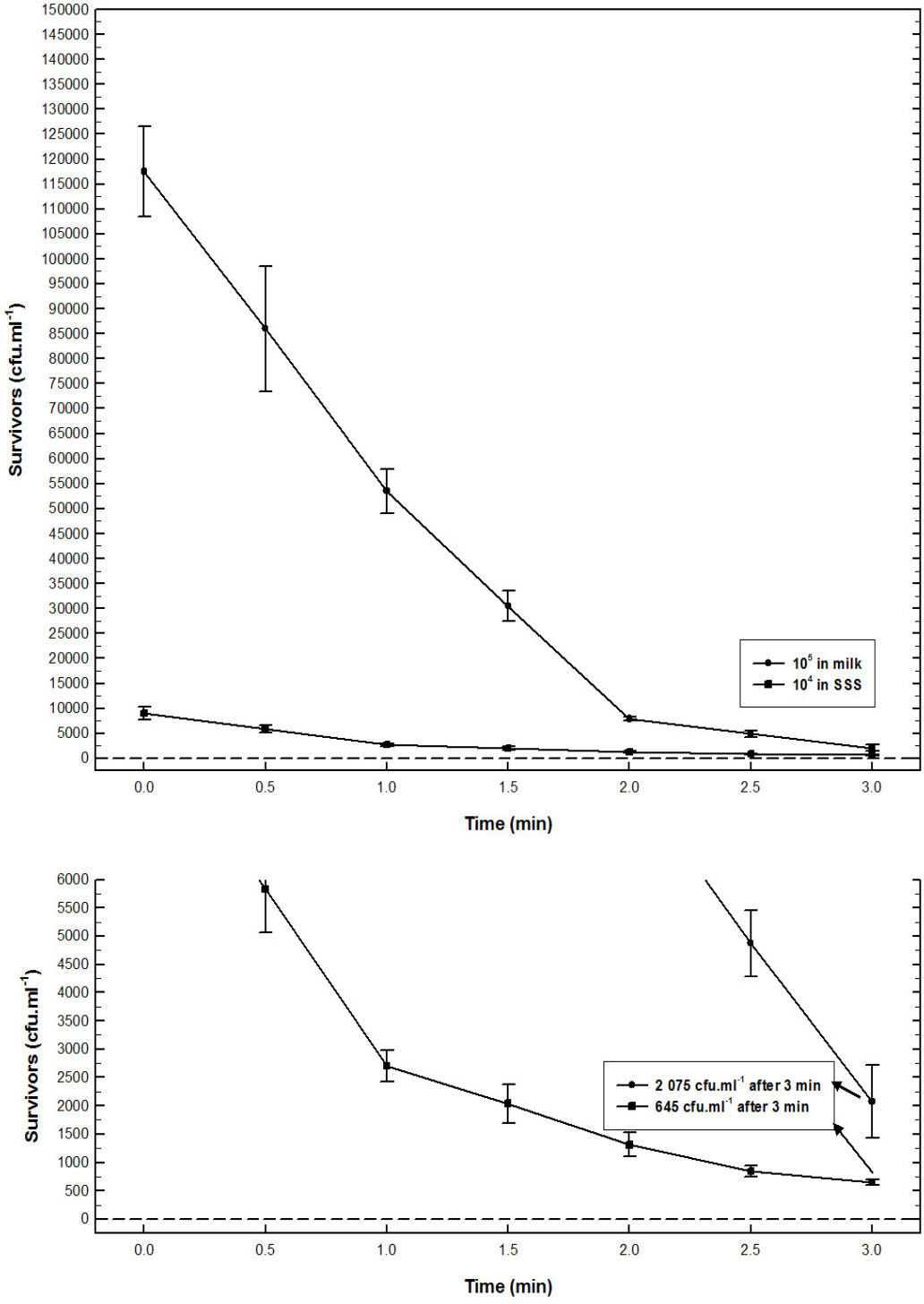

Figure 4 Impact of ultrasonication at 20 kHz on *Escherichia coli* ATCC 11775 at a starting concentration of 1 x 10^5 cfu.ml^{-1} in UHT milk and 1 x 10^4 cfu.ml^{-1} in SSS. The lower graph represents an expanded Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

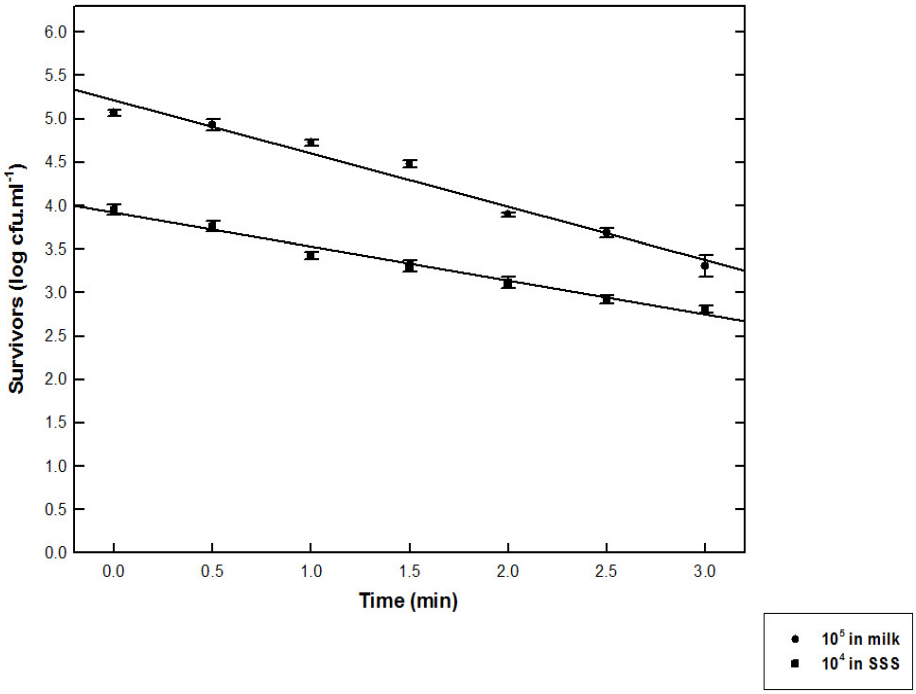

Figure 5 Regression (95% confidence level) of the data showing the impact of ultrasonication at 20 kHz on *Escherichia coli* ATCC 11775 at a starting concentration of 1 x 10^5 cfu.ml^{-1} in UHT milk and 1 x 10^4 cfu.ml^{-1} in SSS (Each data point represents quadruple values. The standard deviation was used as the error-bar).

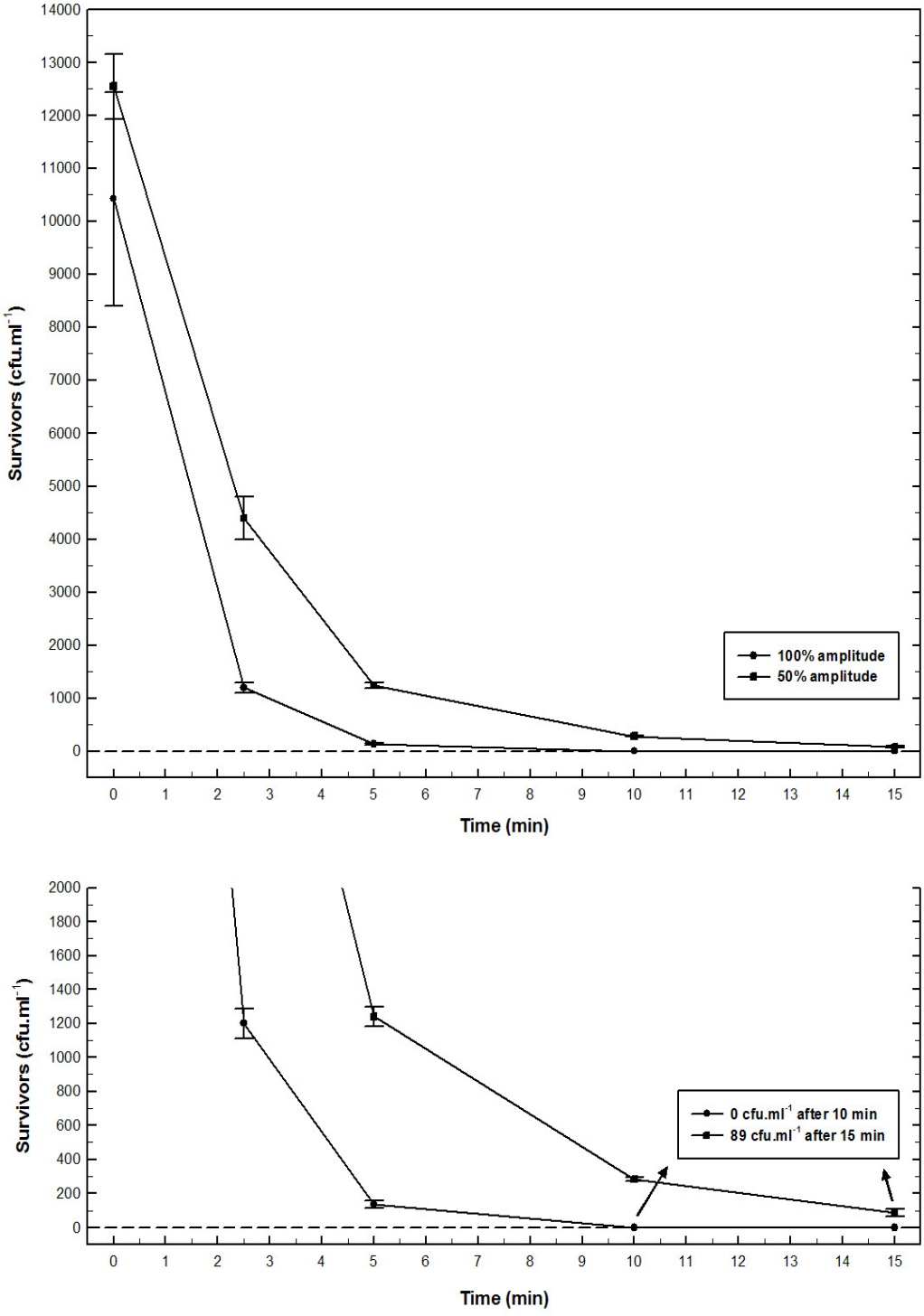

Figure 6 Impact of ultrasonication at 20 kHz and either 100% or 50% displacement amplitude on *Escherichia coli* ATCC 11775 in SSS. The lower graph represents an expanded Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

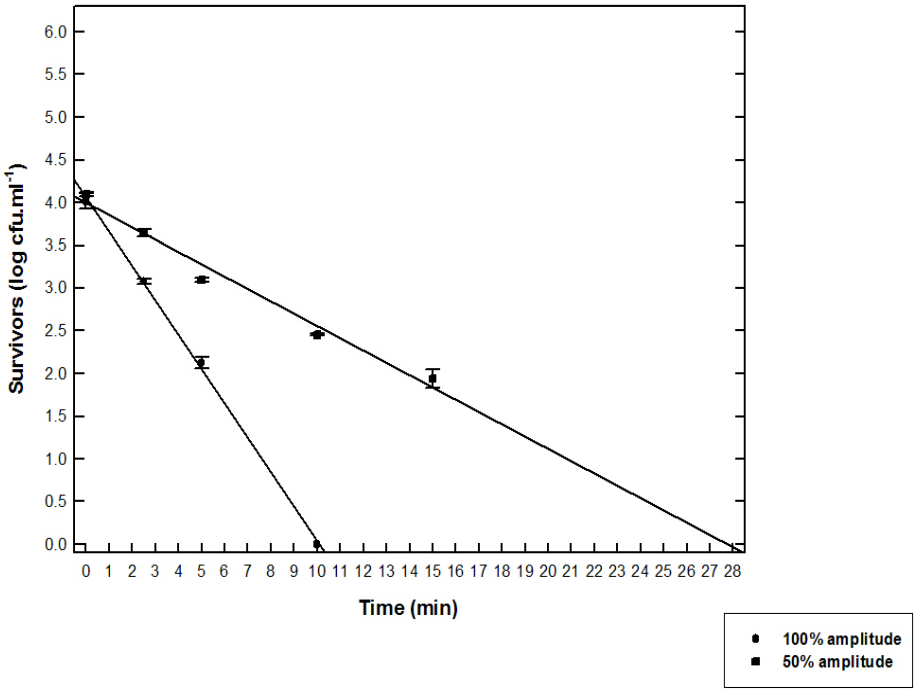

Figure 7 Regression (95% confidence level) of the data showing the impact of ultrasonication, either 100% or 50% displacement amplitude, at 20 kHz and on *Escherichia coli* ATCC 11775 in SSS (Each data point represents quadruple values. The standard deviation was used as the error-bar).

wave amplitude is probably due to the lower power available during ultrasonication, resulting in cavitation at a lower intensity than when 100% wave amplitude was applied.

Study 4 - Impact of ultrasonication for 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude on milk with a low microbial load

The effect of ultrasound on low initial microbial loads was investigated in this study. This was done to determine what effect ultrasound would have on good quality "raw" milk, with an initial *E. coli* load of 2 x 10^3 cfu.ml^{-1}. The actual counts (cfu.ml^{-1}) are given in Fig. 8 and the recalculated data are given in the form of log graphs (Fig. 9).

Ultrasonication of milk containing *E. coli* at lower initial levels proved to be very effective with a 100% elimination (3.38 log cycles) being reached after 5.0 min. The D_{US} was calculated to be 1.6 min (Fig. 9). Thus, it was concluded from the data that the use of ultrasonication for milk with a low initial bacterial load shows great promise for the implementation of this technique with a 100% elimination of the dairy faecal contamination indicator, *E. coli*.

Study 5 - Pulse-ultrasonication for 2.5, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude with on/off pulsing combinations of 5 s/5 s, 10 s/5 s or 10 s/10 s

One question that arose as this study progressed was whether a pulsing scenario would lead to an enhancement of cell destruction. To exclude any possible protective effect of proteins and lipids present in milk, this study was done in SSS only. The actual counts (cfu.ml^{-1}) are given in Fig. 10 and the recalculated data are given in the form of log graphs (Fig. 11).

No pulsing

As described in the previous studies.

Duty cycle: 5 s on/5 s off

The first pulsing combination of the samples was for 5 s, followed by a 5 s off period. This process was repeated for the indicated treatment times: 2.5, 5.0, 6.0, 7.5 and 10.0 min. Pulse-ultrasonication of *E. coli* for 10.0 min in SSS resulted in a 3.39 log reduction (99.96%) of the initial 1 x 10^6 cfu.ml^{-1} inoculum (Fig. 11). The D_{US} was calculated to be 3.0 min.

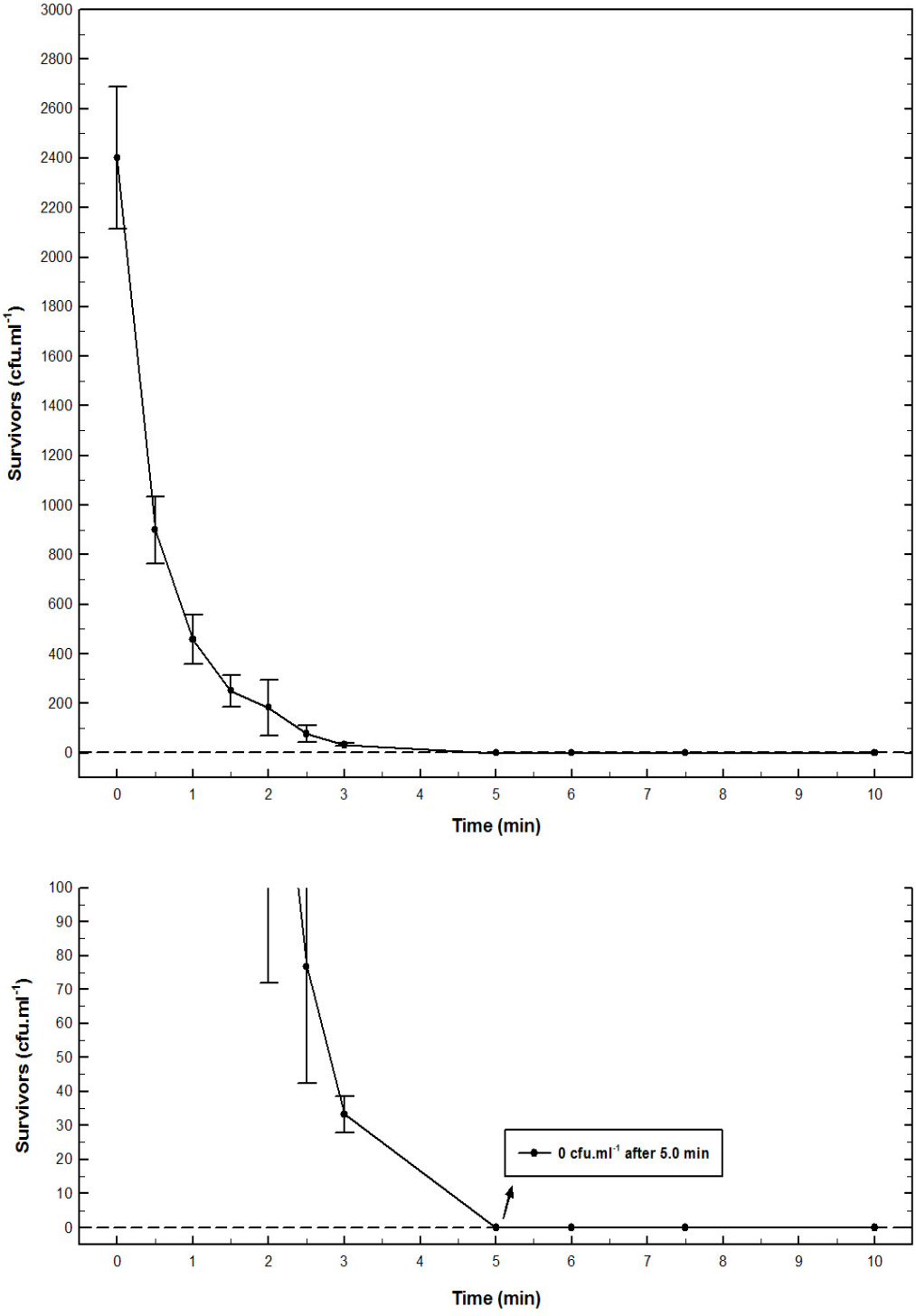

Figure 8 Impact of ultrasonication at 20 kHz on *Escherichia coli* ATCC 11775 at a starting concentration of 2 x 10^3 cfu.ml^{-1} in UHT milk. The lower graph represents an expanded Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

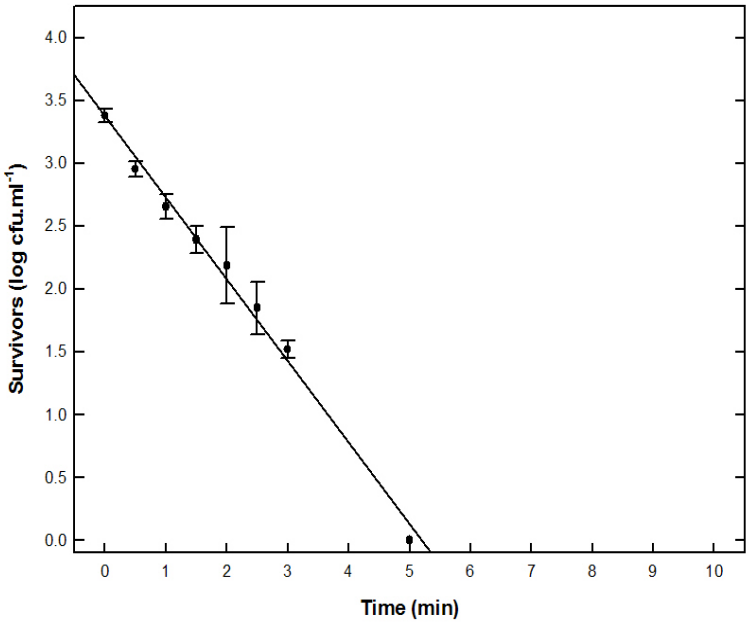

Figure 9 Regression (95% confidence level) of the data showing the impact of ultrasonication at 20 kHz on *Escherichia coli* ATCC 11775 at a starting concentration of 2 x 10^3 cfu.ml^{-1} in UHT milk (Each data point represents quadruple values. The standard deviation was used as the error-bar).

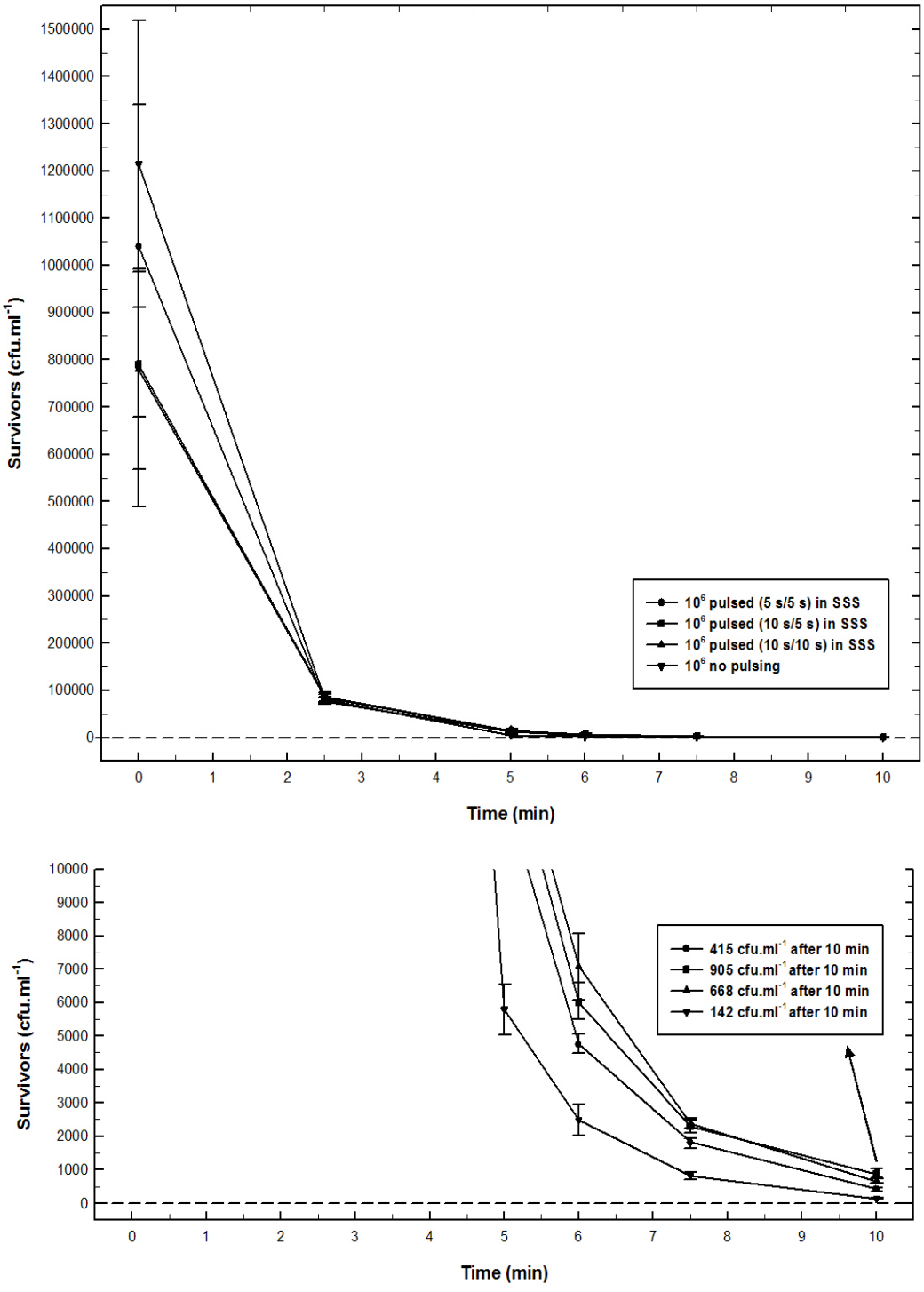

Figure 10 Impact of pulse-ultrasonication at 20 kHz on *Escherichia coli* ATCC 11775 in SSS. The lower graph represents an expanded Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

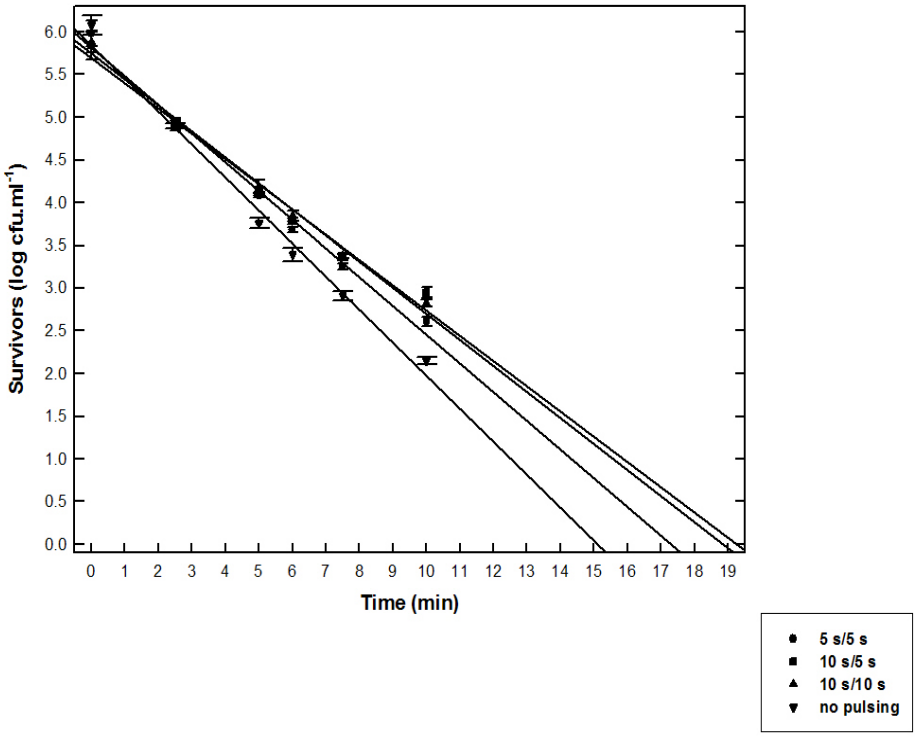

Figure 11 Regression (95% confidence level) of the data showing the impact of pulse-ultrasonication at 20 kHz on *Escherichia coli* ATCC 11775 in SSS (Each data point represents quadruple values. The standard deviation was used as the error-bar).

Duty cycle: 10 s on/5 s off

Pulse-ultrasonication with a 5 s off period, but with a longer ultrasonication period of 10 s achieved similar results over a period of 10.0 min. *Escherichia coli* in SSS with an initial load of 1 x 10^6 cfu.ml^{-1} was reduced by 99.88% (a 2.91 log reduction) after 10.0 min of pulse-ultrasonication (Fig. 11). The D_{US} was calculated to be 3.4 min.

Duty cycle: 10 s on/10 s off

At this pulsing combination, a 99.91% elimination of *E. coli* in SSS was achieved after 10.0 min (Fig. 11). This was equivalent to a 3.07 log reduction of the 1 x 10^6 cfu.ml^{-1} inoculum. The D_{US} was calculated to be 3.3 min.

Discussion of the data from Study 5

The 5 s/5 s pulsing combination was found to be most effective against *E. coli*, with a 99.96% or 3.39 log reduction in viable cells, however, ultrasonication without any pulsing resulted in the lowest D_{US} (2.7 min) (Fig. 11). A pulsing combination of 10 s/5 s was found to be the least effective against the *E. coli* strain tested.

Study 6 - Ultrasonication for 2.5, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude of three different *E. coli* strains

Three different *E. coli* strains were evaluated in SSS to determine whether there is any variation in the sensitivity of different strains. The three strains tested included the reference strain (ATCC 11775), a wild strain isolated from "raw" milk, and the O157:H7 pathogenic strain. The actual counts (cfu.ml^{-1}) are given in Fig. 12 and the recalculated data are given in the form of log graphs (Fig. 13).

In this study a 1 x 10^4 cfu.ml^{-1} inoculum (in SSS) was used, and was reduced to zero viable cells after a 10.0 min treatment for all three strains tested (Fig. 12). This is equivalent to log reductions of 4.08, 4.05 and 3.80 for the reference, wild and O157:H7 strain, respectively. The D_{US} for both the reference and the wild strains was calculated to be 2.5 min and the D_{US} for the O157:H7 strain was 2.4 min.

Conclusions

When all the data generated for the reference strain (1 x 10^4 cfu.ml^{-1} in SSS) were combined, it was found that ultrasonication for 10.0 min reduced *E. coli* levels to zero

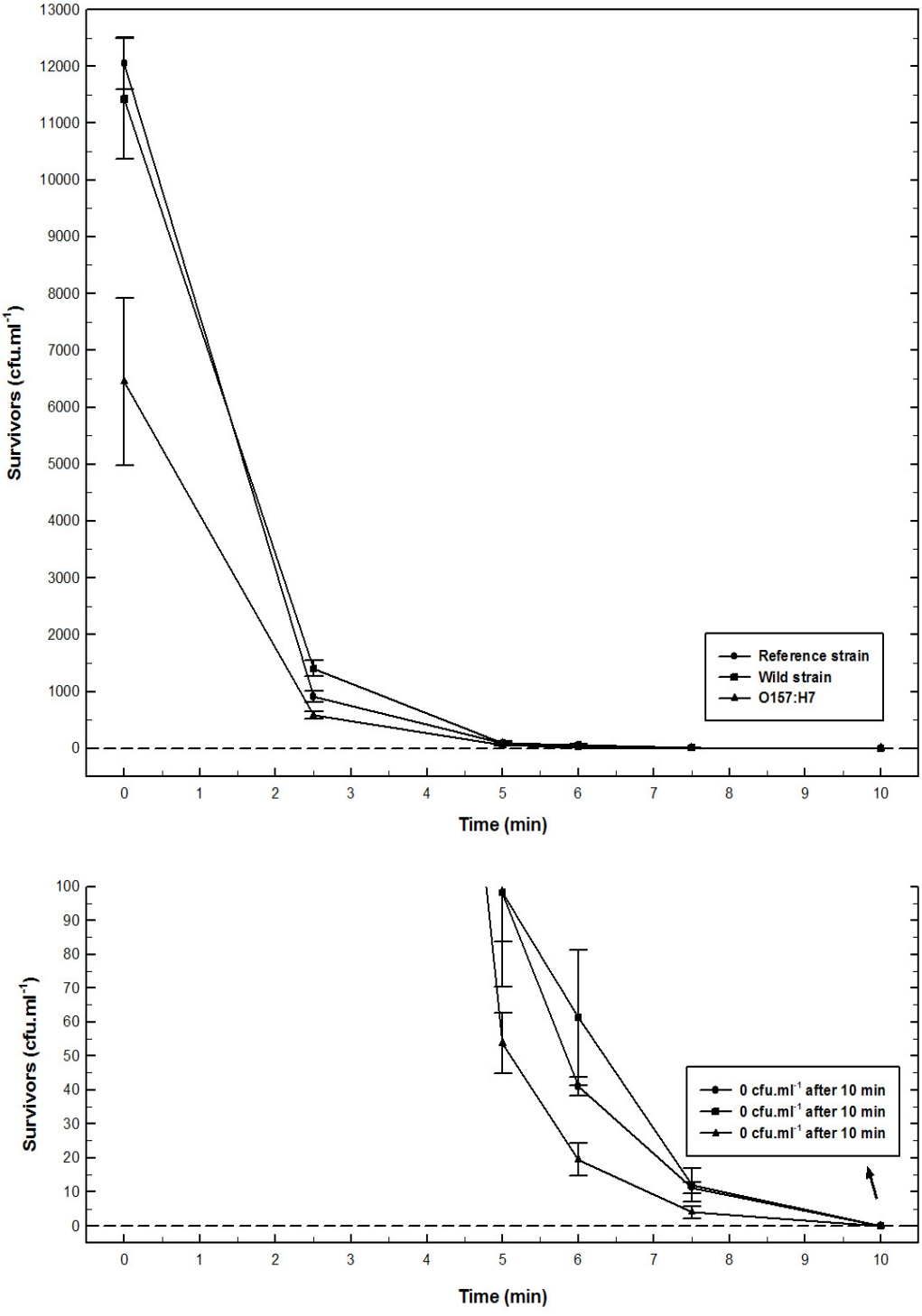

Figure 12 Impact of ultrasonication at 20 kHz on three different strains of *Escherichia coli* in SSS. The lower graph represents an expanded Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

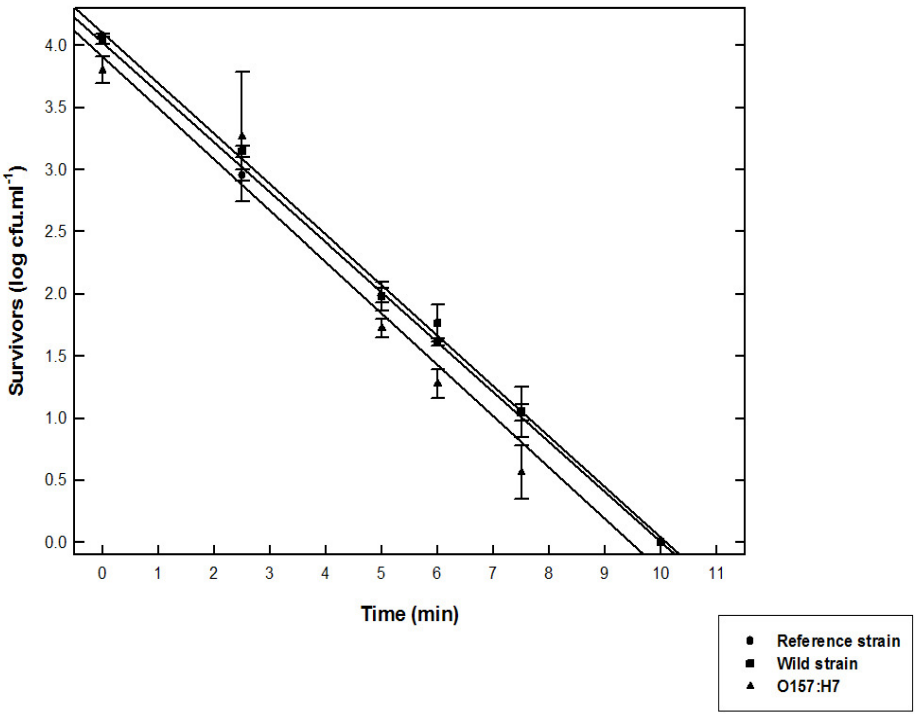

Figure 13 Regression (95% confidence level) of the data showing the impact of ultrasonication at 20 kHz on three different strains of *Escherichia coli* in SSS (Each data point represents quadruple values. The standard deviation was used as the error-bar).

(n = 29). It could therefore be concluded that the "killing effect" of the ultrasonication process is reproducible under standardised and controlled conditions.

From the data generated in this study it is evident that ultrasonication is an efficient method for the elimination of *E. coli* from milk. The data obtained also showed that *E. coli* (at an $N_0 = 1 \times 10^4$ cfu.ml^{-1}) could be reduced to zero, however, when using a higher N_0 in a batch system it is recommended that an extended processing time of up to 15.0 min should be used to ensure total elimination. The data also showed that 100% wave amplitude, and thus the maximum power available to the system, was more effective than 50% wave amplitude.

In this study no difference was found when using either UHT milk or SSS as suspension media. The protective effect of milk claimed in the literature was not observed with the parameters as used in this study. It was also found that pulsing did not improve the efficiency in comparison to an uninterrupted treatment. From a practical point of view, it would be easier to implement a system without the hassle of using different pulsing programs, thereby eliminating possible human errors.

Consideration of the data obtained from the three different *E. coli* strains used in this study showed that the strains responded in a similar way to ultrasonication. No major differences, in terms of the rate of elimination of viable cells, were observed between the different strains tested. It can therefore be concluded that any strain of *E. coli* should respond in a similar way to the effect of ultrasound.

One limit found during this study was that at times one or two microbes remained after the ultrasonic treatment. This was ascribed to the experimental setup used in this study as it was found that when the process was started, unavoidable splashing of the inoculated suspension medium occurred. In time these untreated, "splashed" droplets from the side of the sample holder and probe re-entered the treated sample resulting in the one or two cfu's found on PCA plates. These untreated microbes from the "splashed" droplets would then lead to a false impression that this process was not efficient. In these cases where final counts (one or two of the quadruplicates) were below five, the values were taken as zero. If the ultrasound apparatus could be used as a continuous treatment and the equipment built into a dairy processing line, this sort of problem would be eliminated.

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CHAPTER 4

IMPACT OF ULTRASOUND ON THE SURVIVAL OF A SELECTION OF MICROBES COMMONLY ASSOCIATED WITH MILK

Abstract

Consumers' continued demand for products with improved quality has led to a search for non-thermal processing methods as alternatives to heat pasteurisation. The aim of this study was, therefore, to evaluate the use of ultrasonication as an alternative technique. Eight spoilage and potentially pathogenic microbes previously isolated from pasteurised milk were used as "test" microbes. Saline solution and UHT milk were used as suspension media and were inoculated with exponential growth phase "test" microbes at microbial concentrations of either 1 x 10^4 cfu.ml^{-1} or 1 x 10^6 cfu.ml^{-1}. The samples were subjected to low-frequency high-power ultrasound (20 kHz, 750 W) for different time intervals. The data obtained showed that viable counts of *Pseudomonas fluorescens* were reduced by 100% after 6.0 min. *Saccharomyces cerevisiae* and *Listeria monocytogenes* were reduced by 99.7% and 99%, respectively after 10.0 min. *Lactococcus lactis* was reduced by 97% and *Micrococcus luteus*, *Bacillus cereus* and *Chryseobacterium meningosepticum* by 88%, 87% and 85%, respectively. *Lactobacillus acidophilus* showed the most resistance to ultrasound with only 78% of the viable cells being eliminated after 10.0 min. *Lactococcus lactis* and *B. cereus* were further tested extensively and it was found that pulse-ultrasonication did not enhance the effect of ultrasound. Ultrasonication at low cell inoculations (2 x 10^3 cfu.ml^{-1}) was found to be very effective with 98% of viable *Lc. lactis* cells being eliminated. Ultrasonication, as used in this study, was not found to be effective against *B. cereus* probably as a result of the presence of endospores. The composition of the suspension media (saline solution and milk) did not influence the success of ultrasonication, nor did the cell morphology and size or Gram characteristics influence the effectiveness of ultrasonication.

Introduction

Heat treatment of milk is primarily used as a means of inactivating spoilage or pathogenic microbes (Piyasena *et al*., 2003). There are, however, numerous reports of bacteria surviving pasteurisation. Microorganisms that have been reported to survive commercial pasteurisation include *Pseudomonas* spp. (Ternström *et al*., 1993), *Listeria monocytogenes* (Doyle *et al*., 1987), *Chryseobacterium* spp. (Ternström *et al*., 1993), *Mycobacterium paratuberculosis* (Grant *et al*., 2001) and the heat resistant endospores of *Bacillus cereus* (Griffiths, 1992; Ternström *et al*., 1993; García-Armesto & Sutherland, 1997).

Heat can cause deterioration of the organoleptic properties and also the nutritional value of milk (Efigênia *et al*., 1997; Frõlich, 2002). To avoid the unwanted effects of heat, efforts are being made to find new methods of food preservation, either based on new inactivation procedures (Evrendilek & Zhang, 2005) or by combining existing techniques like heat in combination with ultrasound or pulsed electric fields (Pagán *et al*., 1999a; Sepulveda *et al*., 2005).

The use of ultrasound to inactivate microbes was reported in the late 1920's (Harvey & Loomis, 1929), 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 (Pagán *et al*., 1999a).

Ultrasonic waves are generated by mechanical vibrations of frequencies between 20 kHz and 800 kHz (Hoover, 1997). 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 (Goldman & Lepschkin, 1952). When the bubbles reach a critical size, they collapse violently. This violent collapse is thought to be mechanical forces resulting in the breaking and shearing of cell walls leading to cell death.

In Chapter 3 of this dissertation the impact of ultrasonication on *Escherichia coli* was investigated. The promising results obtained indicated that further research on the response of other microbes frequently associated with pasteurised milk was required. The aim of this study was to determine the effectiveness of high-power, low-frequency ultrasound (20 kHz, 750 W) on the survival of a selection of spoilage and potentially pathogenic microbes.

Materials and methods

Bacterial cultures

The eight different "test" microbes used in this study and their specific growth requirements are summarised in Table 1. A broth subculture was prepared by inoculating 10 ml sterile broth with a specific "test" microbe and incubating for 24 h at the appropriate temperature (Table 1). A 100 ml sterile container, containing 90 ml broth was then inoculated with 5 ml of the 24 h culture and incubated for a further 24 h prior to the ultrasonic treatments.

Standard growth curves

The optical density (OD) of each culture was determined spectrophotometrically at 500 nm (Spectronic 20 Genesys, Spectronic Instruments, Cape Town). Three standard growth curves were obtained for each "test" microbe.

Ultrasonication

Two ml of each batch culture was centrifuged for 10 min at 6 000 x *g* (Eppendorf Centrifuge 5415D, Hamburg). The pellets were suspended in sterile saline solution (SSS) and the data from the standard curves were used to determine the desired cell concentration for inoculation of the suspension medium. The suspension medium, either SSS or full cream (3.4% milk fat) UHT (ultra high temperature) milk, was inoculated with a "test" microbe to give a final concentration of as near as possible to either 1 x 10^4 colony forming units per ml (cfu.ml^{-1}) or 1 x 10^6 cfu.ml^{-1}.

A 750 W, 20 kHz Vibra-Cell High Intensity Ultrasonic Processor VCX 750 (Sonics & Materials, Inc., Newtown, CT USA) was used for ultrasonication. This apparatus was fitted with an autoclavable 13 mm diameter probe with a replaceable titanium tip. A 40 ml sample of the inoculated suspension medium was pipetted into a sterile, jacketed glass sample holder connected to an ice-waterbath (4° - 6°C) to maintain a sample temperature of between 24° and 26°C. With this unit, optimum ultrasonic delivery is ensured by continuous monitoring of feedback from the probe and automatic adjustments made to the frequency and power. Samples were treated at 100% wave amplitude using five different time regimes:

1. 2.5, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude;
2. 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 min at 100% wave amplitude;

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)		
Bacillus cereus	1335	NB[b]/PCA[c]	24	35°	+	Rod (endospores)
Chryseobacterium meningosepticum	1336	NB/PCA	24	37°	-	Rod
Lactobacillus acidophilus	1348	MRS[d]	24	35°	+	Rod (chains)
Lactococcus lactis	315	MRS	24	30°	+	Cocci (chains)
Listeria monocytogenes	1273	NB/PCA	24	35°	+	Rod
Micrococcus luteus	173	NB/PCA	24	35°	+	Cocci (single)
Pseudomonas fluorescens	62	NB/PCA	24	35°	-	Rod
Saccharomyces cerevisiae	462	YDP[e]/MEA[f]	24	25°	NA	Yeast

[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).

NA = not applicable.

3. 2.5, 5.0, 10.0 and 15.0 min at either 50% or 100% wave amplitude;
4. 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude on a low microbial load; and
5. 2.5, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude with duty cycle combinations of 5 s/5 s, 10 s/5 s or 10 s/10 s.

Duplicate ultrasonic treatments were done and duplicate dilutions were made from each treated sample. The pour-plate technique and appropriate media were used for enumeration. Plates with between 30 and 300 colonies were selected for counting (Anon., 1997). UHT milk and SSS samples that were not 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 microbial elimination, was measured by their decimal reduction time (*D*) which, for this study, was defined as the time (min) of a given treatment required to reduce the bacterial population by 90% or by a single log cycle. A minimum of two and a maximum of five (where possible) *D*-values were calculated for each curve. For this study, the *D*-value at 20 kHz/750 W was abbreviated to D_{US}.

Results and discussion

To simplify the discussion of the results generated in this study, some of the data as illustrated in Figs. A1 to A18, were rather included as a separate Appendix (A) and placed at the end of this chapter.

The standard curves for each culture, as given in Figs. A1 and A2 in Appendix A, were used as a reference to determine the cell inoculation size (exponential phase) for the ultrasonication studies.

Study 1 - Ultrasonication for 2.5, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude

In the first part of the ultrasonication study the impact of ultrasound on the eight "test" microbes (Table 1) was investigated. To simplify the comparisons between the cultures, the data illustrating the viable counts vs. time (Figs. A3 to A10 in Appendix A) have been summarised in Figs. 1 (A-D) and 2 (A-D) and Table 2.

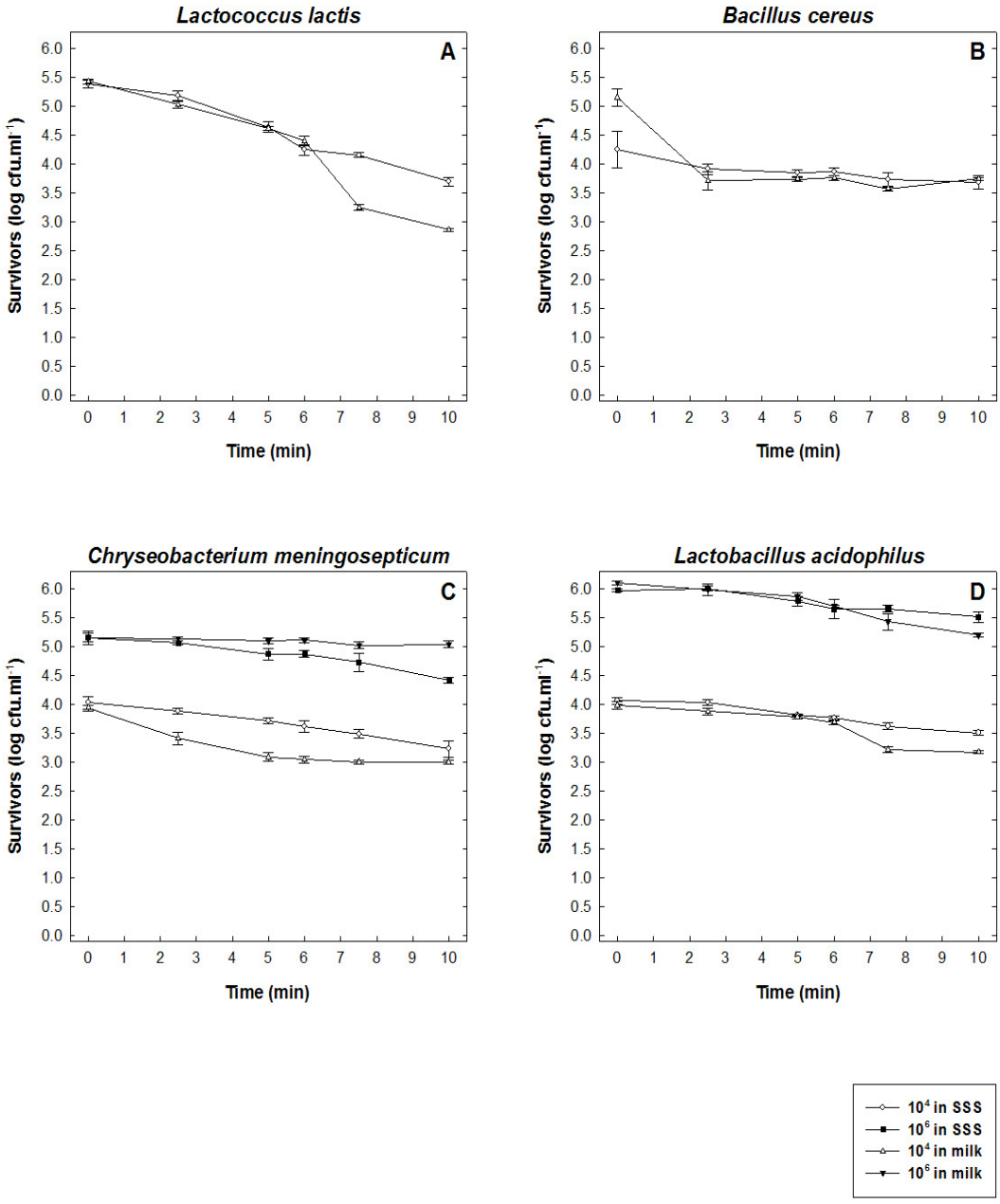

Figure 1 Impact of ultrasonication at 20 kHz on *Lc. Lactis*, *B. cereus*, *C. meningosepticum* and *Lb. acidophilus* at different starting concentrations in either SSS or UHT milk (Each data point represents quadruple values. The standard deviation was used as the error-bar).

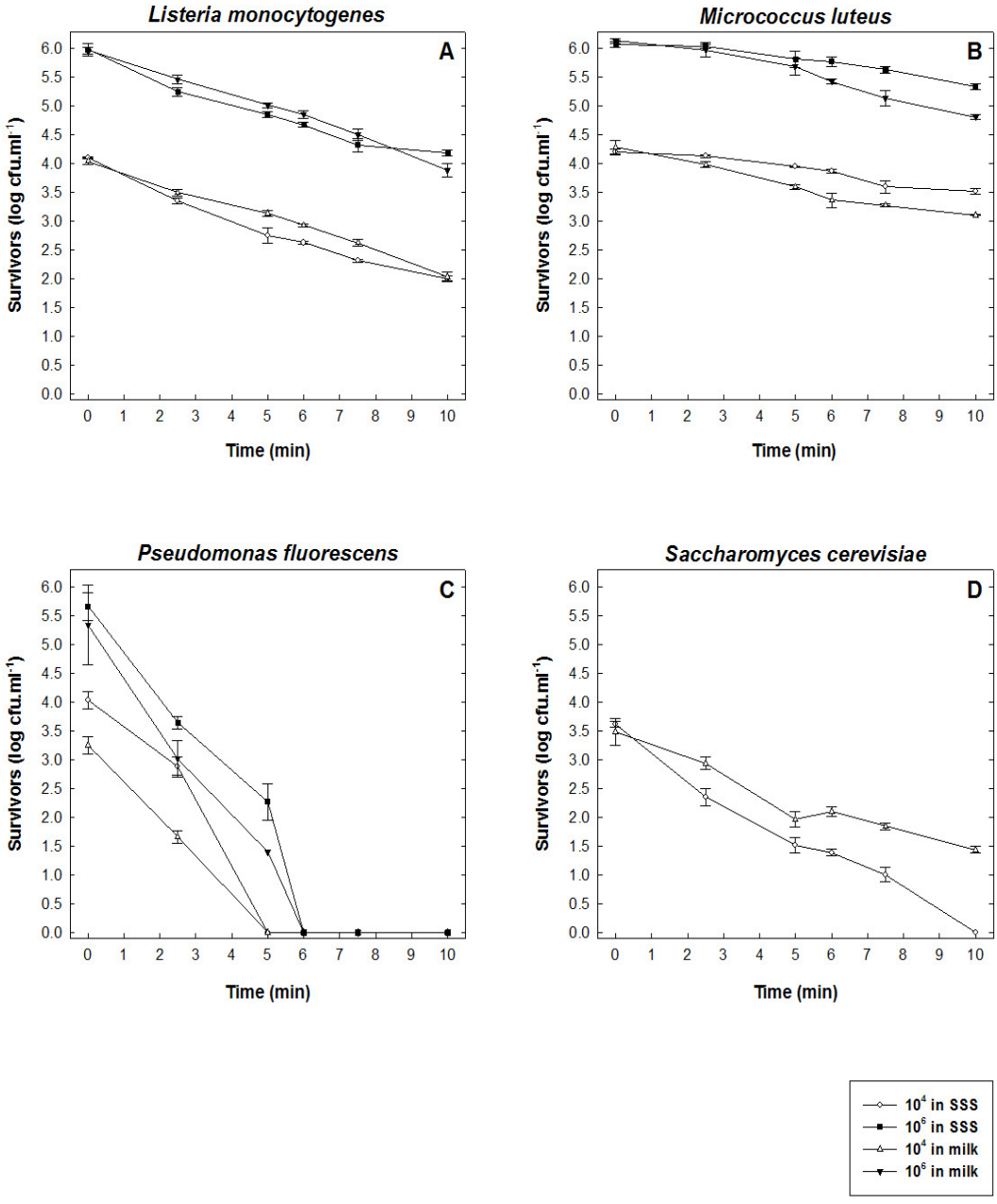

Figure 2 Impact of ultrasonication at 20 kHz on *L. monocytogenes*, *M. luteus*, *P. fluorescens* and *S. cerevisiae* at different starting concentrations in SSS and UHT milk (Each data point represents quadruple values. The standard deviation was used as the error-bar).

Table 2 Summary of the D_{US}-values, and log reductions obtained for the different "test" microbes evaluated in Study 1 over a 10 min treatment time

Study	Microbe	Treatment time (min)	Inoculum (cfu.ml^{-1})	SSS			Milk		
				D_{US} (min)	log red.	% red.	D_{US} (min)	log red.	% red.
1	*Lc. lactis*	10	2 x 10^5	5.0	1.69	97.98%	2.6	2.58	97.34%
1	*B. cereus*	10	2 x 10^4	nc	0.64	77.35%	nc	1.43	96.27%
1	*C. meningosepticum*	10	1 x 10^4	nc	0.79	83.73%	nc	0.94	88.50%
		10	1 x 10^5	nc	0.75	82.07%	nc	0.11	23.08%
1	*Lb. acidophilus*	10	1 x 10^4	nc	0.55	72.08%	nc	0.82	65.17%
		10	1 x 10^6	nc	0.46	84.73%	nc	0.90	87.48%
1	*L. monocytogenes*	10	1 x 10^4	4.4	2.10	99.20%	5.3	2.00	99.00%
		10	1 x 10^6	4.7	1.80	98.42%	4.7	2.07	99.14%
1	*M. luteus*	10	1 x 10^4	nc	0.79	83.70%	8.0	1.18	93.39%
		10	1 x 10^6	nc	0.73	81.18%	6.1	1.32	95.23%
1	*P. fluorescens*	10	1 x 10^4	1.2	4.05	100%	1.5	3.26	100%
		10	1 x 10^5	1.2	5.70	100%	1.2	5.64	100%
1	*S. cerevisiae*	10	1 x 10^4	2.8	3.62	100%	4.3	2.10	99.42%

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

Lactococcus

In milk *Lc. lactis* metabolises lactose to lactic acid with the subsequent acidification of the milk (Frees *et al*., 2003). Therefore, *Lc. lactis* may under certain conditions also be considered a spoilage microbe of fresh milk. In this study, ultrasonication of the *Lc. lactis* strain for 10.0 min in SSS and milk resulted in a 1.69 and 2.58 log reduction, respectively (Fig. 1A). Viable cells in SSS, with an initial bacterial load of 2×10^5 cfu.ml^{-1}, were reduced by 97.98% and in milk the initial bacterial load was reduced by 97.34%. The D_{US} as calculated for these studies using *Lc. lactis* was 5.0 min (non-typical logarithmic death curve) in SSS and 2.6 min in milk (for this specific microbe the D_{US} was taken as the average of two values as a result of a non-typical logarithmic death curve) (Table 2).

Results from this study are in disagreement to those reported by Jacobs & Thornley (1954). They inoculated reconstituted skim milk (8%) with "*Streptococcus lactis*" (200 - 400 cfu.ml^{-1}); treated the strain for 20 min at either 20.5 Kc.s^{-1} or 1 Mc.s^{-1} and found no reduction in viable counts. In this study, however, *Lc. lactis* counts were reduced by more than 97% for both the suspension media tested. This was ascribed to technological advances during the past 50 years, e.g. power output leading to higher wave amplitudes.

Bacillus

Bacillus cereus is a major spoilage and pathogenic microbe in the dairy industry, and the enterotoxins produced may lead to a diarrhoeal illness (Wouters, 1993; Granum & Lund, 1997). It has also been shown by research done elsewhere that conventional pasteurisation processes are not effective against this endospore-former (García-Armesto & Sutherland, 1997).

In this study, ultrasonication of the *B. cereus* strain for 10.0 min resulted in the elimination of 77.35% of the initial 2×10^4 cfu.ml^{-1} in SSS and 96.27% of the initial 1×10^5 cfu.ml^{-1} in milk. This is equivalent to 0.64 and 1.43 log reductions for the SSS and milk, respectively (Fig. 1B). The D_{US} for *B. cereus* in SSS could not be determined as a single log reduction was not reached. When milk was used as suspension medium, a single log reduction was achieved within the first 2.5 min of ultrasonication where after there was no further reduction. It was therefore decided that a D_{US}-value would not give a true reflection of the results (Table 2).

García *et al*. (1989) similarly reported no negative effect on the survival of *Bacillus subtilis* after ultrasonic treatments of 60 min at 20 kHz and 150 W in water,

"whole milk" and glycerol. In this study, 750 W of power and a wave amplitude of 124 µm were used for ultrasonication. More power leads to better cavitation (Alliger, 1975) which would explain why in this study ultrasonication was found to damage *B. cereus*, albeit only to a limited extent. Berger & Marr in 1960, reported that a treatment time of 1 h (75 acoustical watts in a Raytheon 10 kc. sonic oscillator) was necessary to remove most of the exosporium surrounding the spores of some strains of *B. cereus*. They did, however, report a 75% reduction in viable counts after a 2 h treatment. Their aim was not to destroy the cells but rather to make the cells more sensitive to the effect of heat. Sensitivity to heat falls outside the scope of this study, therefore, the results obtained in this study cannot be compared to those of Berger & Marr (1960).

Chryseobacterium

Chryseobacterium meningosepticum is a spoilage bacterium commonly found in raw milk (Muir, 1996) and may cause a variety of defects such as surface taint and an apple flavour in butter (Jooste *et al*., 1986). In this study, ultrasonication of *C. meningosepticum* for 10.0 min in SSS resulted in a 0.79 log reduction (83.73%) for a 1 x 10^4 cfu.ml^{-1} inoculum and a 0.75 log reduction (82.07%) when the initial bacterial load was 1 x 10^5 cfu.ml^{-1} (Fig. 1C). When milk was used as the suspension medium, an 88.50% or 0.94 log reduction in initial viable counts was observed for a 1 x 10^4 cfu.ml^{-1} inoculum. Milk with an initial bacterial load of 1 x 10^5 cfu.ml^{-1} resulted in the elimination of only 23.08% of the viable cells after 10.0 min of ultrasonication. This is equivalent to a 0.11 log reduction. No D_{US} could be determined for *C. meningosepticum* as a single log reduction was not achieved, even after 10.0 min of ultrasonication (Table 2).

Lactobacillus

Lactobacillus acidophilus, when grown in raw milk produces lactic acid which leads to a decrease in pH (Kleerebezem & Hugenholtz, 2003). This microbe can thus be considered as a dairy spoilage microbe in terms of fresh milk production. After ultrasonicating *Lb. acidophilus* for 10.0 min, 72.08% of the initial 1 x 10^4 cfu.ml^{-1} and 65.17% of the initial 1 x 10^6 cfu.ml^{-1} count in SSS were eliminated. This is equivalent to a 0.55 and 0.46 log reduction, respectively (Fig. 1D). In milk, 84.73% or 0.82 log cycles of the initial 1 x 10^4 cfu.ml^{-1} and 87.48% or 0.90 log cycles of the initial 1 x 10^6 cfu.ml^{-1} were eliminated. The D_{US} for *Lb. acidophilus* could not be determined as a single log reduction was not achieved (Table 2).

Listeria

Listeria monocytogenes is considered to be an important dairy pathogen with the ability to grow at refrigeration temperatures (Griffiths, 1989; Pearson & Marth, 1990). In this study ultrasonication of *L. monocytogenes* for 10.0 min in SSS resulted in an elimination of 99.20% of an initial load of 1 x 10^4 cfu.ml^{-1} (a 2.10 log reduction), and a 98.42% reduction for a 1 x 10^6 cfu.ml^{-1} inoculum (a 1.80 log reduction) (Fig. 2A). The D_{US} in SSS was calculated to be 4.4 min (1 x 10^4 cfu.ml^{-1}) and 4.7 min (1 x 10^6 cfu.ml^{-1}) (for this study the D_{US} was taken as the average of two values). When milk was used as the suspension medium, 99% of the initial 1 x 10^4 cfu.ml^{-1} was reduced to only 111 viable cells (a 2.00 log reduction), and 99.14% of the initial 1 x 10^6 cfu.ml^{-1} was reduced to 7 975 viable cells (a 2.07 log reduction) after 10.0 min of ultrasonication. The D_{US} for *L. monocytogenes* in milk was 5.3 min (1 x 10^4 cfu.ml^{-1}) and 4.7 min (1 x 10^6 cfu.ml^{-1}) (for this specific microbe the D_{US} was taken as the average of two values) (Table 2).

Pagán *et al*. (1999b) reported a *D*-value of 4.3 min for *L. monocytogenes* ultrasonicated (20 kHz and an amplitude of 117 μm) at ambient temperature. It was not clear what the initial cell concentration (cfu.ml^{-1}) used by Pagán *et al*. (1999b) had been, and that could explain the slight difference between the *D*-values obtained in this study and those obtained by Pagán *et al*. (1999b). There are a number of factors influencing the efficiency of ultrasonication (strain of microbe, initial concentration, treatment medium, amplitude of sound waves, growth phase, etc.), and omitting or neglecting to mention them makes comparisons between the results of different research groups difficult.

Micrococcus

Micrococcus spp. has been associated with raw milk (Frank, 1997; Sablé *et al*., 1997), and has also been isolated from pasteurised milk (Jay, 1996). In this study, ultrasonication of the *M. luteus* strain for 10.0 min eliminated 83.70% of the initial 1 x 10^4 cfu.ml^{-1} (0.79 log reduction), and 81.18% of the initial bacterial load of 1 x 10^6 cfu.ml^{-1} (0.73 log reduction) in SSS (Fig. 2B). In milk, ultrasonication reduced the initial 2 x 10^4 cfu.ml^{-1} by 93.39% and 1 x 10^6 cfu.ml^{-1} were reduced by 95.23%. This is equivalent to a 1.18 and 1.32 log reduction, respectively. The D_{US} in milk was calculated to be 8.0 min (2 x 10^4 cfu.ml^{-1}) and 6.1 min (1 x 10^6 cfu.ml^{-1}), but the D_{US} in SSS could not be determined as a single log reduction was not reached (Table 2).

Pseudomonas

Pseudomonas is frequently present in raw milk (Frank *et al*., 1993; Jay, 1996), however, it is not reported to survive pasteurisation, and its presence in pasteurised milk is usually ascribed to post-pasteurisation contamination (Aaku *et al*., 2004). In this study, ultrasonication of *P. fluorescens* resulted in a 100% elimination of all viable cells (Fig. 2C). In SSS all viable cells of a 1 x 10^4 cfu.ml^{-1} inoculum were eliminated after only a 5.0 min ultrasonic treatment. This is equivalent to a 4.05 log reduction. When the initial inoculum in SSS was increased to 1 x 10^5 cfu.ml^{-1}, a treatment time of 6.0 min was required to eliminate all viable cells (a 5.70 log reduction). When milk was used as the suspension medium, similar results were obtained with 5.0 min required to eliminate 100% of the 1 x 10^4 viable cells (a 3.26 log reduction), and a treatment time of 6.0 min needed to eliminate 100% of the 1 x 10^5 viable cells (a 5.64 log reduction). The D_{US} was calculated to be 1.2 min (1 x 10^4 cfu.ml^{-1} and 1 x 10^5 cfu.ml^{-1}) in SSS and 1.5 min (1 x 10^4 cfu.ml^{-1}) and 1.2 min (1 x 10^5 cfu.ml^{-1}) in milk (for this specific microbe the D_{US} was taken as the average of at least five values) (Table 2).

The results obtained from this study compares well to some of the results reported by Villamiel & de Jong (2000). They reported log reductions of between 0.6 and 4.2 for *P. fluorescens* in Trypticase Soy Broth with an initial concentration of 6.9 - 7.7 log.cfu.ml^{-1}. The ultrasonication apparatus they used had a fixed frequency of 20 kHz, and a maximum power output of 150 W. They used a continuous system with flow rates of 50 and 33 ml.min^{-1}. In addition to this, they used ultrasound in combination with a heat treatment. The differences in treatment parameters used in this study and those used by Villamiel & de Jong (2000) make it difficult to explain why they obtained such a very low log reduction (0.6).

Saccharomyces

In this study, ultrasonication of the *S. cerevisiae* strain for 10.0 min in SSS resulted in a 100% elimination of the initial 1 x 10^4 cfu.ml^{-1} (a 3.62 log reduction), and 99.42% of the initial 1 x 10^4 cfu.ml^{-1} (a 2.10 log reduction) in milk (Fig. 2D). 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).

Ciccolini *et al*. (1997) reported a *D*-value for *S. cerevisiae* of 1.6 min (100 W) and 2.4 min (180 W) (initial concentration of approximately 1 x 10^8 cfu.ml^{-1} in sterile water). The difference between this study and theirs was that, although they used a frequency of 20 kHz, they combined ultrasound with a heat treatment (55°C) and this accounts for

the differences in *D*-values obtained. Guerrero *et al*. (2001; 2005) also used ultrasound in a hurdle system with three different temperature combinations (35°, 45° and 55°C), and reported *D*-values of 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 comparisons of the results obtained difficult.

Discussion of the data obtained in Study 1

In Study 1, the *P. fluorescens* strain was clearly found to be the most sensitive to ultrasound, followed by *S. cerevisiae*, *L. monocytogenes* and *Lc. lactis*. *Lactobacillus acidophilus*, a Gram-positive rod and dairy spoilage microbe, was found to be the most resistant to the effect of ultrasonication. The Gram-positive microbe that was found to be the most sensitive to ultrasonication was *L. monocytogenes*. *Micrococcus luteus* (a Gram-positive coccus) was also found to be more sensitive to the effect of ultrasonication than the Gram-positive rods, *B. cereus* and *Lb. acidophilus*. *Chryseobacterium meningosepticum*, a Gram-negative rod-shaped dairy spoilage microbe was found to be the more resistant of the two Gram-negative microbes tested.

Jacobs & Thornley reported in 1954 that fresh unhomogenized milk gave more protection to the damaging effect of ultrasonication than homogenized milk, which in turn gave more protection to bacteria than when broth was used as the suspension liquid. The protective effect observed in milk was ascribed to the milk fat molecules and their size. This is contrary to the results obtained in this study where the variation in results between SSS and UHT milk as suspension media was so small that it was considered omissible. In some cases (*C. meningosepticum*, *Lb. acidophilus*, *Lc. lactis* and *M. luteus*) the microbes were less resistant when treated in milk, and therefore less 'protected' in milk, as a larger log reduction was achieved when comparing the results for the same microbes in SSS. No specific explanation can be given for milk being slightly less 'protective' than SSS.

It has been reported that smaller sized bacteria are more resistant to the effect of ultrasound (Jacobs & Thornley, 1954; Alliger, 1975), and that rods are more easily eliminated. Gram-positive bacteria have also been reported to be more resistant (Alliger, 1975; Hülsen, 1999; Villamiel & de Jong, 2000). The results from this study suggest that there was no direct correlation between size or Gram characteristic of bacteria and resistance to ultrasonication. Although the specific strain of *S. cerevisiae* used in this study was found to be more sensitive than most of the bacteria tested, the small (0.5-1.0 x 1.5-5.0 µm) Gram-negative rod, *P. fluorescens*, was found to be most

susceptible to elimination by ultrasonication under the conditions applied in this study. Scherba *et al*. (1991) also reported no difference in ultrasound resistance between Gram-positive and negative bacteria. Although Gram-positive bacteria have a thicker and more tightly adherent peptidoglycan layer than Gram-negative bacteria, Scherba *et al*. (1991) suggested that it is not the cell wall thickness that protects against ultrasound. They proposed that the "target" of ultrasonic damage might rather be the cytoplasmic membrane, which normally consists of a lipoprotein bilayer. Similarly, Ciccolini *et al*. (1997) found that the cavitation field in the microbial suspension generated by ultrasonic waves does not deactivate, break up or kill the cells when using a very high 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. To verify the results of Scherba *et al*. (1991) and Ciccolini *et al*. (1997) it would be advisable to visually examine ultrasonically induced microbial cell damage.

Based on the data obtained in Study 1, where eight "test" microbes were ultrasonicated for at least 10.0 min, it was decided to use only two of the "test" microbes in the subsequent studies. The selected bacteria were: *Lc. lactis*, a Gram-positive coccus and fresh milk spoilage bacterium, and *B. cereus*, a Gram-positive rod, endospore-former and potential pathogen.

Study 2 - Ultrasonication for 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 min at 100% wave amplitude

In Study 1, it was found that the first 2.5 min of ultrasonication often resulted in the highest percentage of viable cells being eliminated (Figs. A3 and A4 in Appendix A). This gave rise to the second study, where the first 2.5 min were divided into 30 s intervals to determine at which time the bacteria were most sensitive to the effect of ultrasound. To simplify comparisons between the cultures used in this study, the data illustrating viable counts vs. time (Figs. A11 and A12 in Appendix A) have been summarised in Fig. 3 (A and B) and Table 3.

Ultrasonication of *Lc. lactis* for 3.0 min resulted in 57.27% of the 1×10^4 viable cells in milk being eliminated. This is equivalent to a 0.37 log reduction (Fig. 3A). The

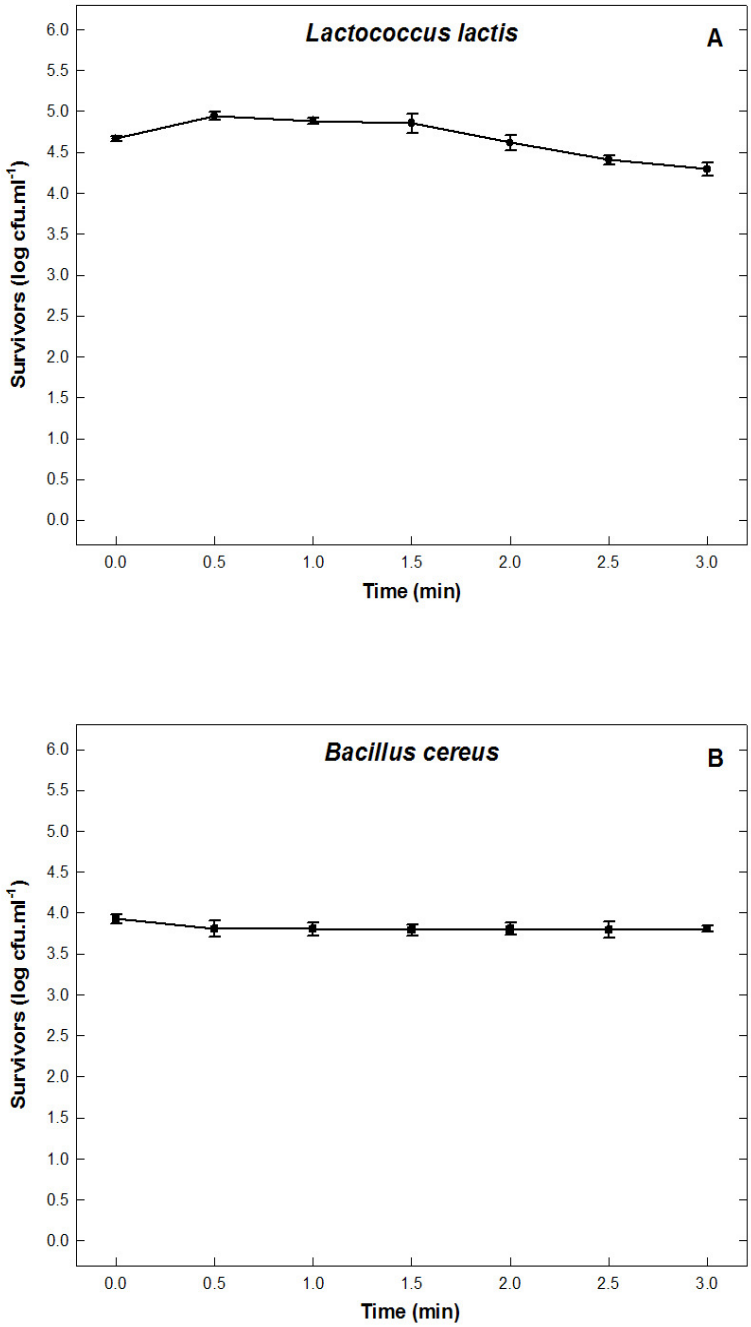

Figure 3 Impact of ultrasonication at 20 kHz on *Lc. lactis* (A) and *B. cereus* (B) in UHT milk (Each data point represents quadruple values. The standard deviation was used as the error-bar).

Table 3 Summary of the D_{US}-values, and log reductions obtained for the different "test" microbes evaluated in Studies 2-5

Study	Microbe	Treatment time (min)	Inoculum (cfu.ml^{-1})	SSS			Milk		
				D_{US} (min)	log red.	% red.	D_{US} (min)	log red.	% red.
2	*Lc. lactis*	3	1 x 10^4	-	-	-	nc	0.37	57.27%
2	*B. cereus*	3	1 x 10^4	-	-	-	nc	0.12	24.18%
3	*Lc. lactis*	(50%, 62 µm) 15	2 x 10^4	nc	0.74	99.54%	-	-	-
		(100%, 124 µm) 15	2 x 10^4	5.3	2.33	81.74%	-	-	-
3	*B. cereus*	(50%, 62 µm) 15	2 x 10^4	nc	0.12	49.17%	-	-	-
		(100%, 124 µm) 15	2 x 10^4	nc	0.29	23.46%	-	-	-
4	*Lc. lactis*	10	2 x 10^3	-	-	-	4.9	1.91	98.77%
4	*B. cereus*	10	2 x 10^3	-	-	-	nc	0.20	37.66%
5	*Lc. lactis*	(5 s/5 s) 10	1 x 10^4	4.3	1.72	98.08%	-	-	-
		(10 s/5 s) 10	1 x 10^4	6.0	1.70	98.01%	-	-	-
		(10 s/10 s) 10	1 x 10^4	5.1	1.93	98.82%	-	-	-
		no pulsing 10	2 x 10^5	5.0	1.69	97.98%	-	-	-
5	*B. cereus*	(5 s/5 s) 10	1 x 10^4	nc	0.17	32.04%	-	-	-
		(10 s/5 s) 10	1 x 10^4	nc	0.19	35.63%	-	-	-
		(10 s/10 s) 10	1 x 10^4	nc	0.07	14.48%	-	-	-
		no pulsing 10	2 x 10^4	nc	0.64	77.35%	-	-	-

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

D_{US} could not be calculated as a single log reduction was not reached within 3.0 min. It is interesting to note that the data in Fig. 3A clearly shows an increase in viable *Lc. lactis* cells after ultrasonication for 0.5 min. In 1997, Ciccolini *et al*. reported the dispersion of microbial clumps/chains through the action of cavitation. Cells that are clumped together in chains in the untreated samples grow as single colonies in pour plates which would lead to an underestimation of the initial microbial load of the inoculum. Based on this it is, therefore, suggested that in this study the initial rise in viable cell counts after 0.5 min of ultrasonication may be ascribed to dispersion of cell chains as can be seen in the micrograph in Fig. 4. This 'dispersion of chains' of *Lc. lactis* was not observed in Fig. 1A, and it was ascribed to the longer ultrasonic treatment times used in Study 1.

After ultrasonication of the *B. cereus* strain for 3.0 min in milk, only 24.18% or 0.12 log cycles of the initial bacterial load of 1 x 10^4 cfu.ml^{-1} (Fig. 3B) was eliminated. A single log reduction was not reached after 3.0 min and, therefore, the D_{US} could not be calculated. It can thus be concluded that an ultrasonic treatment of 3.0 min or less is insufficient for the treatment of *B. cereus* by the dairy industry.

The results from Study 2 are similar to those obtained in Study 1 where it was found that *Lc. lactis* was more sensitive to the effect of ultrasonication than *B. cereus*. The results from Study 2 did not indicate a single treatment time at which the "test" strains were the most sensitive to ultrasonication as viable *Lc. lactis* cells showed an increase after 0.5 min of ultrasonication, whereas for *B. cereus* the highest elimination in viable cells was achieved within the first 0.5 min of ultrasonication.

Study 3 - Ultrasonication for 2.5, 5.0, 10.0 and 15.0 min at either 50% (62 µm) or 100% (124 µm) wave amplitude

The impact of two different wave amplitudes was investigated over a treatment time of 15.0 min. The amount of power used during an ultrasonic treatment is proportional to the wave amplitude; therefore, it is assumed that a higher wave amplitude will result in more intense cavitation and greater cell disruption. To simplify comparisons of the results between the cultures used in this study, the data illustrating viable counts vs. time (Figs. A13 and A14 in Appendix A) have been summarised in Fig. 5 (A and B) and Table 3.

In this study, ultrasonication of the *Lc. lactis* strain in SSS for 15.0 min at 100% wave amplitude (124 µm) eliminated 99.54% of the initial 2 x 10^4 cfu.ml^{-1} (a 2.33 log

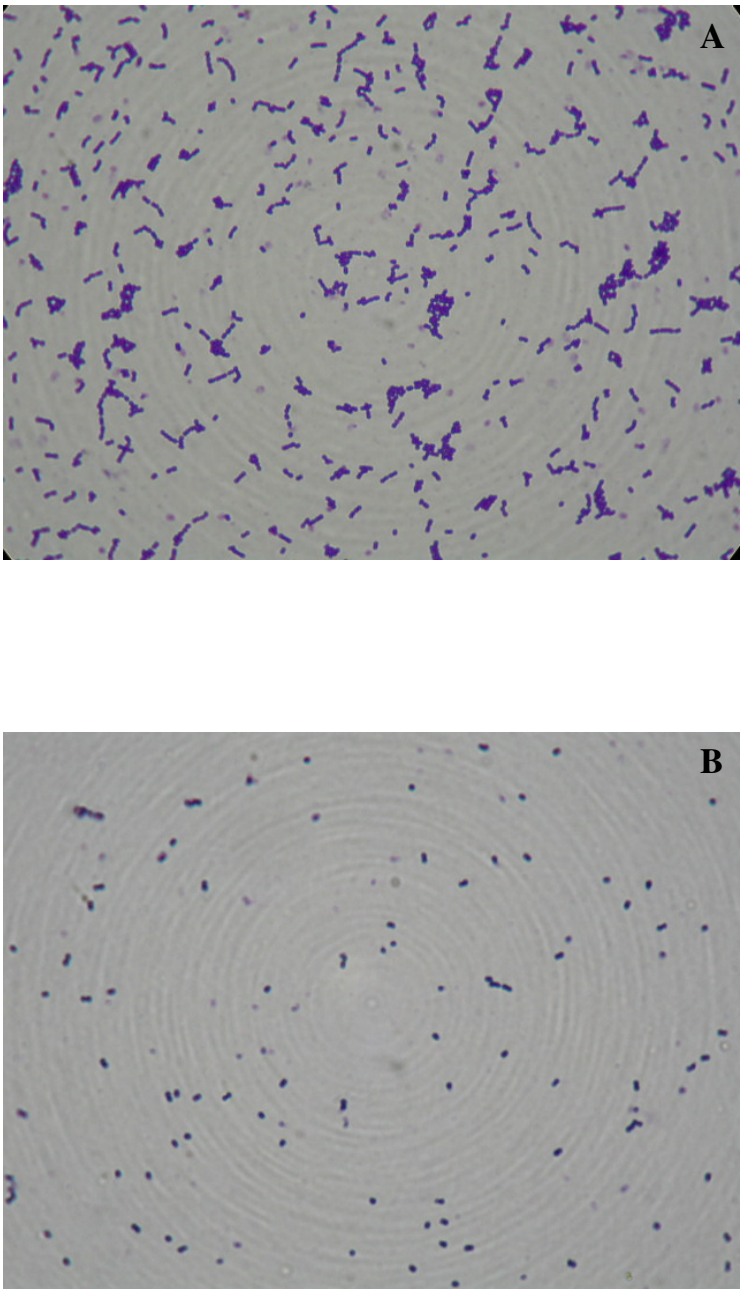

Figure 4 Dispersion of *Lactococcus lactis* chains after ultrasonication (x1000 enlargement) (A = no ultrasonication showing numerous chains; B = 0.5 min ultrasonication with few chains remaining).

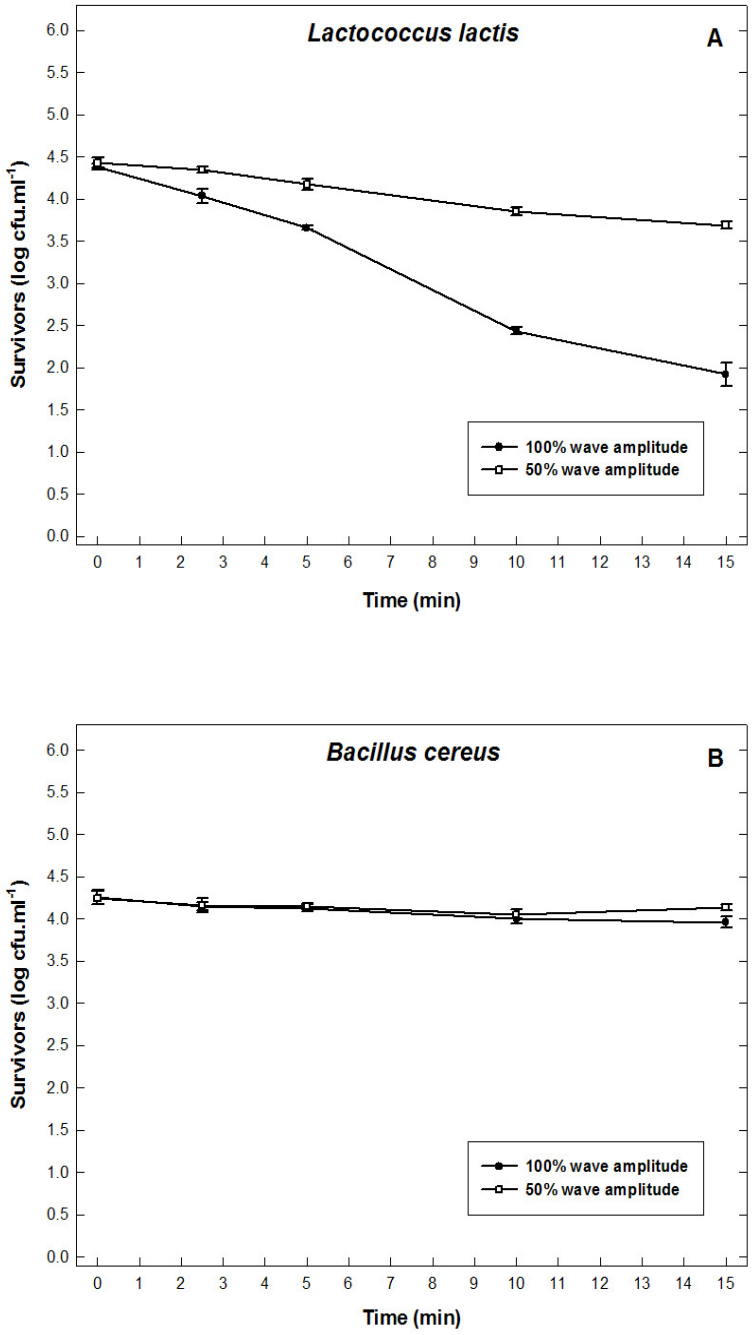

Figure 5 Impact of ultrasonication at 20 kHz and either 100% or 50% wave amplitude on *Lc. lactis* (A) and *B. cereus* (B) in SSS (Each data point represents quadruple values. The standard deviation was used as the error-bar).

reduction). In contrast, only 81.74% of the initial bacterial load of 2 x 10^4 cfu.ml^{-1} (a 0.74 log reduction) was eliminated at 50% wave amplitude (62 µm) after 15.0 min (Fig. 5A). The D_{US} for *Lc. lactis* at 100% wave amplitude (124 µm), was 5.3 min (for this study the D_{US} was taken as the average of three values as a result of a non-typical logarithmic death curve). A single log reduction was not achieved for ultrasonication at 50% wave amplitude (62 µm); therefore, the D_{US} could not be calculated.

A 0.29 log reduction (49.17%) was achieved for *B. cereus* when SSS with an initial bacterial load of 2 x 10^4 cfu.ml^{-1} was ultrasonicated for 15.0 min at 100% wave amplitude (124 µm), and the elimination of only 23.46% (0.12 log cycles) when a 2 x 10^4 cfu.ml^{-1} inoculum was ultrasonicated in SSS for 15.0 min at 50% wave amplitude (62 µm) (Fig. 5B). The D_{US} could not be calculated for *B. cereus* at either of the two amplitudes tested, as a single log reduction in viable counts was not reached. *Bacillus cereus* was again found to be resistant to the effect of ultrasound (Fig. 5B), even with an extended treatment time of 15.0 min, irrespective of the percentage amplitude used.

The importance of power (wave amplitude) for effective ultrasonication is evident from this study, where both "test" microbes showed higher sensitivity to ultrasound delivered at higher power levels (100% wave amplitude rather than 50%). *Lactococcus lactis* was still more sensitive to ultrasonication than *B. cereus*. It is therefore suggested to use the maximum amplitude available for the elimination of microbes from milk. Similar results were obtained for *Escherichia coli* in Chapter 3 where a wave amplitude of 100% proved to be more efficient at eliminating viable cells than a 50% wave amplitude.

Study 4 - Ultrasonication for 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude on milk with a low microbial load

The effect of ultrasound on low initial counts was investigated in this study. This was done to determine what effect ultrasound would have on high quality raw milk (i.e. raw milk with a low initial bacterial load). Tests were performed in milk with an initial bacterial load of 2 x 10^3 cfu.ml^{-1}. To simplify comparisons between the cultures used in this study, the data illustrating viable counts vs. time (Figs. A15 and A16 in Appendix A) have been summarised in Fig. 6 (A and B) and Table 3.

A 1.91 log reduction (98.77%) of viable *Lc. lactis* cells was achieved after ultrasonication for 10.0 min (Fig. 6A). The D_{US} for *Lc. lactis* was calculated to be

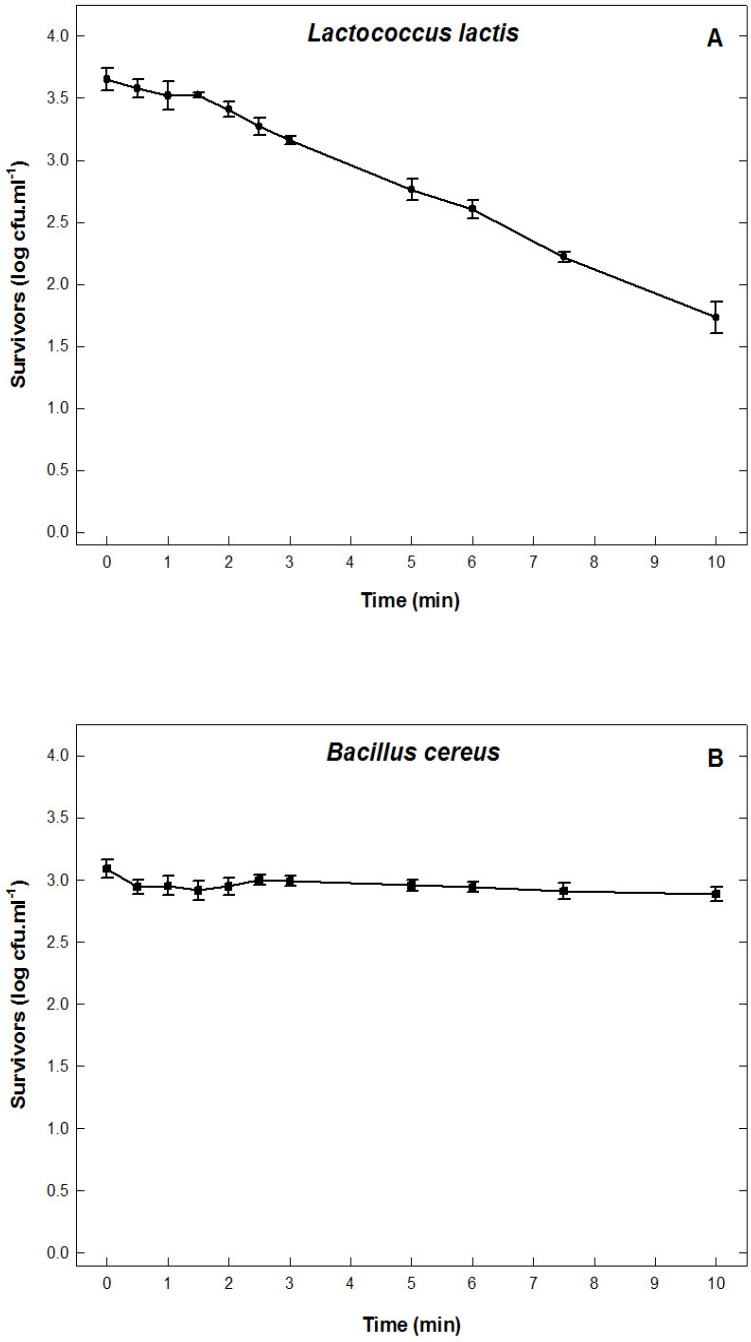

Figure 6 Impact of ultrasonication at 20 kHz on *Lc. lactis* (A) and *B. cereus* (B) at a starting concentration of 2 x 10^3 cfu.ml^{-1} in UHT milk (Each data point represents quadruple values. The standard deviation was used as the error-bar).

4.9 min (for this study the D_{US} was taken as the average of two values as a result of a non-typical logarithmic death curve).

Bacillus cereus was again found to be resistant to the effect of ultrasonication, even at low counts. Only a 0.20 log reduction (37.66%) could be reached after 10.0 min of ultrasonication, therefore, the D_{US} could not be calculated (Fig. 6B).

Lactococcus lactis at a low microbial load proved to be a bit more sensitive to the effect of ultrasonication. However, even at low inoculation concentrations the data showed *B. cereus* to be of some concern with the endospores being very resistant to ultrasound. These results are in accordance with those obtained for *E. coli* in Chapter 3 of this dissertation which also confirmed that ultrasonication is very effective against low microbial loads.

Study 5 - Pulse-ultrasonication for 2.5, 5.0, 6.0, 7.5 and 10.0 min at 100% wave amplitude with on/off pulsing combinations of 5 s/5 s, 10 s/5 s or 10 s/10 s

One question that arose as this study progressed, was whether a pulsing scenario might not enhance the effect of ultrasonication, especially on *B. cereus* endospores. Bearing in mind the dispersion of clumps/chains, pulsing might also eliminate the increase in survivors noted after 0.5 min of ultrasonication, as was found with *Lc. lactis* in Study 2. To simplify comparisons between the cultures used in this study, the data illustrating viable counts vs. time (Figs. A17 and A18 in Appendix A) have been summarised in Fig. 7 (A and B) and Table 3.

During ultrasonication the cells that are being treated may become suspended in an ultrasonic standing wave. Ultrasonic standing waves reduce the energy efficiency of the system by forming cavitationally active and passive zones (Tatake & Pandit, 2002). The cells experience time-independent radiation forces that move them to preferred regions of the field separated by half-wavelength intervals (Limaye & Coakley, 1998) where they are protected from cavitation.

Ultrasonic standing waves are beneficial when the system involves applications of physical phenomena such as particle separation, mixing, or emulsification, etc. (Holl, 1978; Kuhn *et al*., 1985) as it creates a pressure gradient resulting in efficient mixing or emulsification due to particle/droplet migration (Tatake & Pandit, 2002). However, when ultrasound is applied as a means of "sterilisation", the formation of standing waves is not desirable. Ultrasound in the mega-range (MHz), which does not promote the

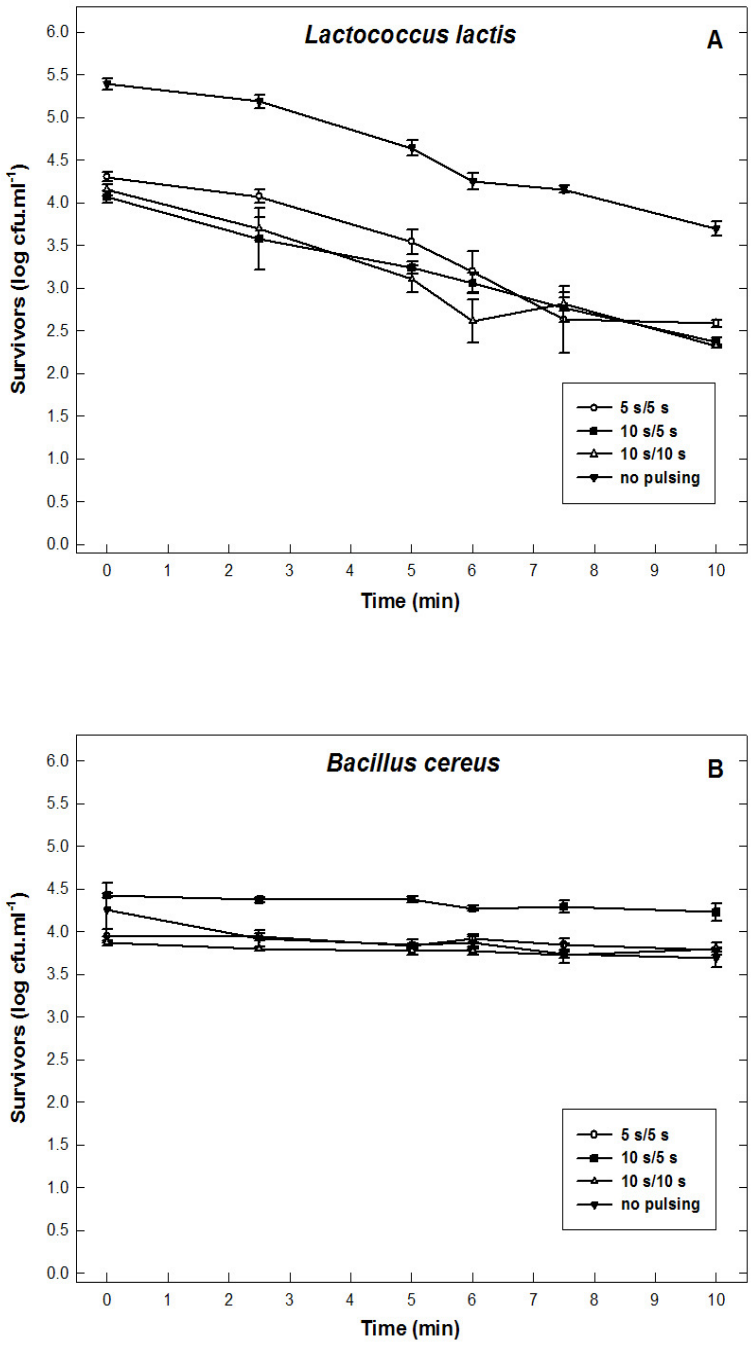

Figure 7 Impact of pulse-ultrasonication at 20 kHz on *Lc. lactis* (A) and *B. cereus* (B) in SSS (Each data point represents quadruple values. The standard deviation was used as the error-bar).

formation of cavitating bubbles, is frequently used to create standing waves (Kilburn *et al*., 1989; Whitworth *et al*., 1991). The use of pulse-ultrasonication would eliminate the aggregation of viable cells in standing waves, should they form at the frequency used in this study. The aim of Study 5 was therefore to indirectly determine the possible existence of standing waves.

No pulsing

As described in Study 1. Viable *Lc. lactis* cells were reduced by 97.98% (1.69 logs). The initial inoculum was 2 x 10^5 cfu.ml^{-1} in SSS. The D_{US} was calculated to be 5.0 min (non-typical logarithmic death curve).

A 0.64 log reduction (77.35%) was described in Study 1 for *B. cereus* in SSS with an initial concentration of 2 x 10^4 cfu.ml^{-1}. The D_{US} could not be calculated for *B. cereus* as a single log reduction was not reached (Table 3).

Duty cycle: 5 s on/5 s off

The first pulsing combination of the samples was for 5 s, followed by a 5 s off-period. This process was repeated for the above mentioned treatment times.

Pulse-ultrasonication of *Lc. lactis* in SSS with an initial bacterial load of 1 x 10^4 cfu.ml^{-1} for 10.0 min resulted in a 98.08% reduction (1.72 log cycles) (Fig. 7A). The D_{US} was found to be 4.3 min.

The data showed that *B. cereus* was more resistant to pulse-ultrasonication than *Lc. lactis*, with only a 32.04% (0.17 log cycles) of the initial 1 x 10^4 cfu.ml^{-1} being eliminated after 10.0 min of pulse-ultrasonication (Fig. 7B). The D_{US} for *B. cereus* could not be calculated (Table 3).

Duty cycle: 10 s on/5 s off

Pulse-ultrasonication with a 5 s off-period, but with a longer ultrasonication period of 10 s achieved similar results over a period of 10.0 min. A 1.70 log reduction (98.01%) of a 1 x 10^4 cfu.ml^{-1} inoculum was achieved after pulse-ultrasonicating *Lc. lactis* in SSS for 10.0 min (Fig. 7A). The D_{US} as calculated for *Lc. lactis* for this pulse-ultrasonic treatment was 6.0 min.

Bacillus cereus in SSS with an initial bacterial load of 1 x 10^4 cfu.ml^{-1} was reduced by 35.63% (0.19 log cycles) after 10.0 min of pulse-ultrasonication (Fig. 7B). The D_{US} for *B. cereus* could not be calculated as a single log reduction was not reached (Table 3).

Duty cycle: 10 s on/10 s off

In this study an initial *Lc. lactis* load of 1 x 10^4 cfu.ml^{-1} in SSS was reduced by 98.82% (1.93 logs) after 10.0 min of pulse-ultrasonication (Fig. 7A). The D_{US} was found to be 5.1 min.

The D_{US} for *B. cereus* could not be determined as only a 0.07 log reduction (14.48%) was achieved after 1 x 10^4 viable cells in SSS were pulse-ultrasonicated for 10.0 min (Fig. 7B) (Table 3).

Discussion of the data from Study 5

In this study, *Lactococcus lactis* was found to be the most sensitive to a pulsing combination of 10 s/10 s with a 1.93 log reduction (98.82%), followed by the 5 s/5 s (98.08%) and 10 s/5 s (98.01%) pulsing combinations.

The pulsing of ultrasound did not affect the survival of *B. cereus,* however, a pulsing combination of 10 s/5 s with a 0.19 log reduction (35.63%) was slightly more effective than the 5 s/5 s (32.04%) or 10 s/10 s (14.48%) combinations. The results from this study suggest that 10 s/5 s was the best average pulsing combination for both "test" microbes. As with *Lc. lactis*, a pulse-treatment did not enhance the destructive effect of ultrasound on *B. cereus*. These results are similar to those obtained for *E. coli* in Chapter 3 where pulsing of ultrasound was also not found to enhance the lethality of ultrasonication.

Standing waves will lead to the aggregation of viable cells in regions where they are protected from the lethality of ultrasound. By pulsing the ultrasound, the formation of standing waves is eliminated, which should lead to an increase in the efficiency of ultrasonication. As pulsing did not enhance the efficiency of ultrasound, it was thus concluded that ultrasonic standing waves are not formed at a frequency of 20 kHz.

Conclusions

There are numerous reports in the literature suggesting "pasteurisation failures" in the dairy industry as a possible cause for an end-product with a poor quality. A wide range of microbes have attributed to a decrease in shelf-life. Cronje (2003) reported on pasteurised milk with high counts and isolated *B. cereus*, *C. meningosepticum*, *Lb. acidophilus* and *Lc. lactis*. Therefore these isolates, as well as other spoilage and potential pathogenic cultures were evaluated in this study.

From the data obtained in this study it is clear that each microbe evaluated showed a unique response to ultrasonication and that the microbe size, morphology and the Gram characteristics should not be used as a guaranteed indication of sensitivity to ultrasonication.

One of the problems encountered while using cultures with the ability to form chains, was that an increase in viable cells was noted after a short ultrasonic treatment. In 1997, Ciccolini *et al*. reported that ultrasound could be employed to disperse chains. In cases where this happens, it would lead to a false impression that either the number of cells are increasing after an ultrasonic treatment, or that there is no reduction in the number of viable cells. In Study 2 it was showed that the number of viable cells increased after the first 30 sec of ultrasonication. This phenomenon was ascribed to the dispersion of clumps.

In Chapter 3 of this dissertation it was found that *E. coli* behaved linearly as the time of treatment was lengthened. It is generally accepted that the number of survivors after ultrasonication is an exponential function of time (Davies, 1959; Mett *et al*., 1988), and therefore should produce a linear logarithmic curve. In this study, however, all the microbes used did not show a linear death curve. The presence of endospores explains the non-linear behaviour of *B. cereus*. The disruption of *Lc. lactis* chains also led to a non-linear death curve obtained in Study 2 for this microbe. As with heat inactivation, it can thus be concluded that there is some deviation from linearity when ultrasound is used.

When ultrasonication data is graphically illustrated in logarithmic format, the formation of 'tails' has been reported. Some authors (Jacobs & Thornley, 1954; Lee *et al*., 1989) were of the opinion that this is not due to cell recovery or a more resistant fraction of the population, but rather to the progressive loss of efficacy of an ultrasonic treatment. According to these authors dissolved gas, and also bacteria would stop acting as cavitation nuclei due to the destructive effect of ultrasound. If this statement was true, it should be seen for all the microbes tested and not only for *B. cereus*.

The SA “milk law” (Anon., 1997) states that raw milk with contamination levels of 200 000 cfu.ml^{-1} or less must be reduced to less than 50 000 cfu.ml^{-1} prior to selling as pasteurised milk. This is equivalent to a 75% reduction in viable counts. This study indicated that the number of viable cells for all microbes evaluated were reduced by more than 75% with *P. fluorescens* (100% elimination) and *S. cerevisiae* (99.7% elimination) being the most sensitive. *Listeria monocytogenes* (99% elimination) and *Lc. lactis* (97% elimination) were also very sensitive to the destructive effect of

ultrasound. *Bacillus cereus* (87% elimination), *C. meningosepticum* (85% elimination) and *M. luteus* (88% elimination) were more resistant to ultrasonication. The data showed that *Lb. acidophilus* (78% elimination) was the most resistant to low-frequency high-power ultrasound. Based on the results from this study, it is recommended that the dairy industry consider low-frequency high-power ultrasound as an alternative to thermal pasteurisation as this could lead to the elimination of the entire microbial population.

To achieve complete elimination of all microbes and to extend the shelf-life beyond that of normal pasteurised milk, it might be feasible to consider using a mild heat treatment in combination with ultrasound. It is also recommended that the effect of ultrasonication on different milk components (proteins and enzymes) should be determined. These different milk components are very important when pasteurised milk is intended to be further processed (cheese, ice-cream, yogurt, etc.).

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APPENDIX A

To Chapter 4

To simplify the discussion of the results, the data illustrated in Figs. A1 - A18 have been included in this Appendix.

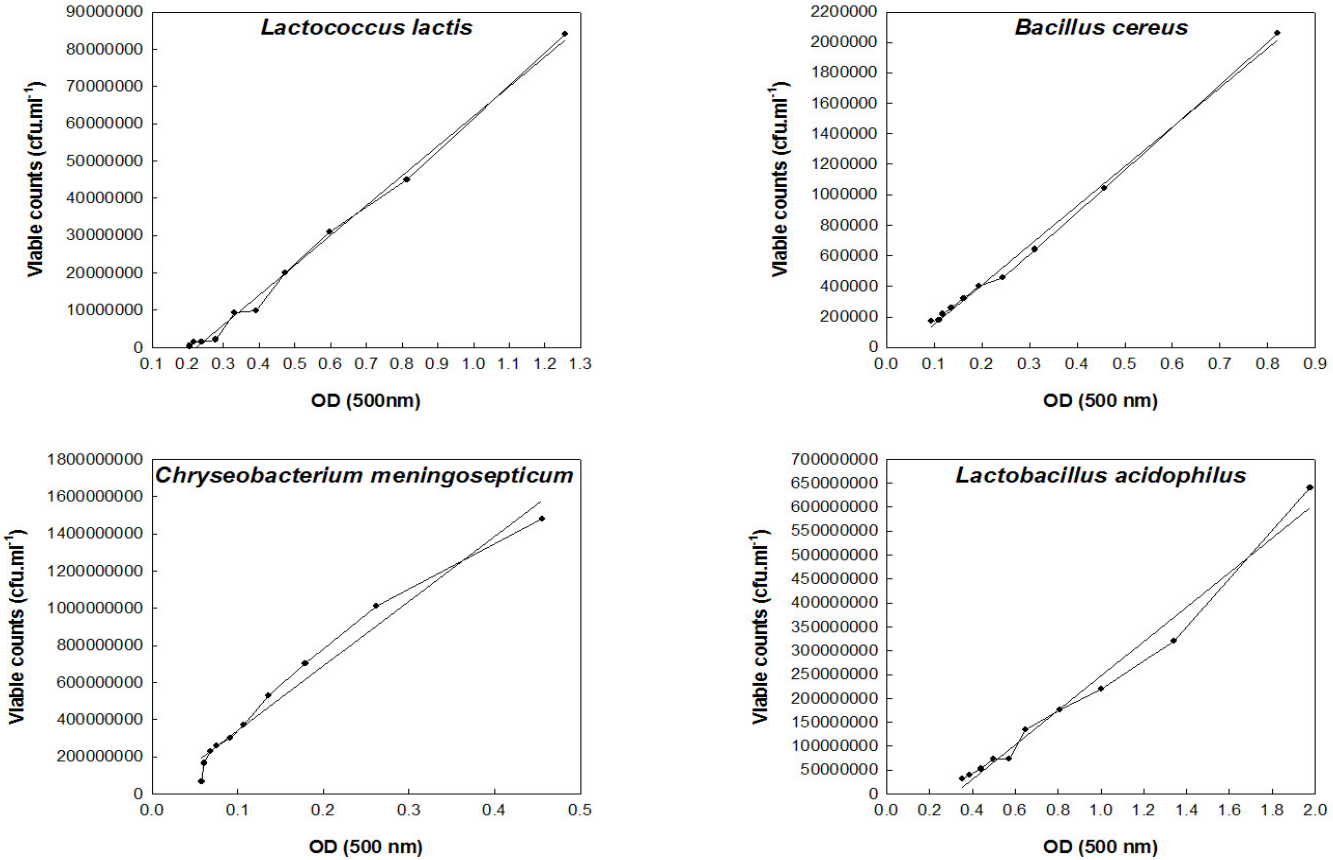

Figure A1 Standard growth curves of 24 h pure cultures of *Lc. lactis*, *B. cereus*, *C. meningosepticum* and *Lb. acidophilus* (Data points represents averages of three experiments).

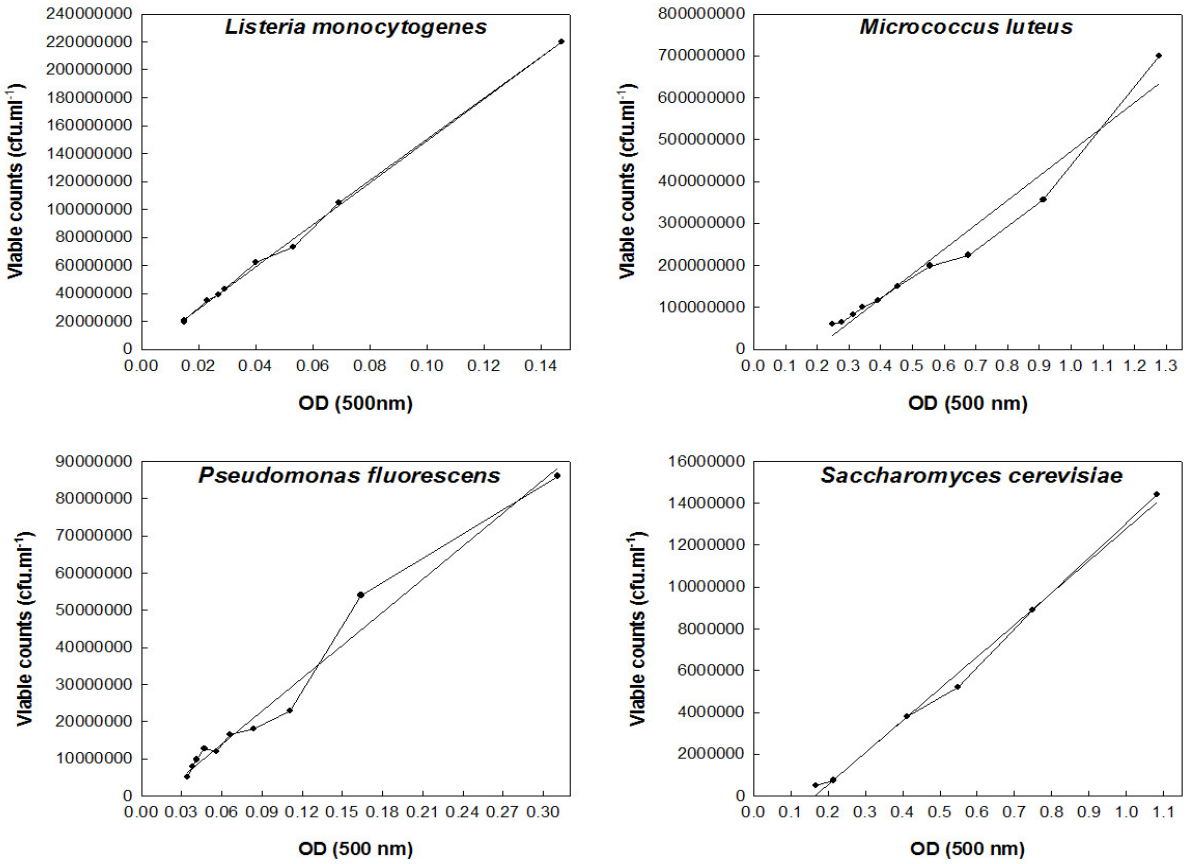

Figure A2 Standard growth curves of 24 h pure cultures of *L. monocytogenes*, *M. luteus*, *P. fluorescens* and *S. cerevisiae* (Data points represents averages of three experiments).

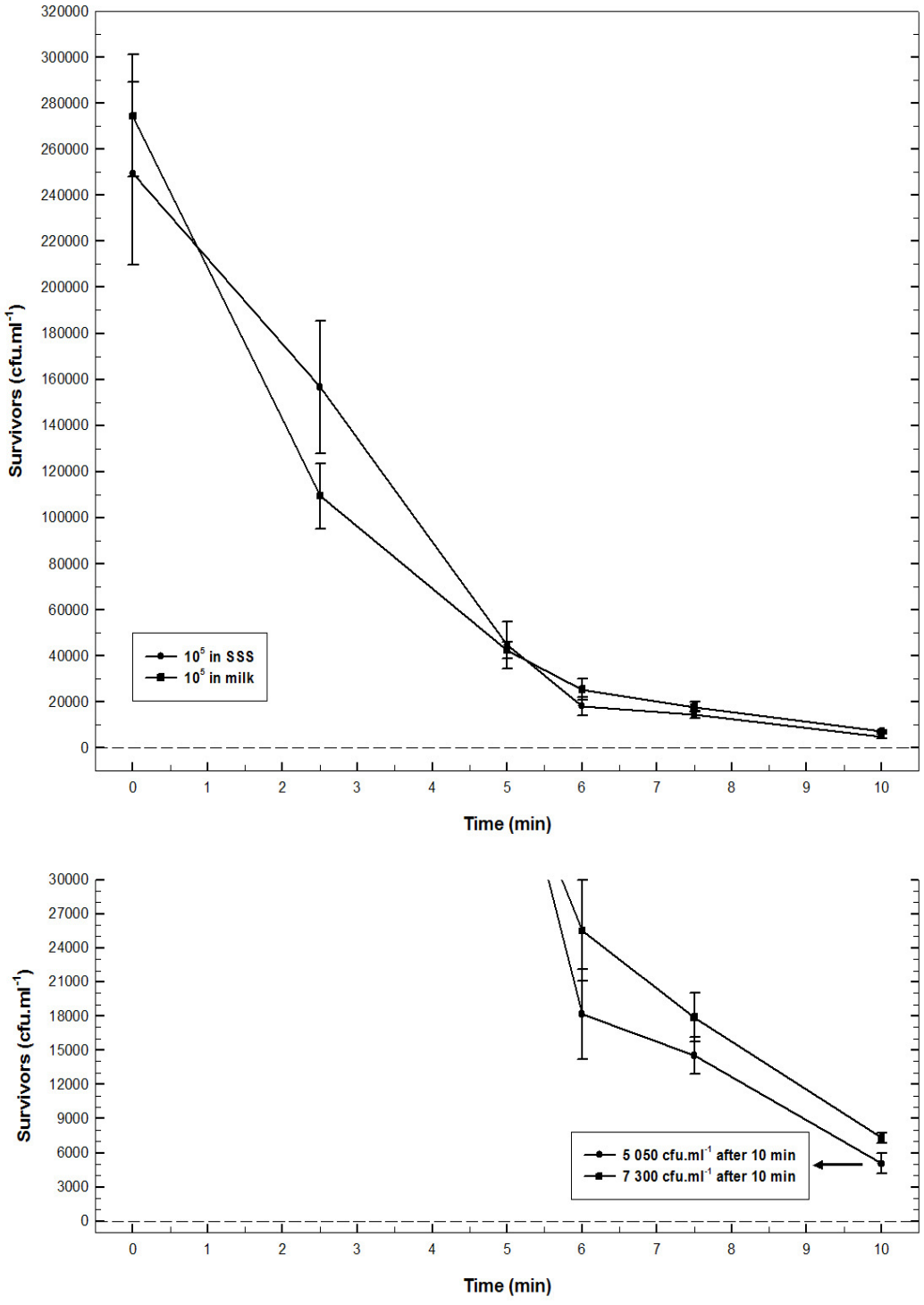

Figure A3 Impact of ultrasonication at 20 kHz on *Lactococcus lactis* at a starting concentration of 1 x 10^5 cfu.ml^{-1} in either SSS or UHT milk. The lower graph represents an extended Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

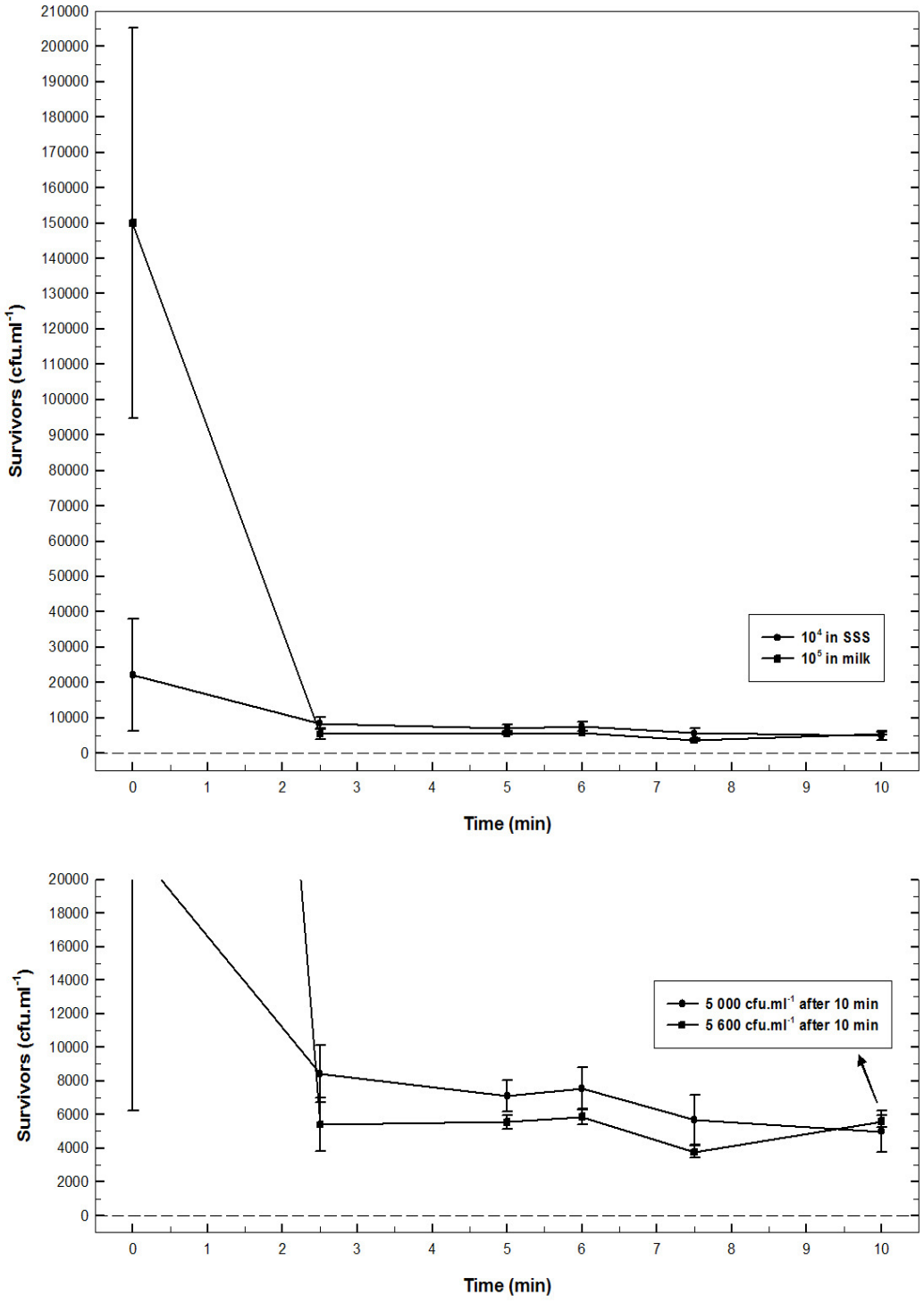

Figure A4 Impact of ultrasonication at 20 kHz on *Bacillus cereus* at different starting concentrations in either SSS or UHT milk. The lower graph represents an extended Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

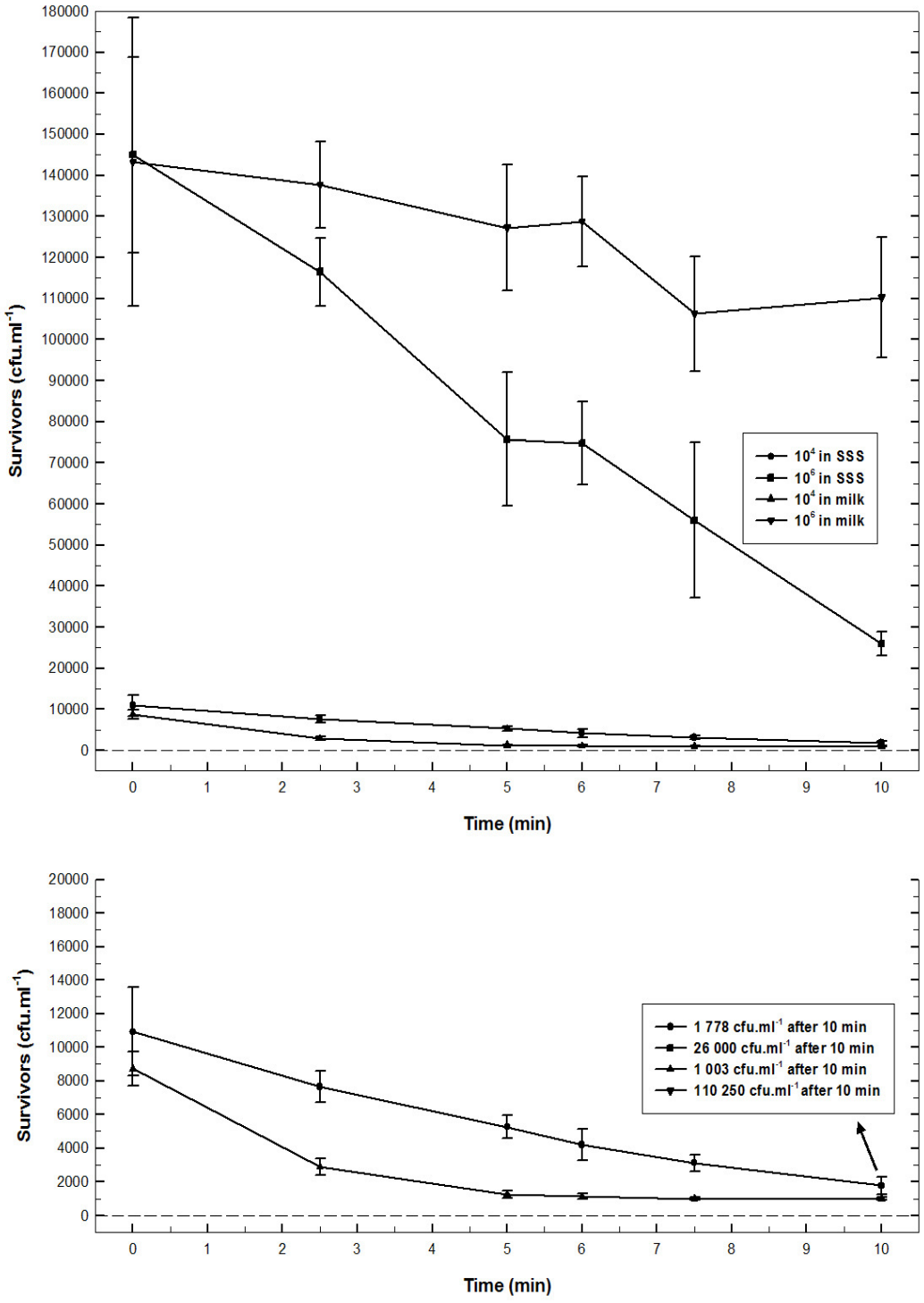

Figure A5 Impact of ultrasonication at 20 kHz on *Chryseobacterium meningosepticum* at different starting concentrations in either SSS or UHT milk. The lower graph represents an extended Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

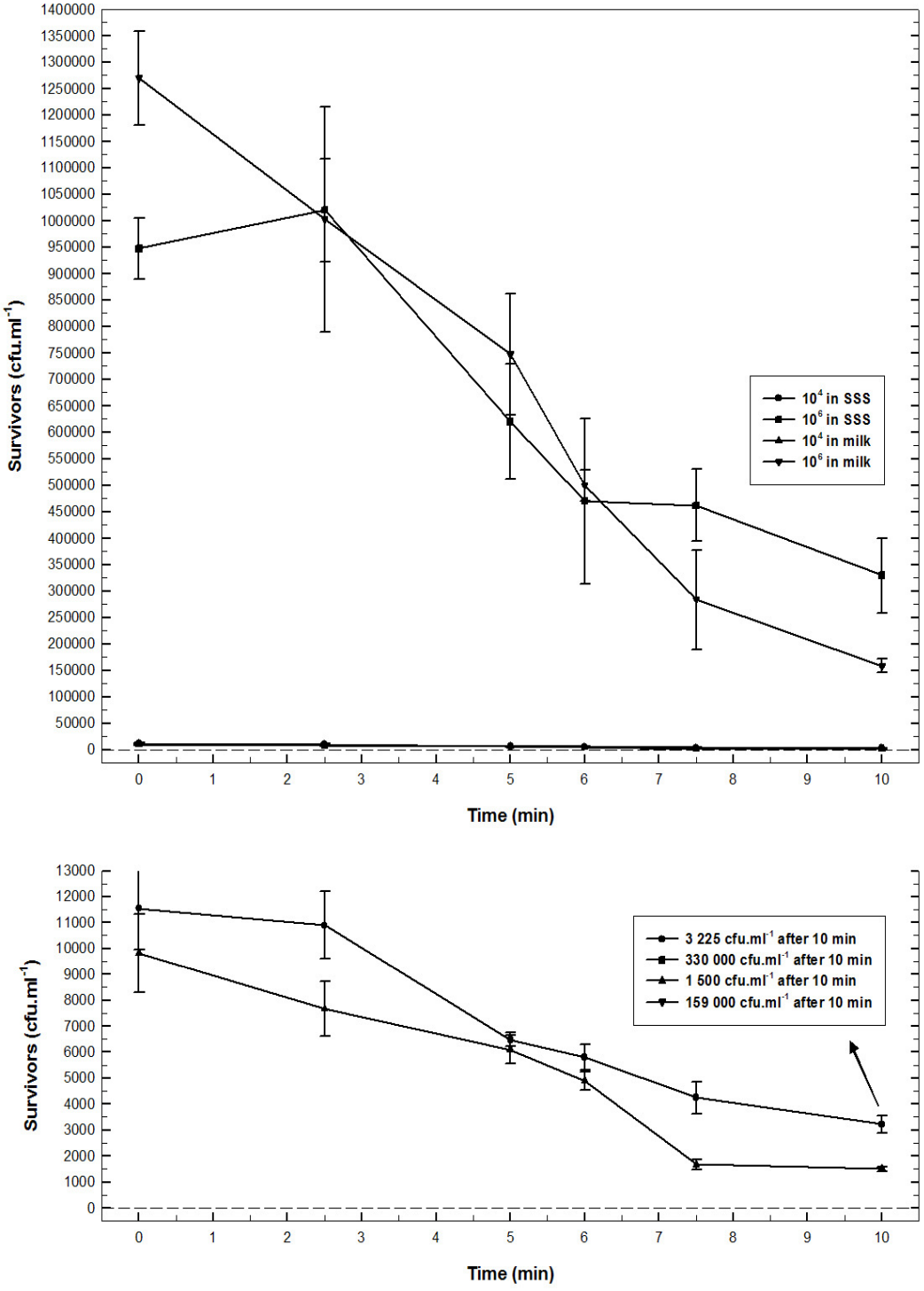

Figure A6 Impact of ultrasonication at 20 kHz on *Lactobacillus acidophilus* at different starting concentrations in either SSS or UHT milk. The lower graph represents an extended Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

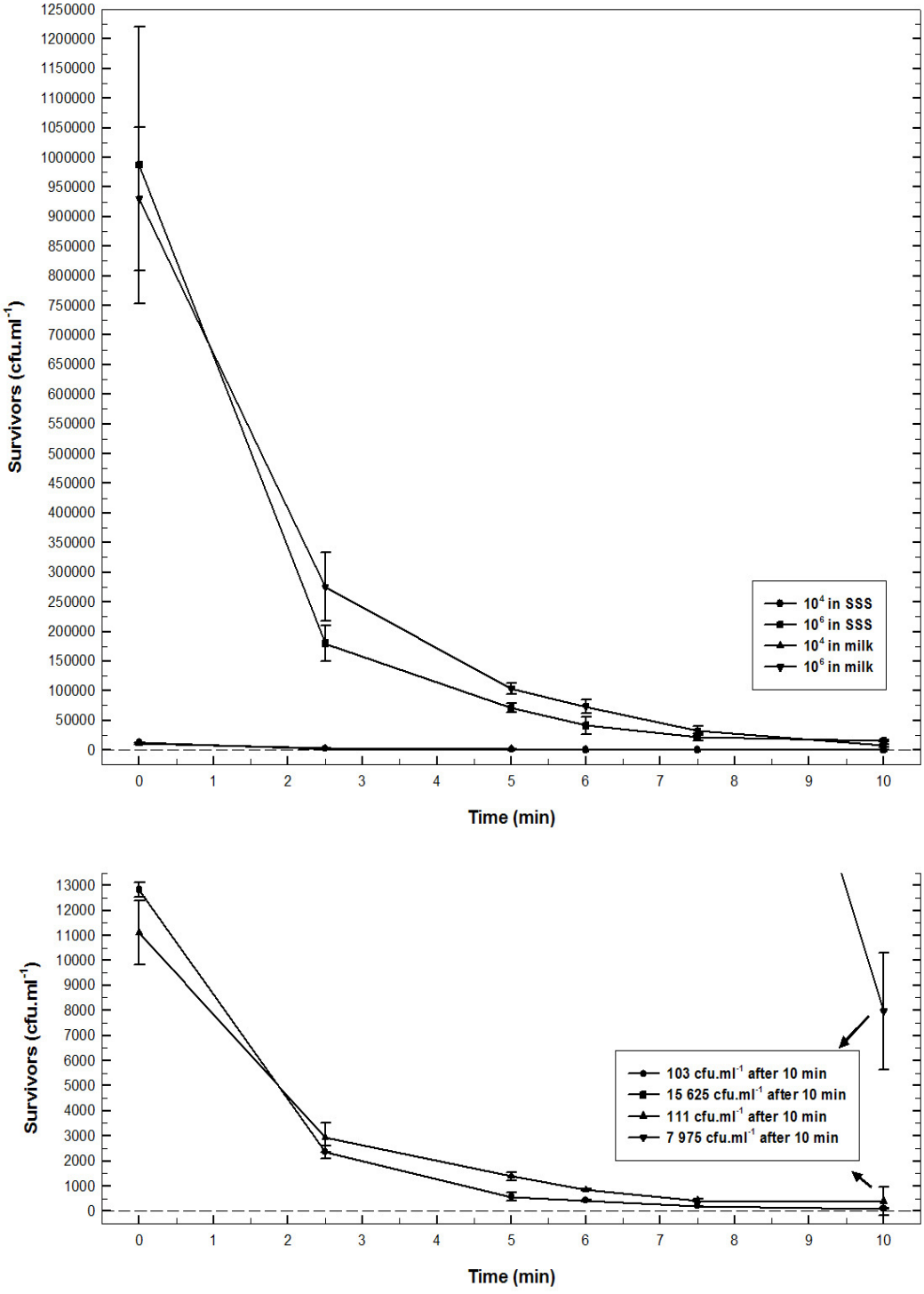

Figure A7 Impact of ultrasonication at 20 kHz on *Listeria monocytogenes* at different starting concentrations in either SSS or UHT milk. The lower graph represents an extended Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

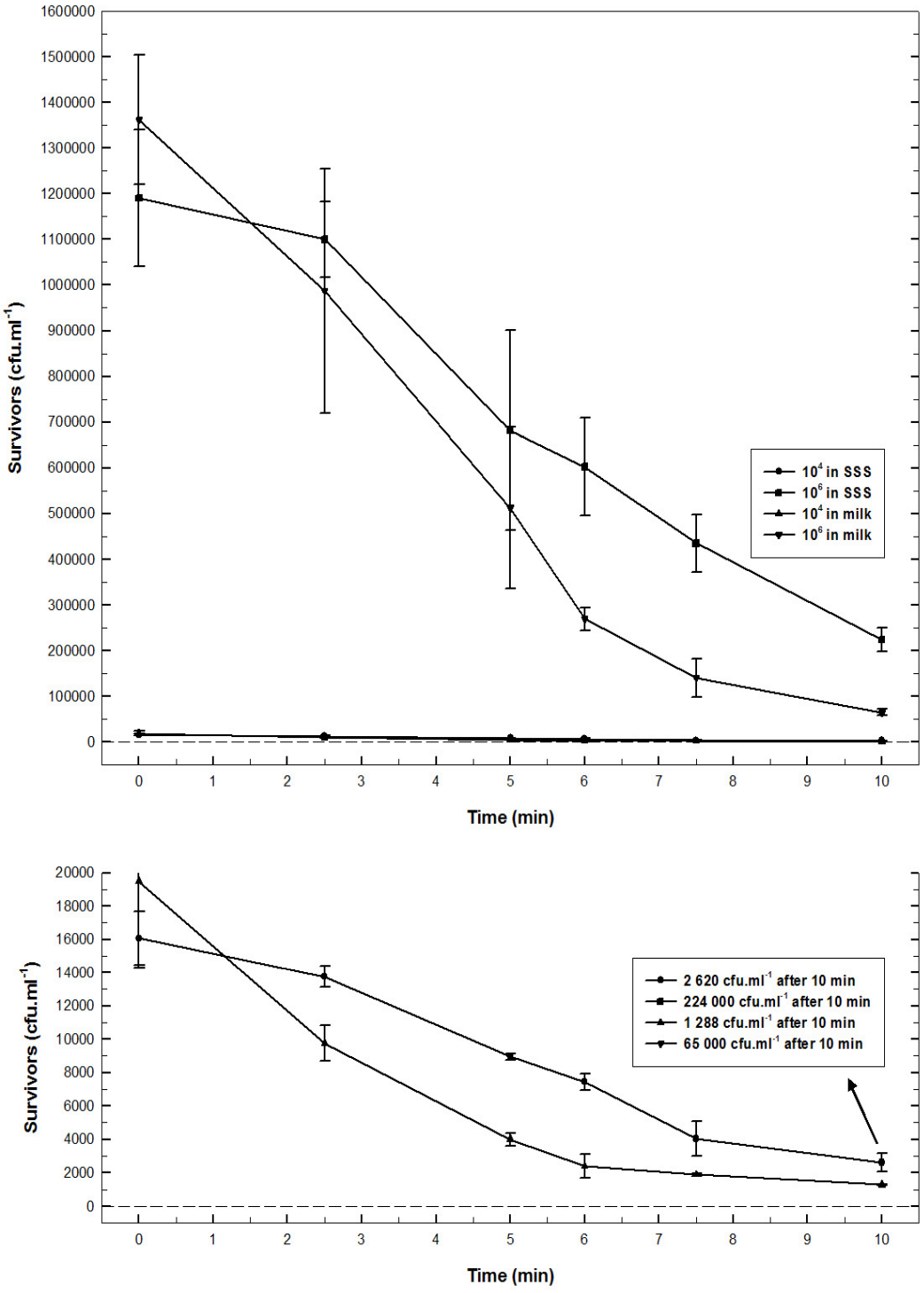

Figure A8 Impact of ultrasonication at 20 kHz on *Micrococcus luteus* at different starting concentrations in either SSS or UHT milk. The bottom graph represents an extended Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

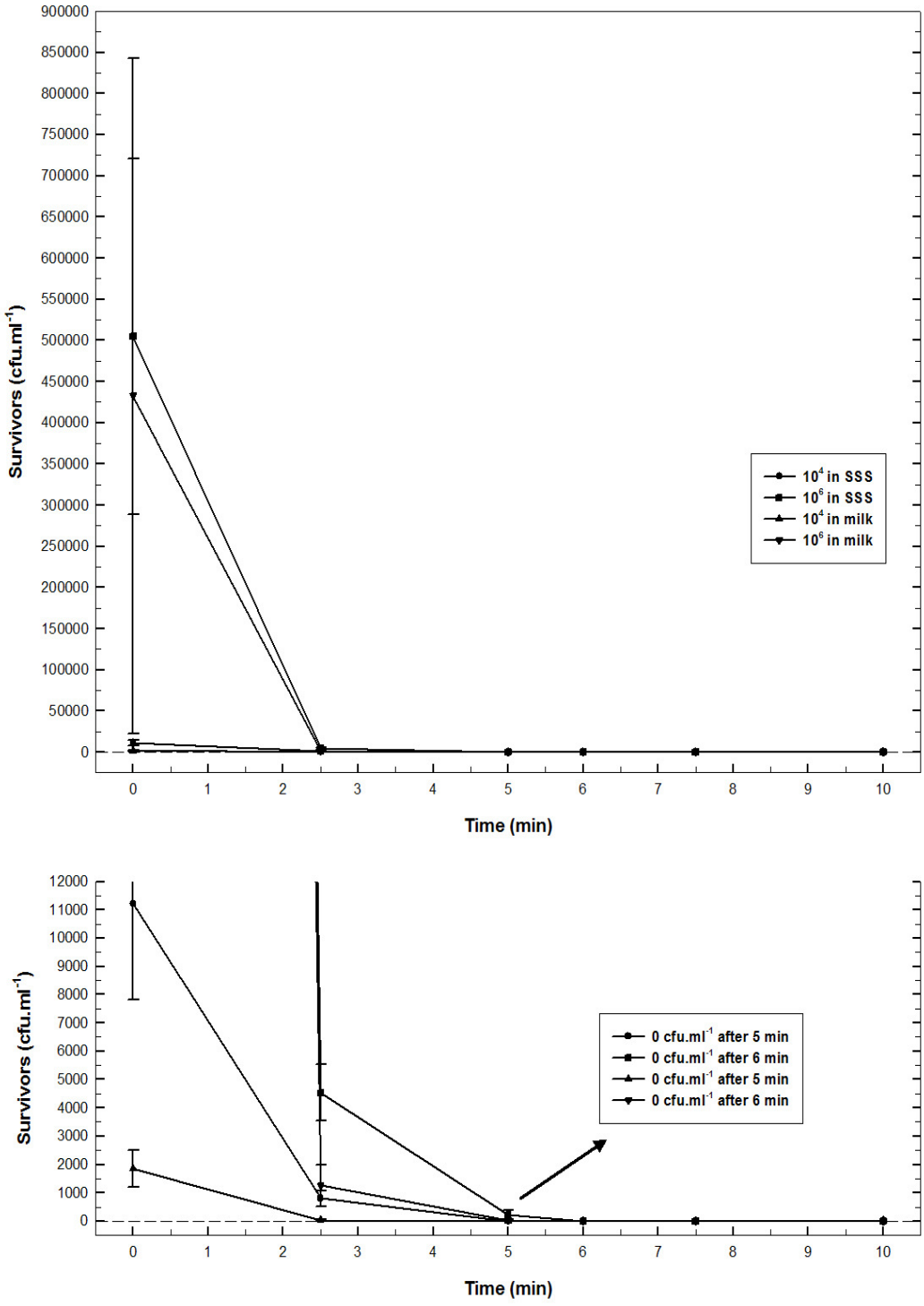

Figure A9 Impact of ultrasonication at 20 kHz on *Pseudomonas fluorescens* at different starting concentrations in either SSS or UHT milk. The lower graph represents an extended Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

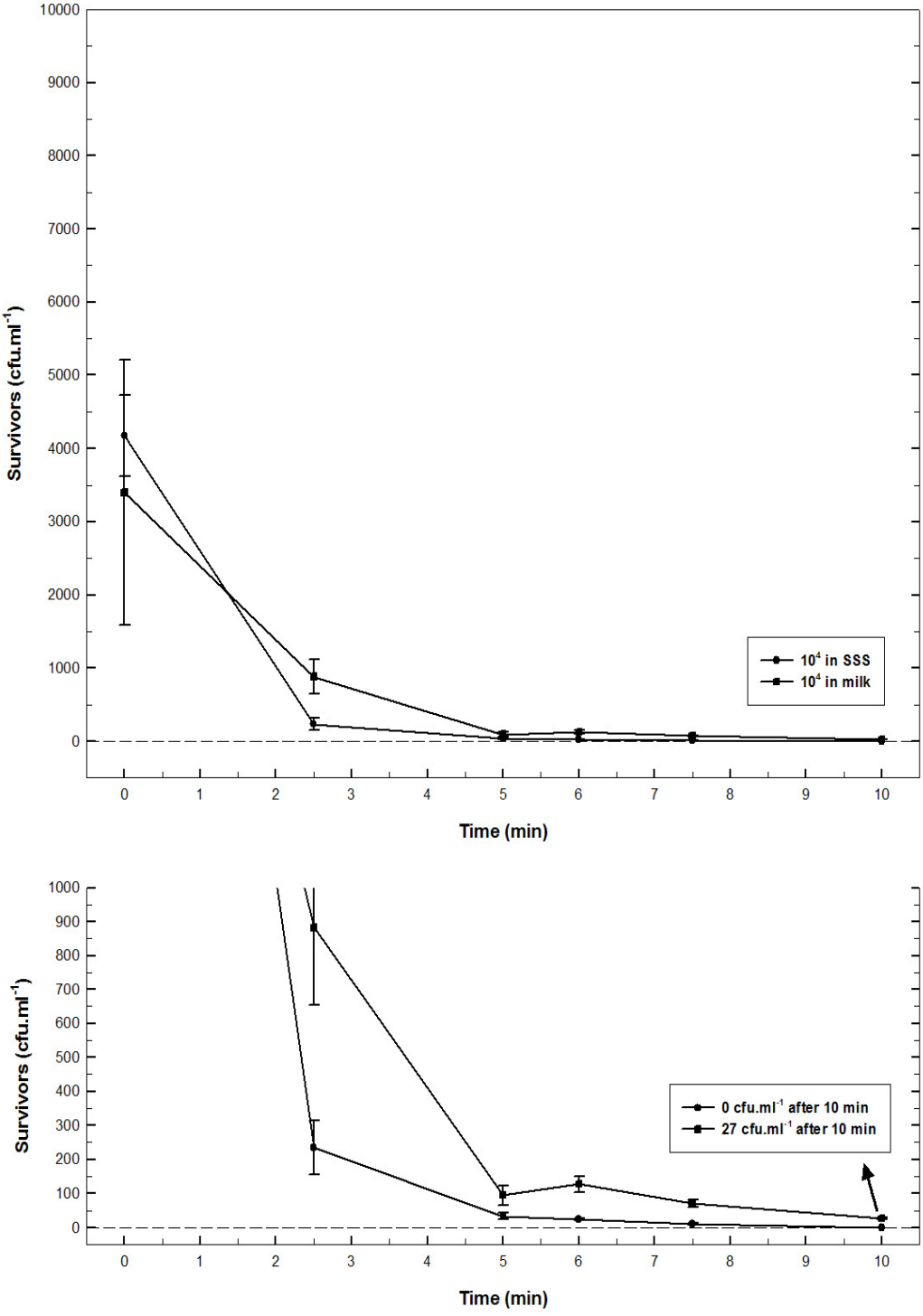

Figure A10 Impact of ultrasonication at 20 kHz on *Saccharomyces cerevisiae* at a starting concentration of 1 x 10^4 cfu.ml^{-1} in either SSS or UHT milk. The lower graph represents an extended Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

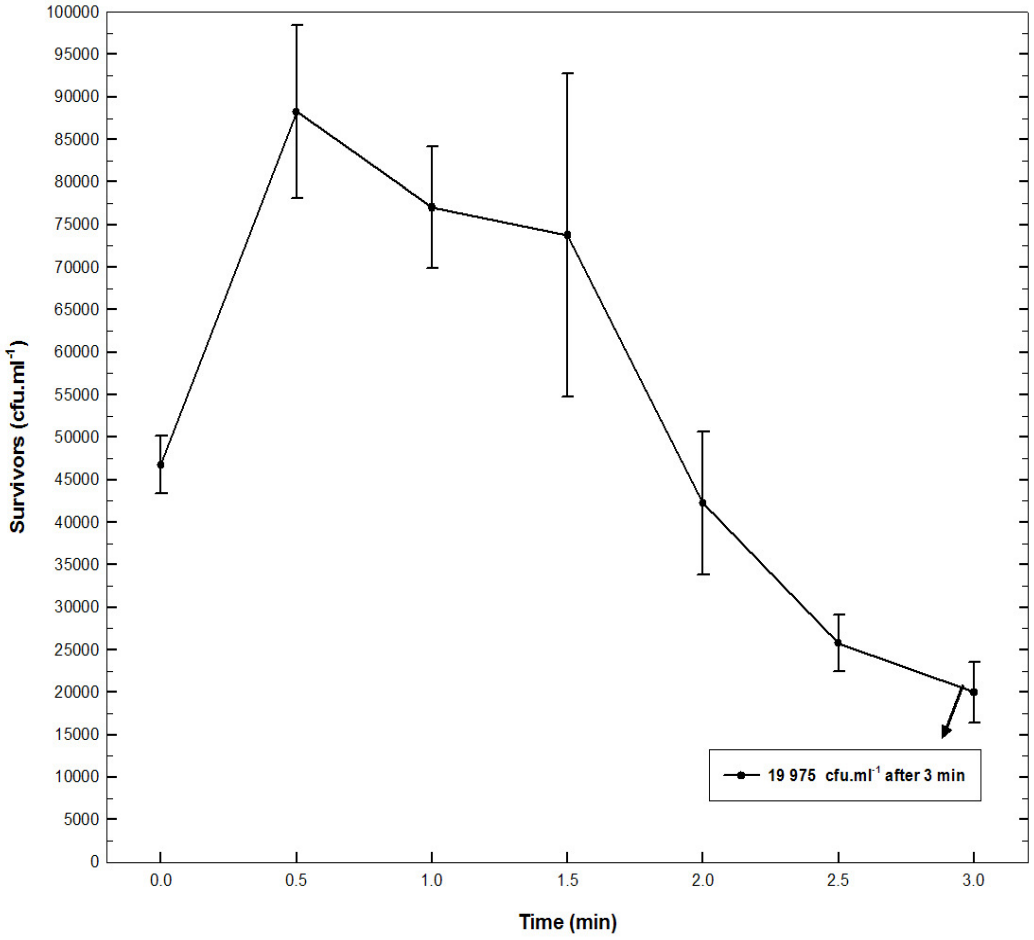

Figure A11 Impact of ultrasonication at 20 kHz on *Lactococcus lactis* at a starting concentration of 1 x 10^4 cfu.ml^{-1} in UHT milk (Each data point represents quadruple values. The standard deviation was used as the error-bar).

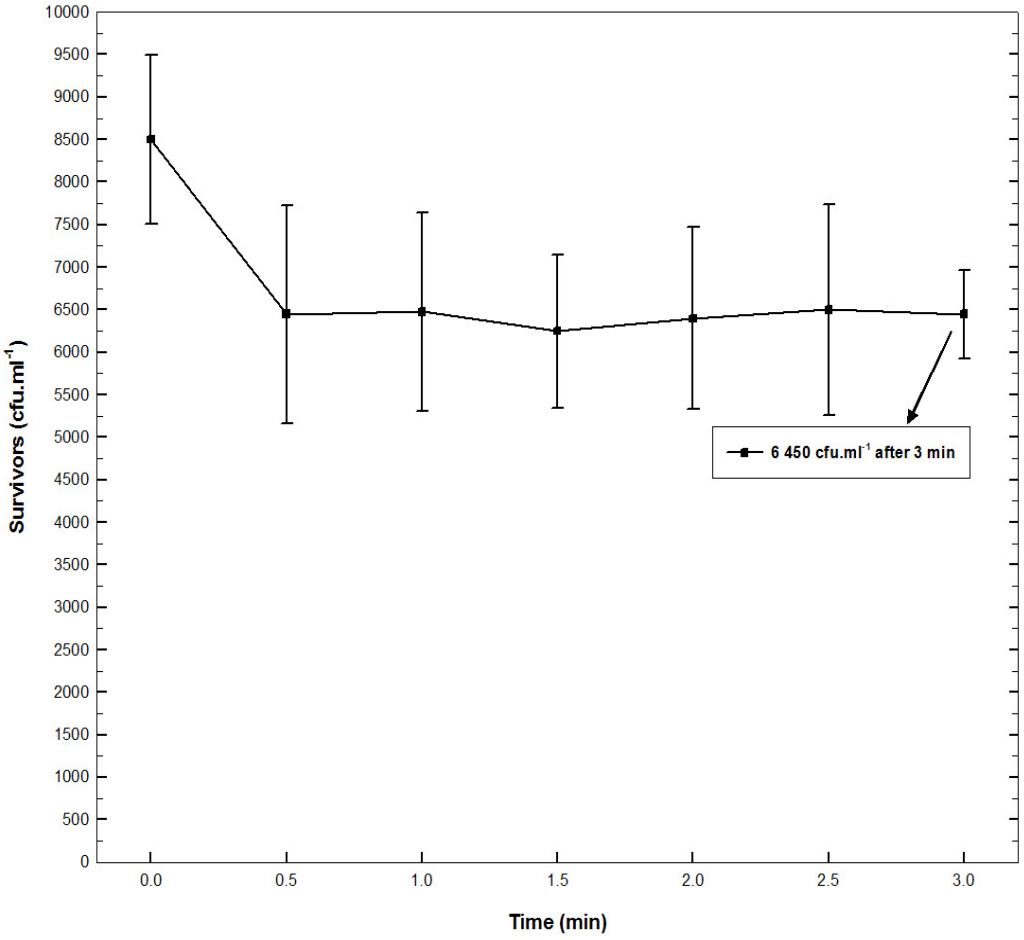

Figure A12 Impact of ultrasonication at 20 kHz on *Bacillus cereus* at a starting concentration of 1 x 10^4 cfu.ml^{-1} in UHT milk (Each data point represents quadruple values. The standard deviation was used as the error-bar).

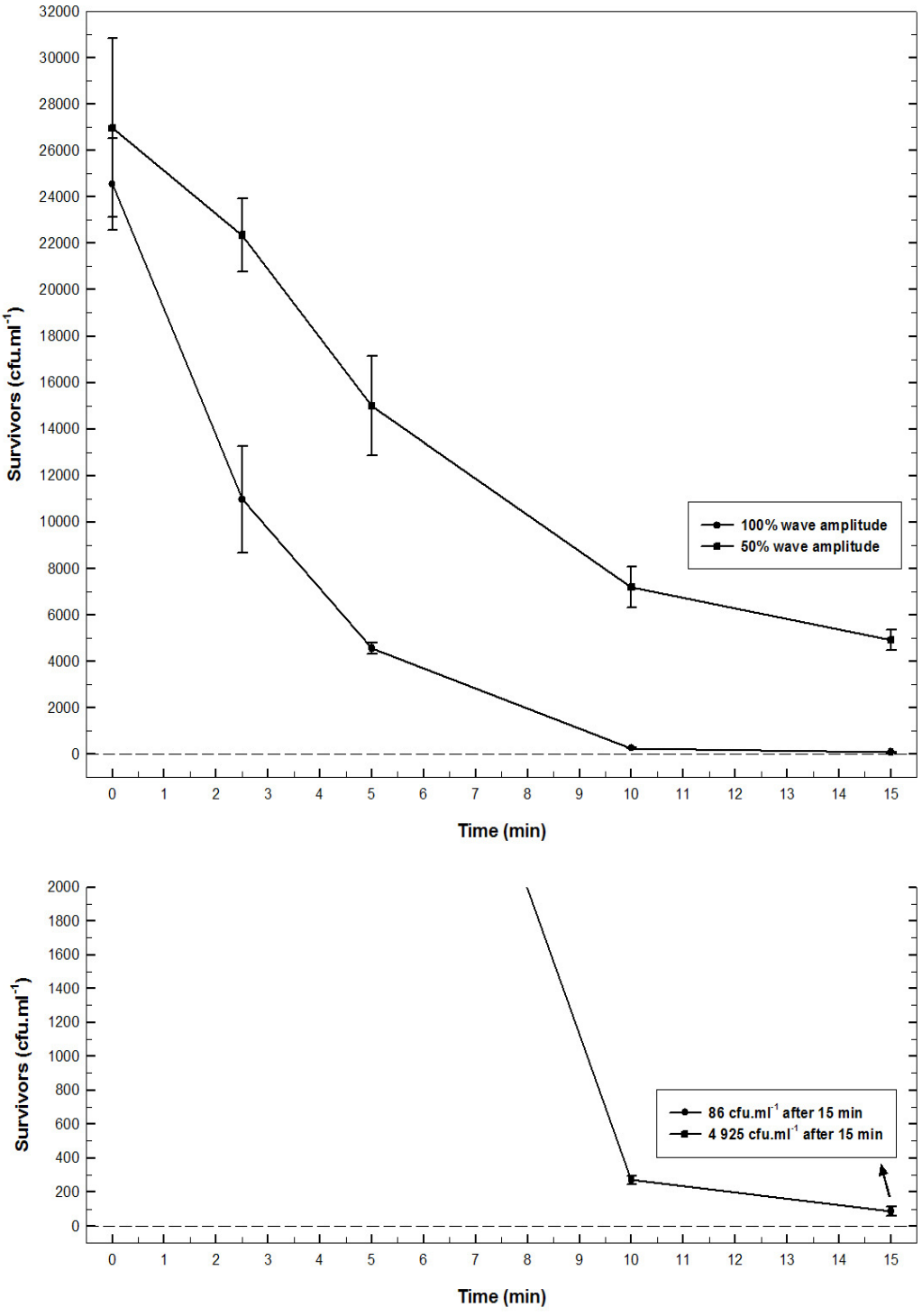

Figure A13 Impact of ultrasonication at 20 kHz and either 100% or 50% wave amplitude on *Lactococcus lactis* in SSS. The lower graph represents an extended Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

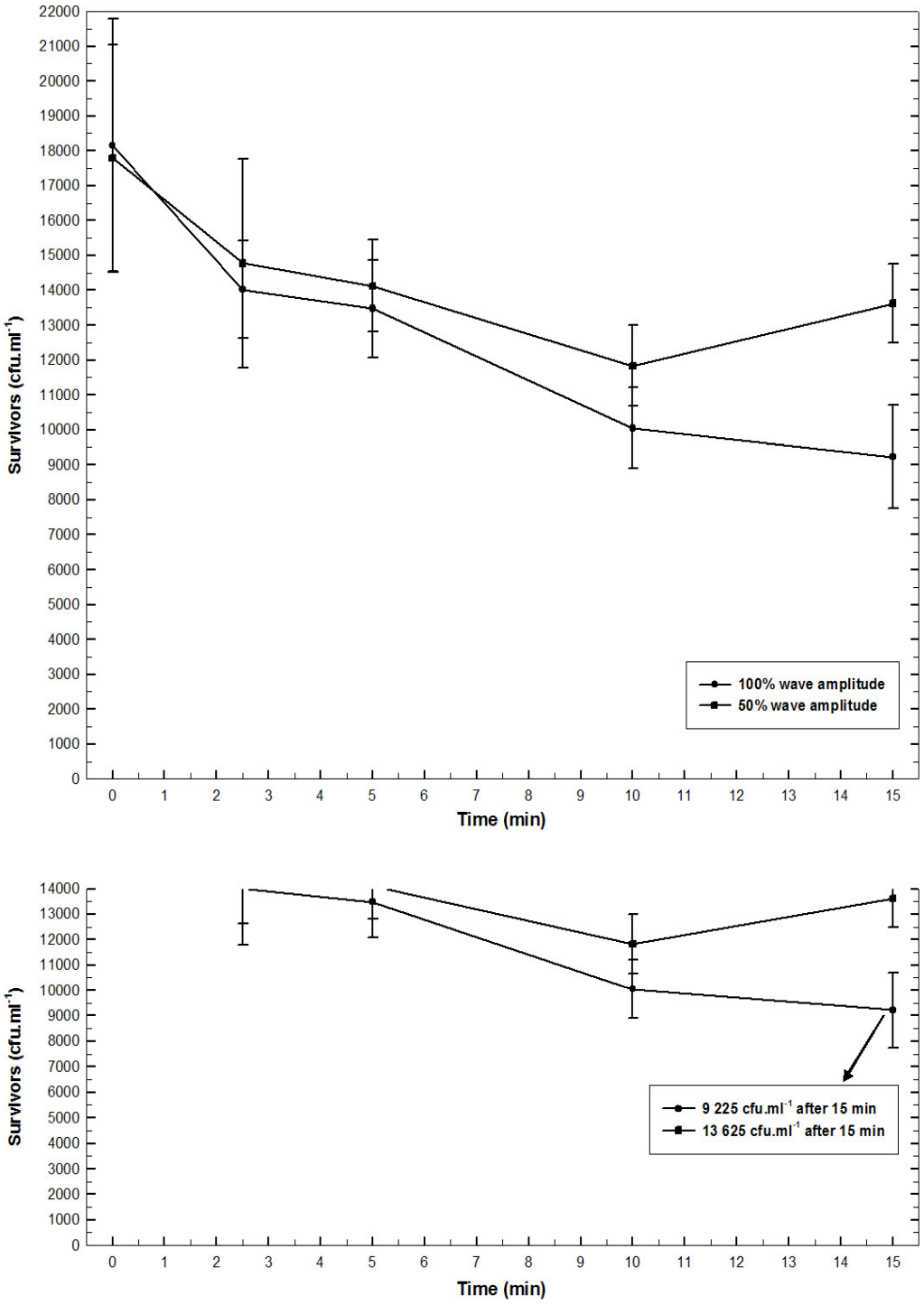

Figure A14 Impact of ultrasonication at 20 kHz and either 100% or 50% wave amplitude on *Bacillus cereus* in SSS. The lower graph represents an extended Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

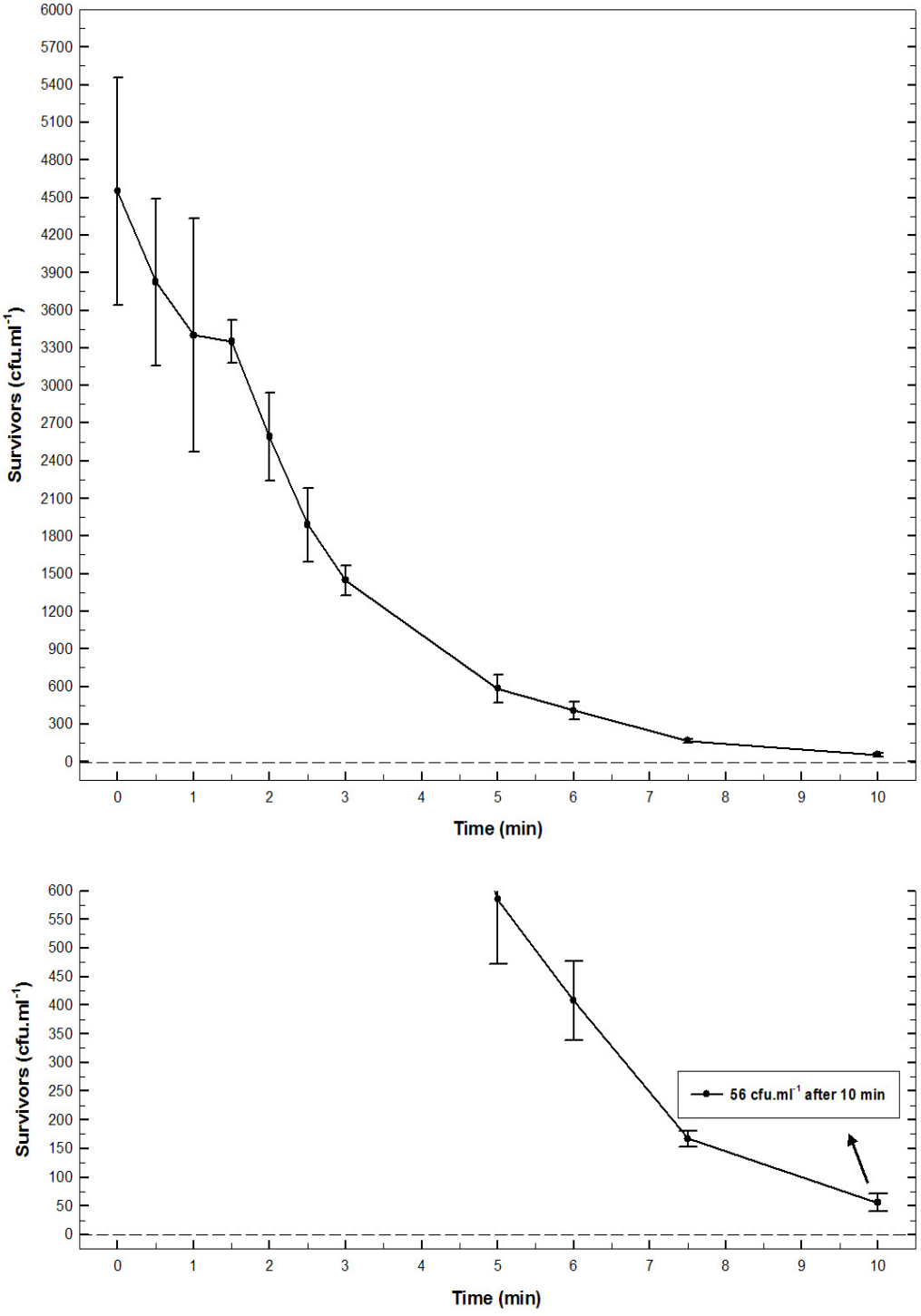

Figure A15 Impact of ultrasonication at 20 kHz on *Lactococcus lactis* at a starting concentration of 2 x 10^3 cfu.ml^{-1} in UHT milk. The lower graph represents an extended Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

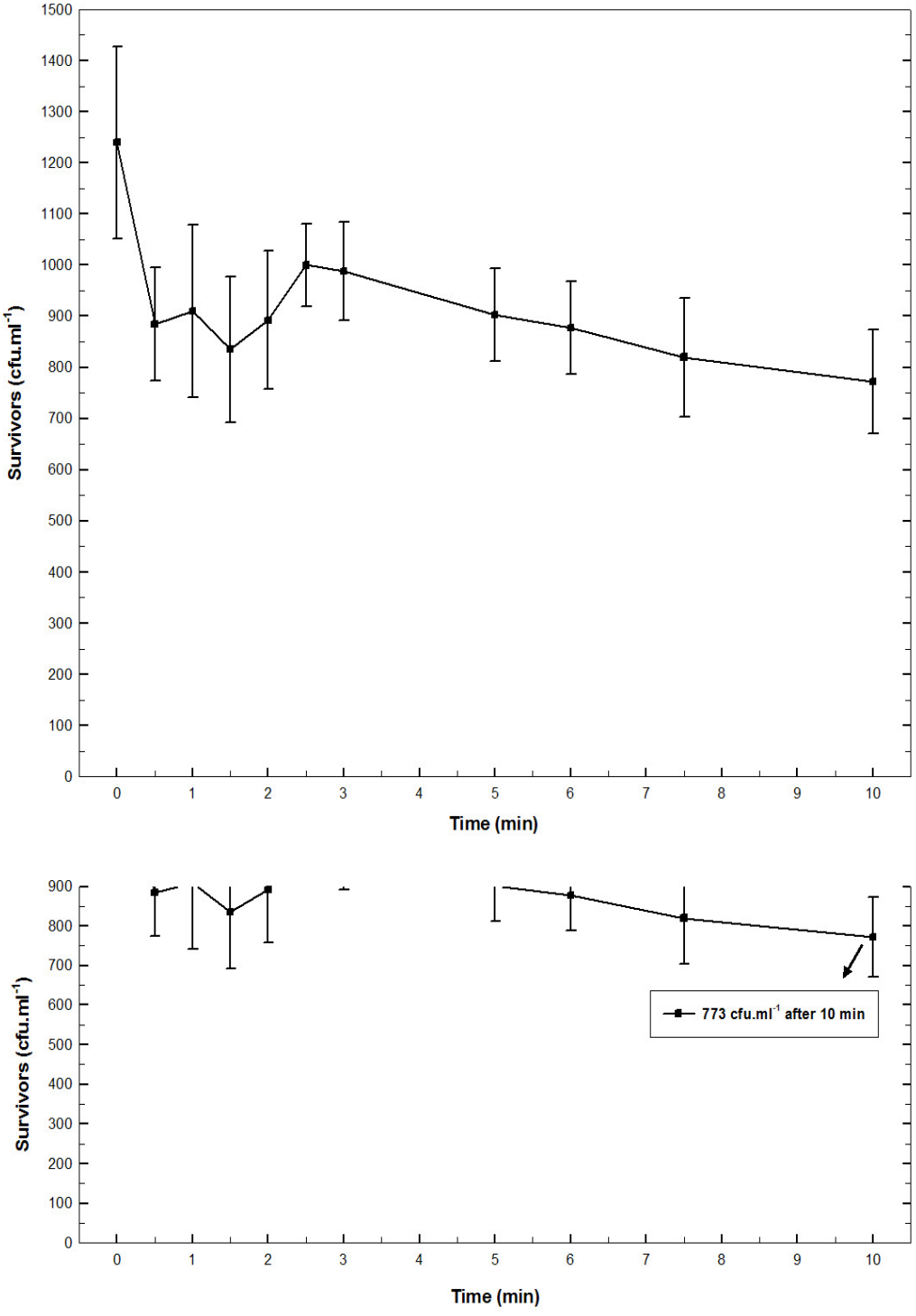

Figure A16 Impact of ultrasonication at 20 kHz on *Bacillus cereus* at a starting concentration of 2 x 10^3 cfu.ml^{-1} in UHT milk. The lower graph represents an extended Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

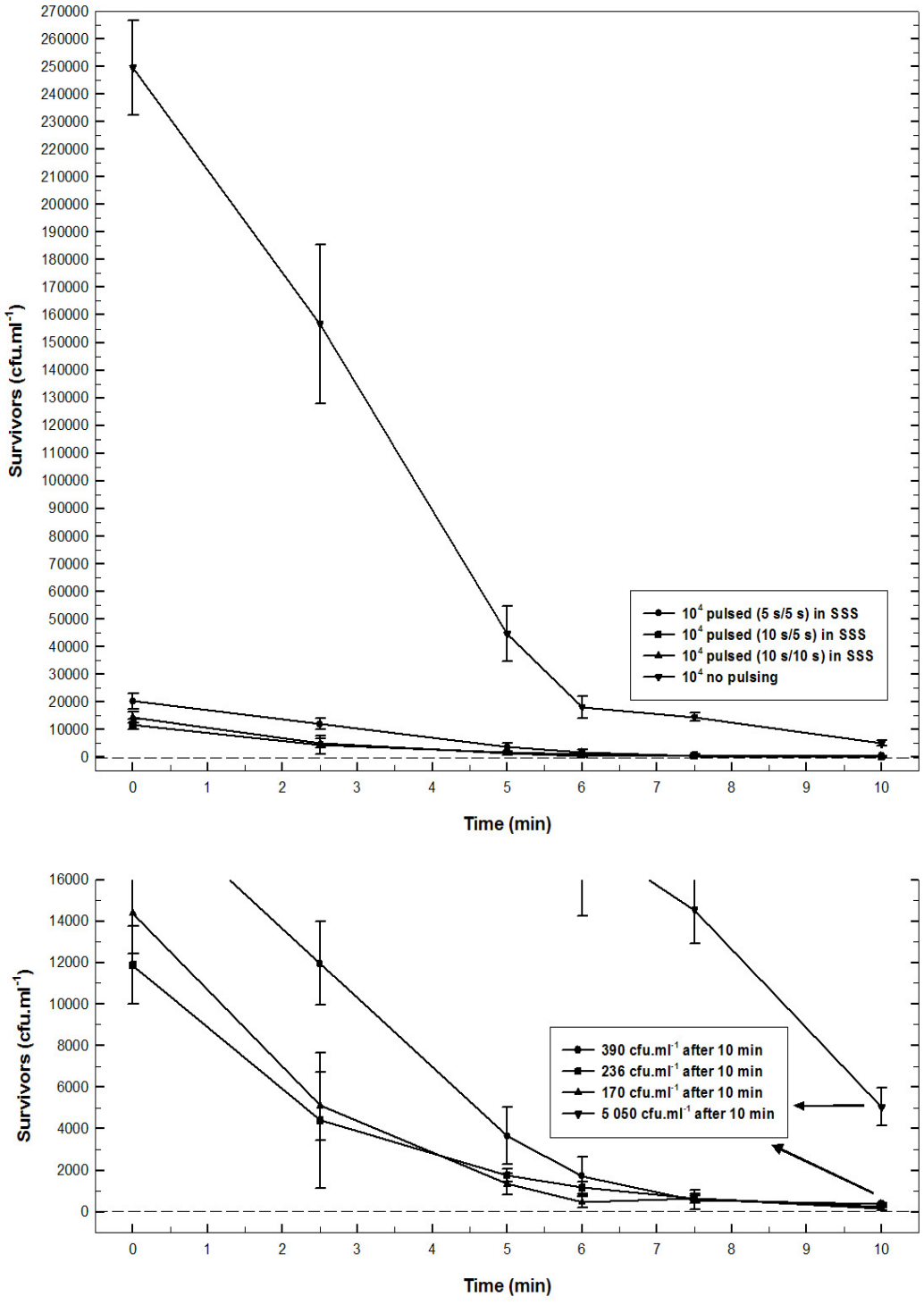

Figure A17 Impact of pulse-ultrasonication at 20 kHz on *Lactococcus lactis* in SSS. The lower graph represents an extended Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

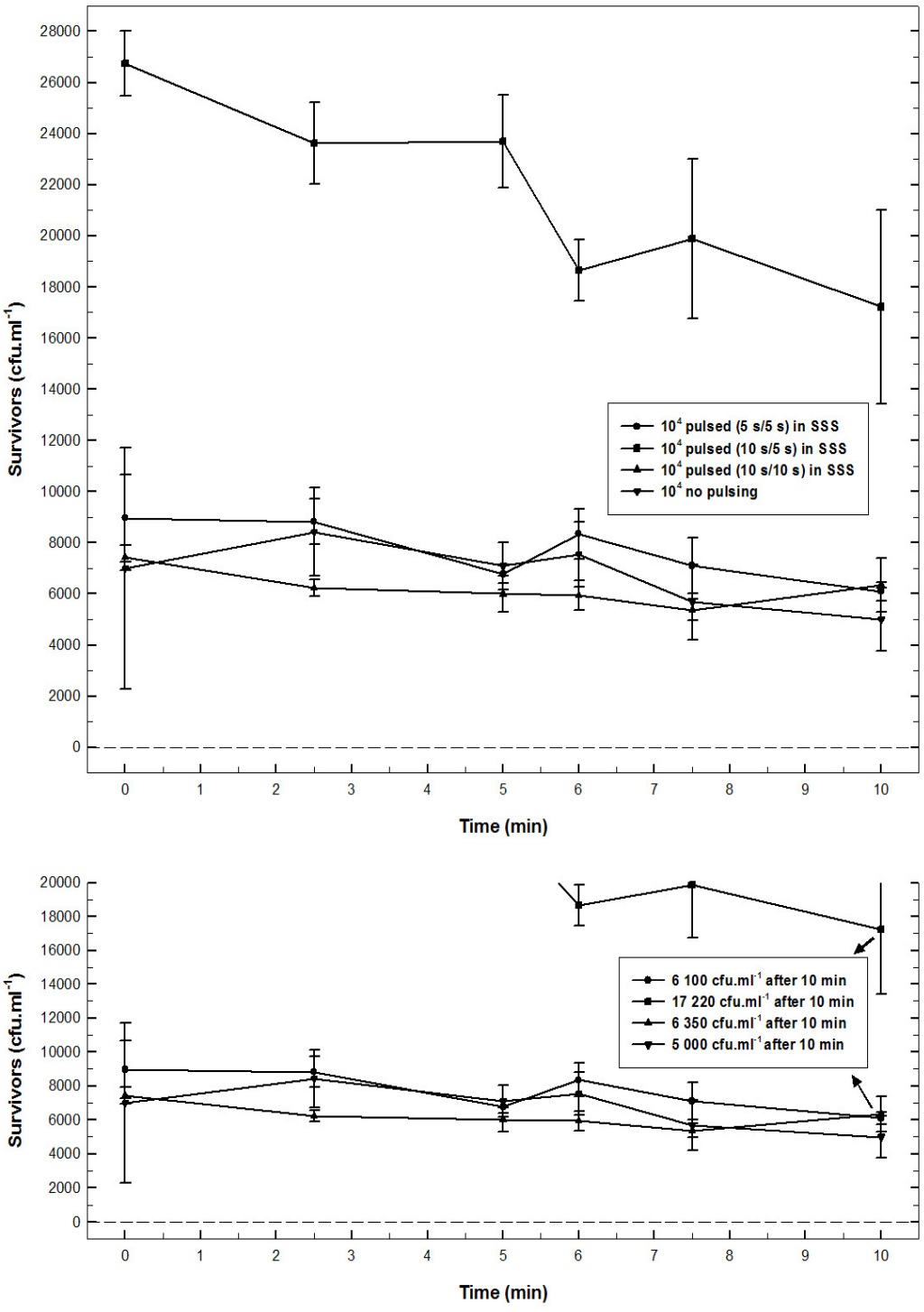

Figure A18 Impact of pulse-ultrasonication at 20 kHz on *Bacillus cereus* in SSS. The lower graph represents an extended Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

CHAPTER 5

QUANTIFICATION OF ULTRASONICALLY INDUCED CELL DAMAGE

Abstract

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, such as nutrient and flavour loss. The aim of this study was to determine if microbial protein and DNA are released from cells after ultrasonication, and if the concentrations released give an indication as to the "degree" of microbial damage as a result of ultrasonication. Electron microscopy (SEM and TEM) was used to gather visual information as to the type and extent of structural damage inflicted on microbial cells after an ultrasonic treatment. It was found that *Escherichia coli* and *Lactococcus lactis* protein and DNA are released and indicate microbial damage. It was, however, found to be impossible to correlate the protein or DNA concentrations released to the exact number of viable cells eliminated. Scanning electron microscopy micrographs showed that ultrasonication damages the cell wall of *E. coli*, whilst with TEM it was possible to observe both internal as well as external damage inflicted on *E. coli*, *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* cells.

Introduction

Milk is generally given some form of a heat treatment to control microbial growth. Heat processing is not effective against all microbes (Ternström *et al*., 1993; Larsen & Jørgensen, 1997; Salmerón *et al*., 2002) associated with milk and may trigger unwanted reactions such as loss of flavour, nutrient and vitamins (Aronsson *et al*., 2001). These short-comings have led to renewed interest in non-thermal preservation methods that can effectively eliminate microbial activity (Piyasena *et al*., 2003). 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.

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 (Foegeding & Ray, 1992). 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 extremely useful insight on the cell wall and cell organelles. 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 (Hajmeer *et al*., 2006).

The aim of this study was to determine if an ultrasonic treatment would lead to increases in released microbial protein and DNA, and also to determine if these increases could be used to quantify ultrasonically induced cell damage. Transmission (TEM) and scanning electron microscopy (SEM) was furthermore used to gain visual information on ultrasonically inflicted cell damage.

Materials and methods

Bacterial cultures

The four "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 ml 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 prepared as described in Chapters 3 and 4 and were used to determine the inoculum size of the samples to be ultrasonicated.

Sample preparation

The growth medium (150 ml) containing specific levels of cells in the exponential phase were centrifuged at 5 000 x *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 sterile saline solution (SSS) (0.85% m/v) followed by centrifugation.

Table 1 Growth media, incubation times and temperatures used for the four "test" microbes

Microbe	USFSCC[a]	Medium	Incubation	
			Time (h)	Temperature (°C)
Escherichia coli	11775	NB[b]/PCA[c]	24	37°
Lactobacillus acidophilus	1348	MRS[d]	24	35°
Lactococcus lactis	315	MRS	24	30°
Saccharomyces cerevisiae	462	YDP[e]/MEA[f]	24	25°

[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).

The cell pellet was washed to remove any traces of growth medium which may interfere with protein determinations. The final pellet was resuspended in 500 ml saline solution and this was used as the sample to determine changes in released microbial protein and DNA. An inoculum of (as near as possible to) either 1 x 10^6 colony forming units per ml (cfu.ml^{-1}) or 1 x 10^8 cfu.ml^{-1} was used for *Escherichia coli*. The *Lactococcus lactis* strain was treated at an initial concentration of either 1 x 10^5 cfu.ml^{-1} or 1 x 10^7 cfu.ml^{-1}.

For the TEM and SEM studies, the samples were prepared as described for protein and DNA determinations, except the final pellets were resuspended in 150 ml SSS.

Ultrasonication

Forty ml of the sample was placed in a jacketed glass sample holder that were connected to an ice-water bath (4° - 6°C) to maintain the sample temperature at 24° - 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 and a replaceable titanium tip, was used for ultrasonication. Samples were treated at 100% wave amplitude for different times (0 - 10 min). All ultrasonic treatments were done in duplicate as part of three sets.

Determination of released microbial protein and DNA

Four ml of each treated sample, as well as an untreated sample (control), was centrifuged for 10 min at 5 000 x *g* (Force 7 Microcentrifuge, Denver Instrument Company, USA). Increases in released microbial protein and DNA were measured as possible indicators of cell damage.

To determine the DNA concentration of the treated samples, 1.5 ml of the supernatant was pipetted into matched quartz cuvettes and the absorbance read at 260 nm (Beckman Coulter DU 530 Life Sciences UV/Vis Spectrophotometer, Beckman Instruments Inc., USA). The value obtained was divided by 20 to convert the concentration from molarity to mg.ml^{-1} [Concentration (mg.ml^{-1}) = A_{260}/20] (Johnson, 1994).

The Bio-Rad protein assay (Bio-Rad Protein Assay manual, 2005) was used to quantify the concentration of released microbial protein in the supernatant. This protein assay is a dye-binding assay based on the colour change of the Coomassie Brilliant

Blue dye in response to the concentration of protein (Bradford, 1976). The absorbance of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when it is bound to protein (Sedmak & Grossberg, 1977). A standard curve, using bovine serum albumin, was prepared for each protein assay (Fig. 1). A total of 12 standard curves were prepared during this study.

Scanning and transmission electron microscopy

Scanning electron microscopy

Four ml of the ultrasonicated sample was centrifuged (5 000 x *g*; 10 min) (Force 7 Microcentrifuge, Denver Instrument Company, USA). Cells were fixed by suspending the pellet in 10% glutaraldehyde (BDH) (in 0.1 M K_2HPO_4, KH_2PO_4 buffer; pH 7.0) (BDH) and this was stored at 4°C. Cells that received no ultrasonication served as controls. After fixation, cells were collected on 12 mm diameter Nucleopore membranes (pore diameter 0.2 µm). The cells on the membranes were dehydrated in a series of ethanol solutions for 10 min each: 35%, 50%, 70%, 80%, 90%, 95% and 100%. The samples were fixed onto aluminium stubs using carbon glue. Before drying, 3 x 100 µl hexamethyldisilazane (Sigma) were added to each membrane and left to dry in a fume hood. The samples were coated with a gold/palladium alloy (40:60) to a thickness of 10-20 nm using a Polarium sputter coater. Samples were viewed with a LEO 440 Fully Analytical scanning electron microscope using the secondary detector at 10.00 kV with a working distance of 15 mm. This was done at the Electron Microscope Unit at the University of Cape Town (UCT) (M. Waldron, Electron Microscope Unit, University of Cape Town, Cape Town, South Africa, personal communication, 2006).

Transmission electron microscopy

Eight ml of each of the ultrasonicated samples were centrifuged (5 000 x *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 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

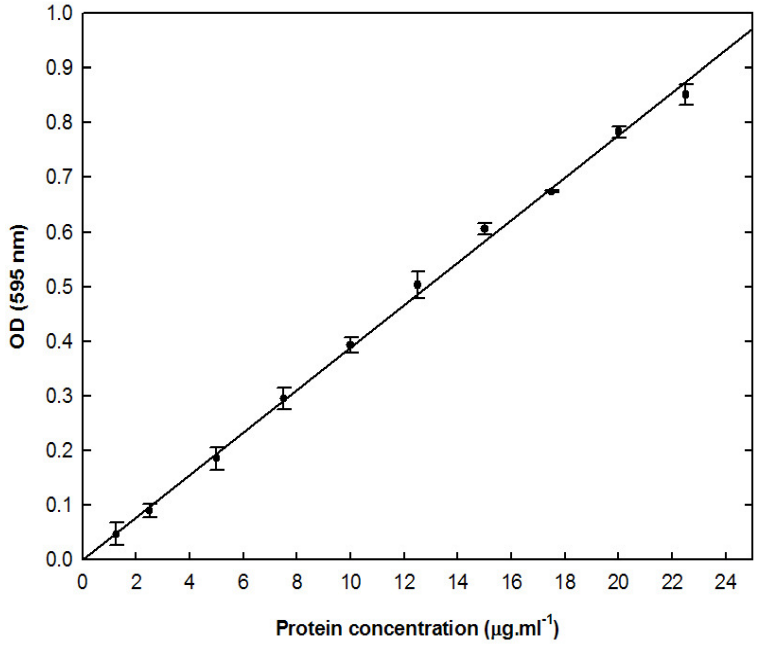

Figure 1 Standard protein concentration curve for bovine serum albumin using the Bio-Rad microassay (Each data point represents twelve values. The standard deviation was used as the error-bar).

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 (Spurr, 1969). 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 (Reynolds, 1963), and viewed with a Leo 912 transmission electron microscope operating at 120 kV. This was done at the Electron Microscope Unit at UCT under the supervision of M. Jaffer (M. Jaffer, Electron Microscope Unit, University of Cape Town, Cape Town, South Africa, personal communication, 2006).

Enumeration of bacteria

To determine the number of viable cells present before and after each ultrasonic treatment, a sample was serially diluted. The pour-plate technique was used, and the plates incubated according to each microbe's requirements as specified in Table 1.

Results and discussion

The four "test" microbes used in this study were chosen for different reasons. *Escherichia coli* was included in the protein and DNA, SEM and TEM studies as it is considered to be an indicator of faecal contamination by the dairy industry. Furthermore, this small Gram-negative rod grows rapidly and well on simple, non-specific growth media, is easy to detect and was found, in Chapter 3, to be sensitive to ultrasonication. *Lactococcus lactis* was included in the protein and DNA study because it is a Gram-positive coccus and was found to be more resistant to the effect of ultrasound than *E. coli* (Chapter 4). *Lactobacillus acidophilus* was included in the TEM study as it is a Gram-positive rod and was found to be resistant to ultrasonication as described in Chapter 4. This microbe produces lactic acid, and may therefore, be considered a potentially spoilage microbe of fresh milk. In Chapter 4, *Saccharomyces cerevisiae* was found to be sensitive to the effect of ultrasonication. This, coupled with the considerable size of this eukaryote led to its inclusion in the TEM study.

Increase of released microbial protein and DNA

When cells in a liquid are physically damaged, the cell contents leak out and into the suspension medium. This "disintegration" of cells can be observed by measuring an increase in intracellular components released into the supernatant. As the number of survivors decreases during a treatment such as ultrasonication at 20 kHz, it should be possible to measure an increase in both the protein and DNA concentrations of the supernatant. The data obtained for released microbial protein and DNA from *E. coli* and *Lc. lactis* cells resulting from the ultrasonic treatment is shown in Fig. 2.

One technical problem encountered during this study was that it was "impossible" to obtain the same initial cell concentration for the three sets of data used for each culture and as a result, the differences in the starting inoculum led to unacceptable variations. Therefore, for obvious reasons, the triplicate data sets were not shown in the figures.

In the first study using *E. coli*, an initial inoculum of about 1.5 x 10^6 cfu.ml^{-1} was used but it was found (as shown in Fig. 2A) that when a sample with a low *E. coli* inoculum was ultrasonicated, no changes in the protein concentrations could be detected in the supernatant. It could be argued that either the method was not sensitive, or that the amount of protein released was too low to be quantified by the protein assay (protein detection range of 1 - 25 µg.ml^{-1}) (Bio-Rad Protein Assay manual, 2005) used in this study. Similarly, spectrophotometric results also showed that no increase in released microbial DNA could be detected. It was thus decided to repeat the ultrasonically released microbial protein and DNA determinations using a sample with a higher initial inoculum of 1 x 10^8 cfu.ml^{-1}.

Ultrasonication of the higher initial *E. coli* inoculum (1 x 10^8 cfu.ml^{-1}) resulted in a measurable increase in both the protein and DNA concentrations of the supernatant. This was accompanied by a concurrent decrease in viable cell numbers (Fig. 2A). The protein concentration was found to increase from 0.2 µg.ml^{-1} before ultrasonication to 17.5 µg.ml^{-1} after 4 min of ultrasonication. The results showed that after 4 min of ultrasonication, the protein concentration stabilised and only small variations in the protein concentration were observed during the remainder of the treatment.

Similarly, the DNA concentration in the supernatant was found to increase from 1.85 µg.ml^{-1} at 0 min to 11.85 µg.ml^{-1} after 4 min of ultrasonication. As was found with the protein concentration, the DNA concentration varied only slightly after the ultrasonic treatment from 5 to 10 min (Fig. 2A).

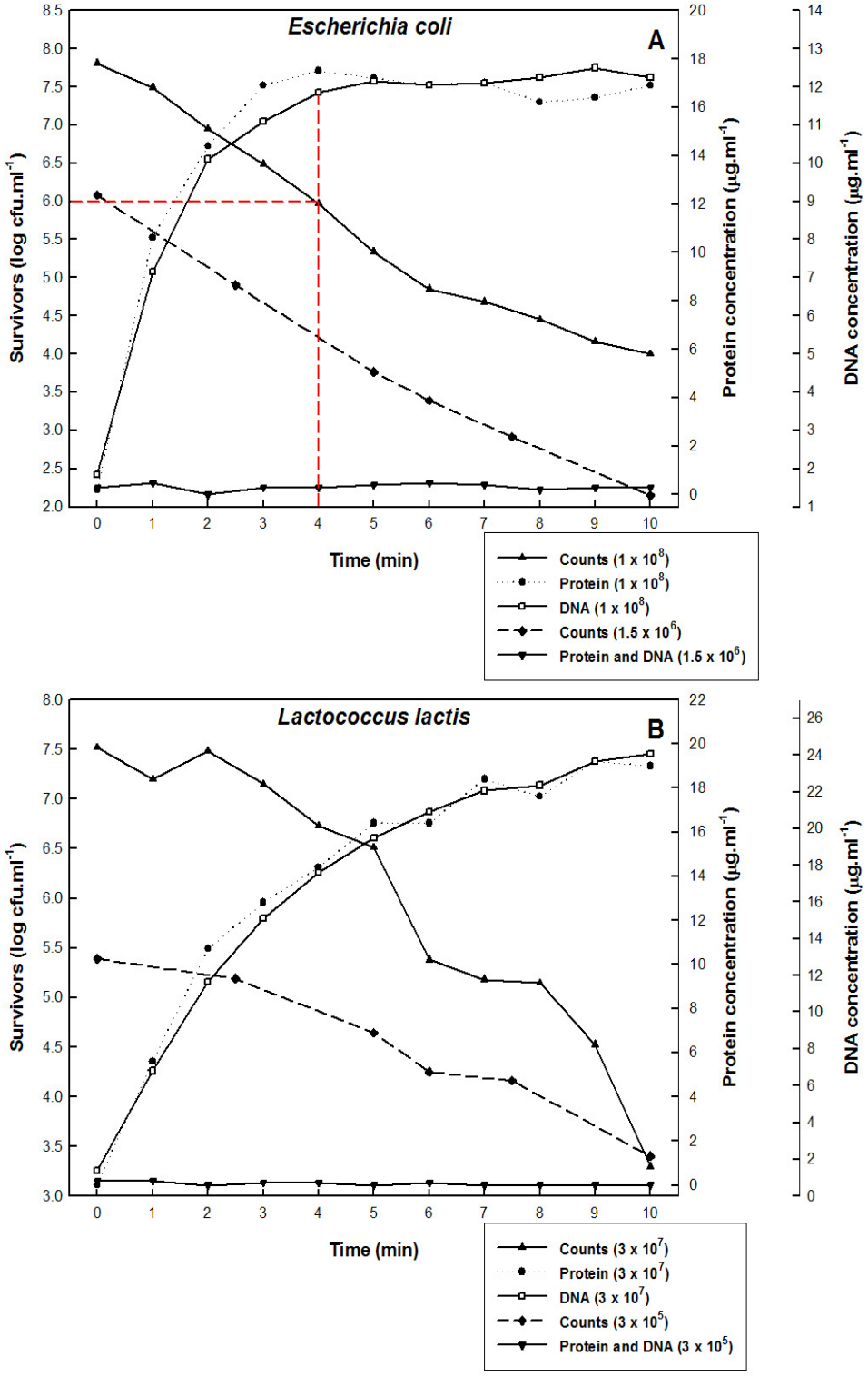

Figure 2 Impact of ultrasound (20 kHz) on the concentrations of protein and DNA released from *E. coli* (A) and *Lc. lactis* (B) cells (red dash lines were used to indicate the protein and DNA limitations at the lower initial cell concentration).

After 4 min of ultrasonication the *E. coli* protein and DNA concentrations stabilised with only slight variation for the remainder of the ultrasonic treatment. It is clear from the data in Fig. 2A that the number of viable cells had decreased to less than 1 x 10^6 cfu.ml^{-1} when the protein concentration stabilised after 4 min of ultrasonication. In the first trial, when a low inoculum (1 x 10^6 cfu.ml^{-1}) was used, no released protein or DNA could be detected, and it is therefore concluded that the amount of protein released when there were less than 1 x 10^6 viable cells (after 4 min) was too low to be quantified with the methods described in this study.

In the first trial with *Lc. lactis*, a sample with an initial inoculum of 3 x 10^5 cfu.ml^{-1} was ultrasonicated. Similar to *E. coli*, no increase in either the protein or DNA concentrations of the supernatant could be observed (Fig. 2B). It was concluded that the amount of protein and DNA released from damaged *Lc. lactis* cells when using a low inoculum (1 x 10^5 cfu.ml^{-1}) was insufficient and could not be quantified by the methods used in this study. When a higher *Lc. lactis* inoculum (3 x 10^7 cfu.ml^{-1}) was used, increases in both protein and DNA concentrations were observed (Fig. 2B). The data showed that the increase in both the protein (0 µg.ml^{-1} - 16.4 µg.ml^{-1}) and DNA (1.4 µg.ml^{-1} - 19.5 µg.ml^{-1}) concentration during the first 5 min of ultrasonication was higher than the increase observed from 5 to 10 min of ultrasonication (16.4 µg.ml^{-1} - 19.0 µg.ml^{-1} for protein and 20.9 µg.ml^{-1} - 24.05 µg.ml^{-1} for DNA). This reduced rate of microbial protein and DNA release after ultrasonication was attributed to a continued lowering in the number of viable cells as the treatment progressed. A problem experienced with *Lc. lactis* was that the logarithmic death curves were "wobbly" (unpredictable). After numerous repetitions, the logarithmic death curves remained non-linear. As was discussed and shown photographically in Chapter 4, the increase in viable cells from 1 min to 2 min of treatment was ascribed to dispersion of the chains of *Lc. lactis* as a result of ultrasonication (Fig. 3). Furthermore, in Chapter 4, 2.5 min ultrasonication intervals were used, while in this study 1 min intervals were used, and this lead to a more prominent "wobbly" effect that was noticeable in the logarithmic death curves.

It was hypothesised that by measuring an increase in microbial protein and DNA released after ultrasonication, it would give an indication as to the occurrence of cell damage. This was shown in Fig. 2, and it is therefore evident that ultrasonication physically "breaks" the microbial cells, resulting in the proteins and DNA to leak out of the cells, and ultimately cell death.

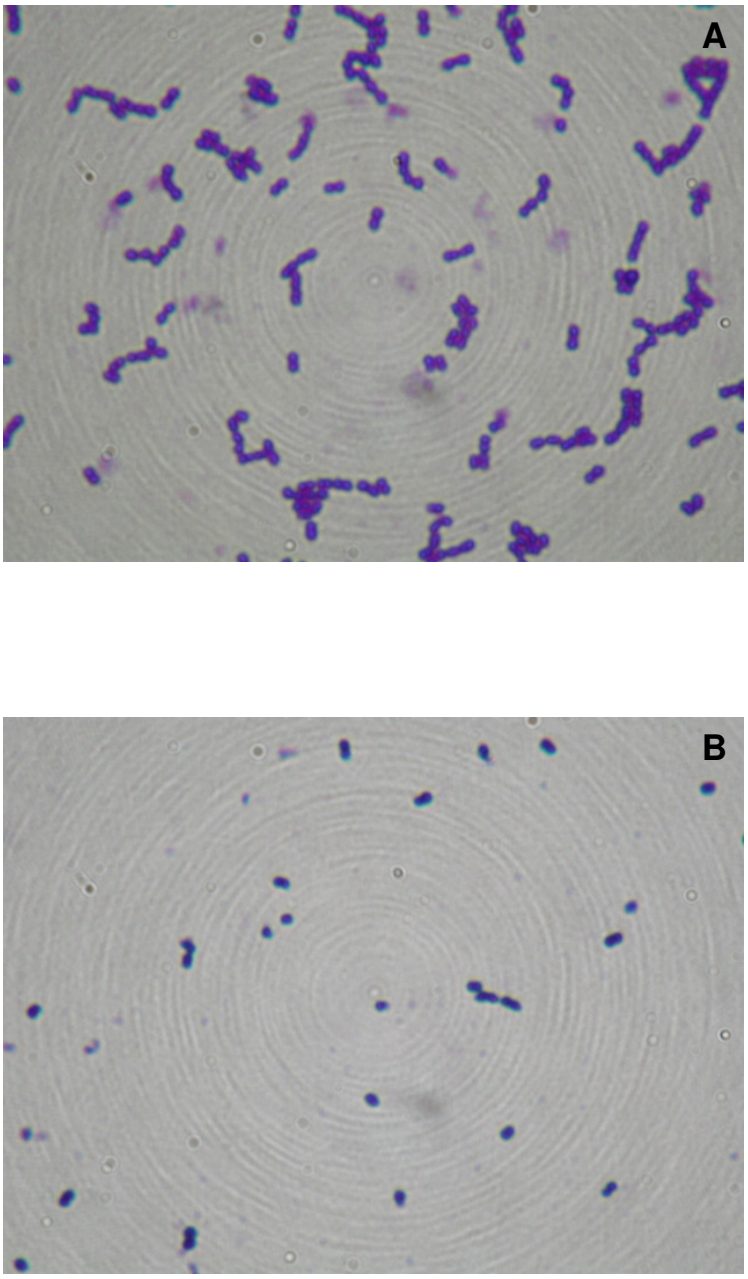

Figure 3 Dispersion of *Lactococcus lactis* chains after ultrasonication (x1000 enlargement) (A = no ultrasonication showing numerous chains; B = 1 min ultrasonication with few chains remaining).

Scanning and transmission electron microscopy

Scanning electron microscopy

Scanning electron microscopy is used as a valuable tool to observe the presence of superficial damage of microbial cells. In this study the SEM was used to show the external cell damage inflicted on *E. coli* cells during the ultrasonic treatment (Fig. 4). In the micrographs, *E. coli* cells which had received no ultrasonic treatment are shown (Figs. 4A and B), and it was clear that there was no visual damage to the external surfaces of the cells.

Escherichia coli cells that had received a 2 min ultrasonic treatment are shown in Fig. 4 (A-F). In Fig. 4C specifically, the whole cell had a "wrinkled" and "fuzzy" appearance. Figure 4D shows a cell with an indented cell wall. The cell in Fig. 4E appears badly damaged and the cell content has probably leaked out with just the cell wall remaining. In Fig. 4F it is clear that the cell wall ruptured during ultrasonication and that a fragment of the cell had probably been removed by cavitational forces.

Transmission electron microscopy

Transmission electron microscopy is generally used to investigate the internal structure of microbial cells. For this study, thin (<120 nm) sections were made from cells embedded in resin to observe any possible microstructural changes resulting from ultrasonication. Untreated (Figs. 5, 7 and 9) and treated (Figs. 6, 8 and 10) *E. coli*, *Lb. acidophilus* and *S. cerevisiae* cells were examined using TEM. Both external and internal cell damage caused by ultrasonication was visible in the micrographs.

Viable *E. coli* cells before ultrasonication, with both the cell wall and cell membrane intact are shown in Fig. 5 (A-F). An array of badly damaged cells after a 2 min ultrasonic treatment is shown in Fig. 6 (A-H). The cell wall of one of the cells in Fig. 6B has the same "wrinkled" and "fuzzy" appearance as was observed with the SEM (Fig. 4C). Some of the cells appear to be empty with only the cell wall remaining (Figs. 6C, E and F). It is clear that ultrasonication is an effective method for eliminating viable *E. coli* cells.

Viable untreated *Lb. acidophilus* cells with the distinct Gram-positive cell wall (Kandler & Weiss, 1986) are shown in Fig. 7 (A-E), and ultrasonicated cell are shown in Fig. 8 (A-H). An ultrasonic treatment of 5 min proved to be damaging to viable *Lb. acidophilus* cells, although not all the cells were extensively damaged as can be seen in the micrographs in Figs. 7A, D, E, F, G, and H. In keeping with cfu counts, the

presence of cells appearing intact after a 5 min ultrasonic treatment indicated that a

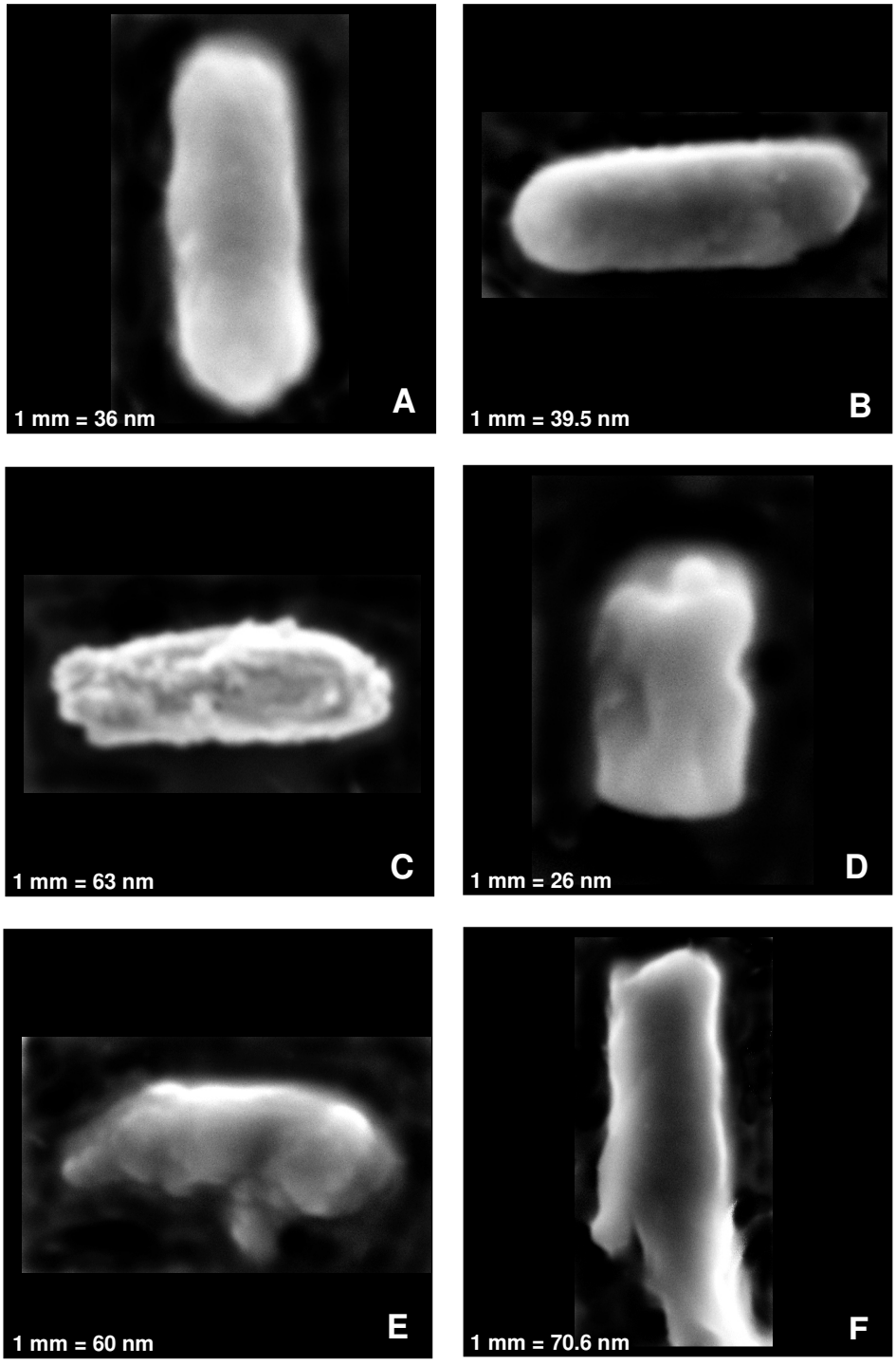

Figure 4 SEM micrographs of *E. coli* before and after ultrasonication for 2 min (A and B = normal cells no ultrasonication; C to F = external cell damage after 2 min of ultrasonication).

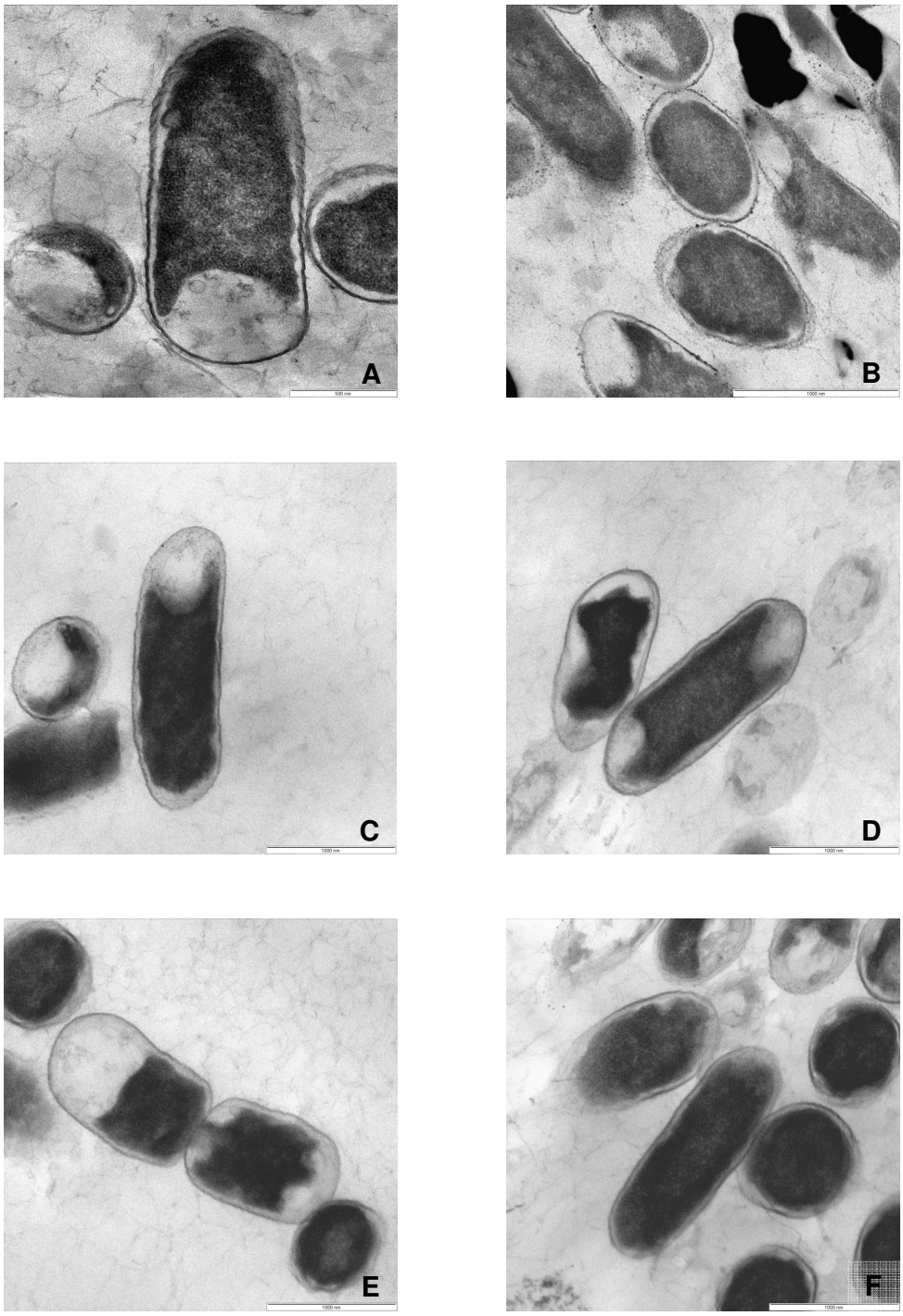

Figure 5 TEM micrographs of *E. coli* showing normal cells before ultrasonication (bar = 1 000 nm).

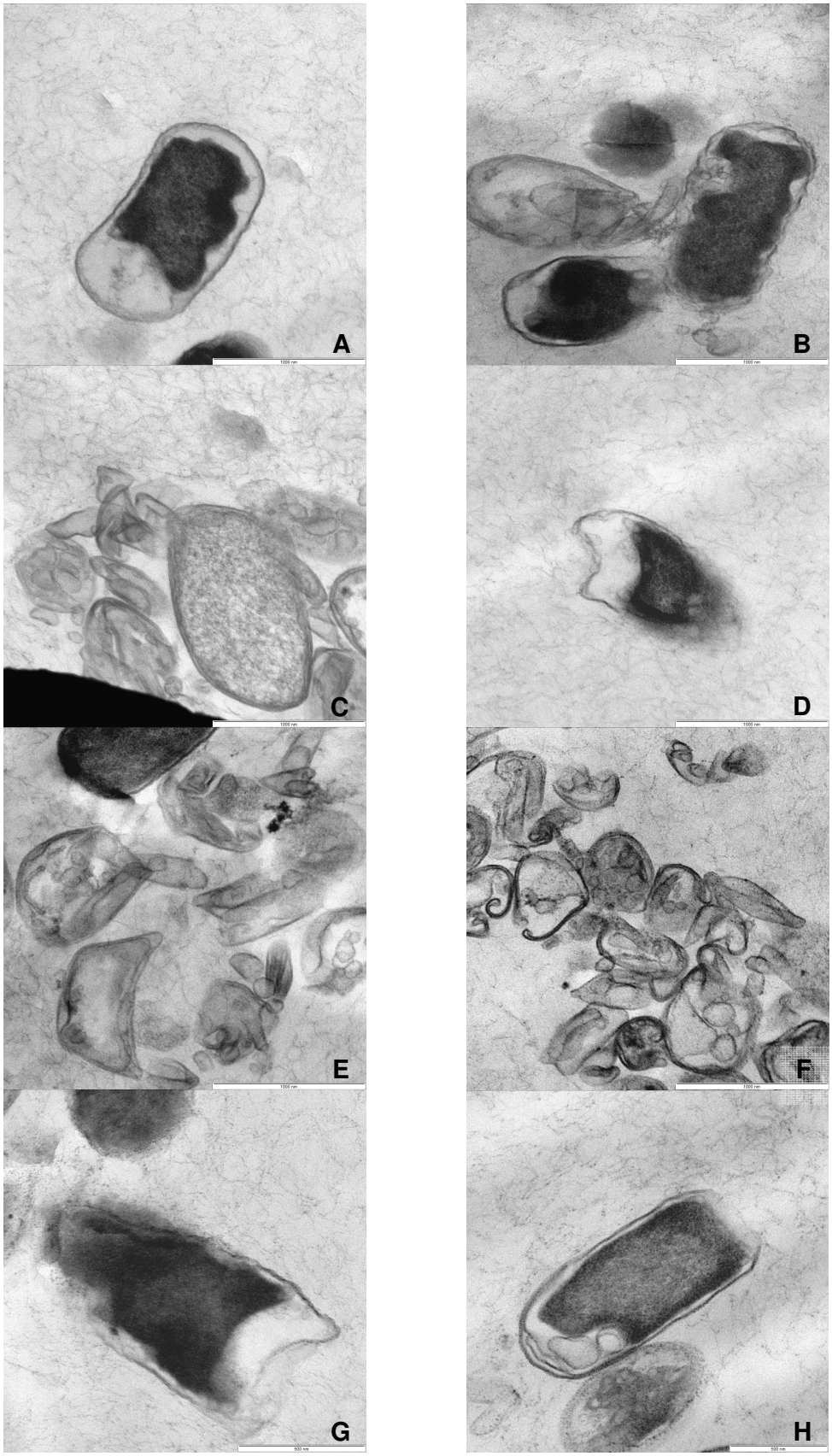

Figure 6 TEM micrographs of *E. coli* showing cell damage after 2 min of ultrasonication (bar = 1 000 nm).

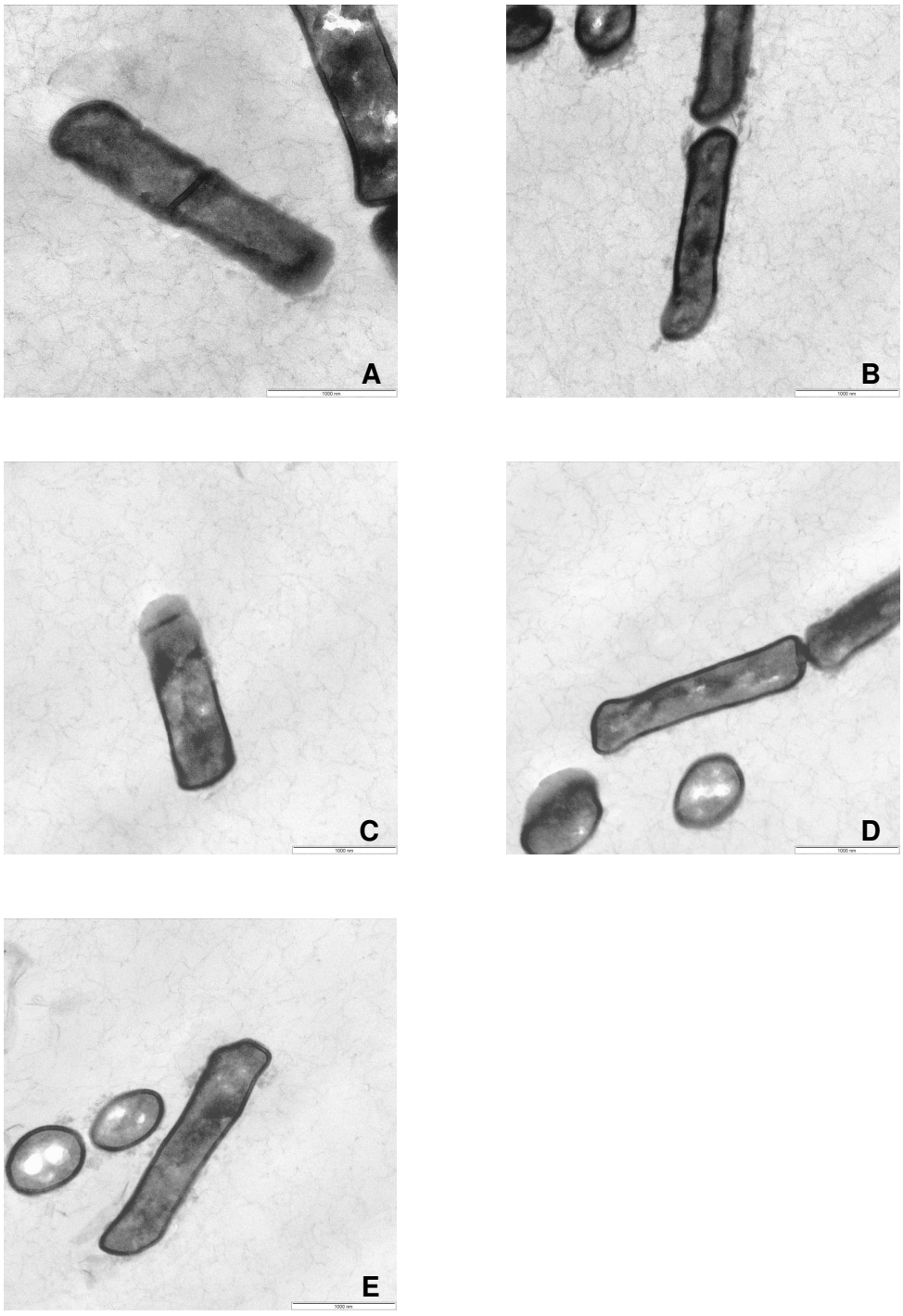

Figure 7 TEM micrographs of *Lb. acidophilus* showing normal cells with no ultrasonication (bar = 1 000 nm).

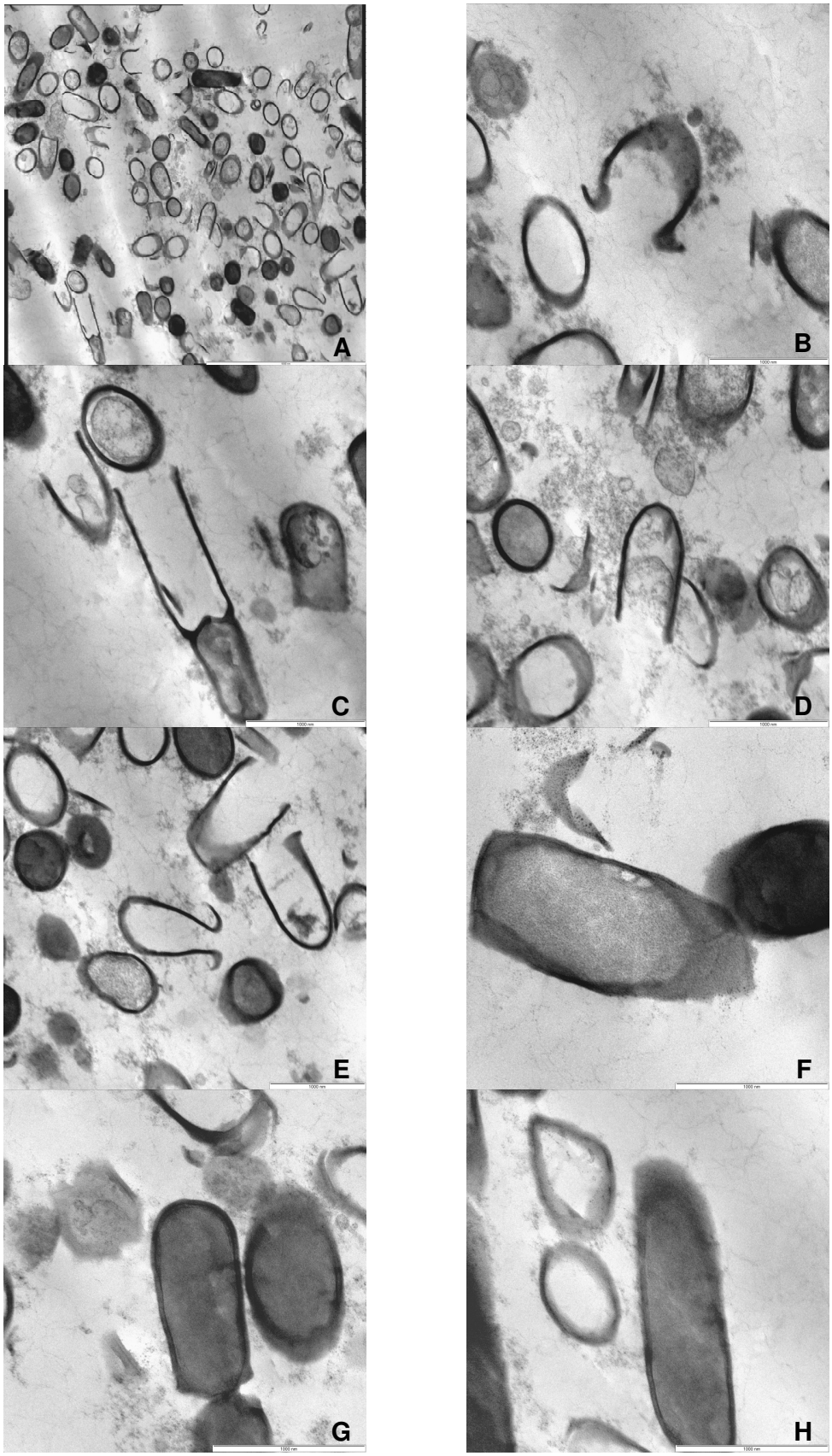

Figure 8 TEM micrograph of *Lb. acidophilus* showing cell damage after 5 min of ultrasonication (bar = 1 000 nm).

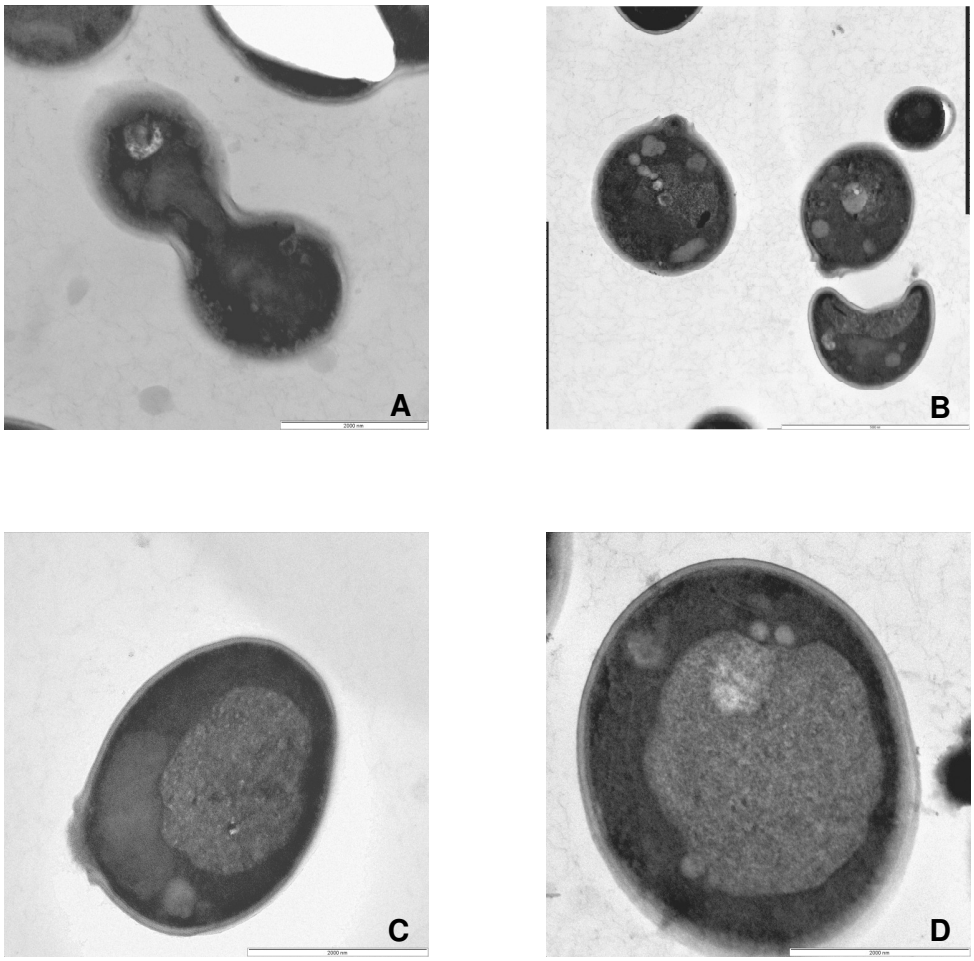

Figure 9 TEM micrographs of *S. cerevisiae* showing normal cells with no ultrasonication (bar = 2 000 nm).

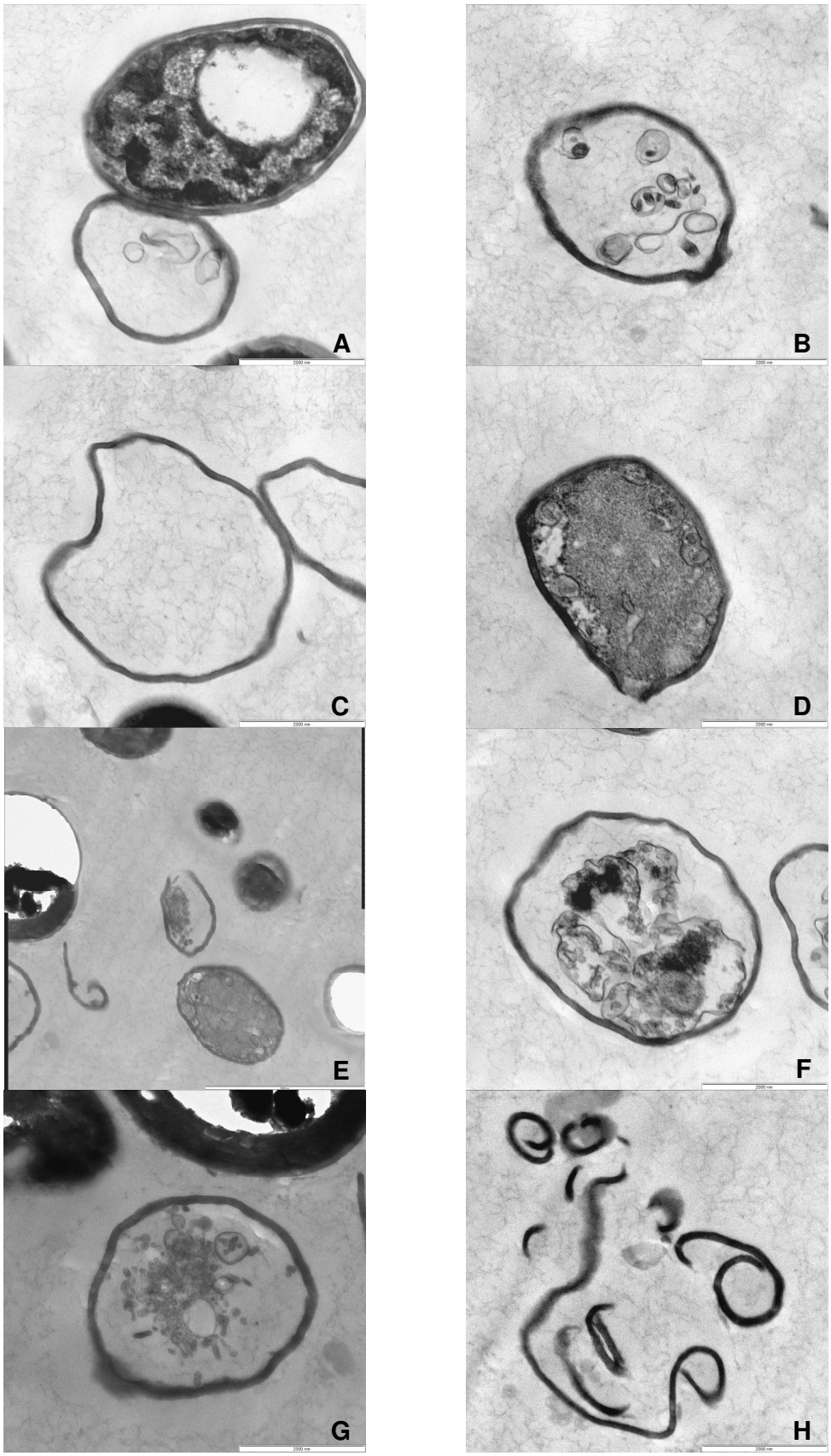

Figure 10 TEM micrographs of *S. cerevisiae* showing cell damage after 2 min of ultrasonication (bar = 2 000 nm).

5 min treatment is still insufficient to render the sample used in this study free from any viable cells. In Fig. 8C it can be seen that the end of one of the rod-shaped cells was "sheared off" during ultrasonication. The entire cell content had leaked out, leaving just the cell wall. Similar cell damage is shown in Figs. 8D and E.

Yeast cells that were subjected to ultrasonication provided visual results comparable to those obtained for *E. coli* and *Lb. acidophilus*. In Fig. 9 (A-D) viable untreated *S. cerevisiae* cells are shown, and cells that received an ultrasonic treatment of 2 min are shown in Fig. 10 (A-H). In Fig. 10A an enlarged vacuole, probably as a result of the ultrasonic treatment (2 min), is visible. In this micrograph, the adjacent cell is devoid of content except for a few membrane fragments. In Figs. 10B, C, F and G the cells are badly damaged with the cell walls having a "wrinkled" appearance, and the cell organelles damaged or completely missing. In Figs. 10E and H only fragments of the cell wall are visible.

The TEM studies indicate that the cells have been severely damaged both internally and externally. It is thus evident that ultrasonication kills microbial cells by damaging the cell wall and cell membrane and probably the microstructures of the cells.

Conclusions

The visual information (by means of SEM and TEM) obtained in this study showed that ultrasound structurally damages microbial cells. SEM is a relatively easy technique to show surface damage as little sample preparation is needed. However, no internal damage can be observed. With TEM the destructive effect of ultrasound on both the cell wall, as well as the different cell organelles is visible, thus providing insight on the type and magnitude of damage due to the treatment. It can be concluded, based on the visual information, that due to the destructive power of ultrasound, this technique could be employed as a successful pasteurisation/sterilisation method.

During this study it was found that the amount of microbial protein and DNA released during ultrasonication does give an indication as to the occurrence of cell damage. The aim of this study, however, was to try and accurately measure the release of microbial protein and DNA and thereby quantify the exact "degree" of cell damage. This study showed that it is only possible to observe, but not quantify, the cell damage. One limitation that was evident from this study was that it must be ensured that the inoculum is large enough. When the cell inoculum was too low ($<1 \times 10^6$ cfu.ml^{-1}) it became more difficult and even impossible to observe cell damage

as the protein assay used in this study was only sensitive enough to detect protein concentrations between 1 and 25 $\mu g.ml^{-1}$. It was also found to be impossible to detect any variations in the DNA concentration at such low inoculum levels.

Results from this study indicated that it was impossible to correlate the amount of released microbial protein and DNA with a specific number of inactivated cells. Because of the low levels of protein and DNA in microbial cells, it is therefore suggested that when this method is used as an indicator of cell damage, to always ensure that the inoculum is high enough to be able to detect changes in the concentration of released protein and DNA.

When applying ultrasound to milk it is possible that native milk proteins may interfere, rendering this method of measuring cell damage unsuitable. A question which does arise from this study is what the impact of ultrasonication would be on the much higher concentration of native milk proteins and other milk components. When milk is intended for further processing (cheese, yogurt, etc.), the effect of a given treatment on the milk components are important, as a deterioration in some components (especially proteins), may lead to yield losses.

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CHAPTER 6

IMPACT OF ULTRASOUND ON MILK COMPONENTS

Abstract

Ultrasonication offers the dairy industry a non-thermal alternative to pasteurisation. The effect of a "pasteurisation" method on the different milk components is important as it can impact the yield of processed milk products. The aim of this study was to determine if ultrasonication has any detrimental effect on the different milk components. An infra-red based apparatus was used to analyse raw and pasteurised milk after an ultrasonic treatment. Ultrasonication was found to have no impact on the protein or lactose content of both raw and pasteurised milk. This study indicated that ultrasonication has a homogenising effect on the fats of raw milk, but no effect on pasteurised/homogenised milk. Kjeldahl nitrogen determinations confirmed that ultrasonication had no detrimental effect on the total protein or casein content of pasteurised milk. Alkaline phosphatase and lactoperoxidase activity was also investigated as potential indicators of an effective ultrasonic treatment. Ultrasonication was, however, found to be ineffective in deactivating both enzymes used regularly by the dairy industry as indicators of effective thermal processes.

Introduction

Milk, being such a rich source of nutrients, has been an important part of the human diet for thousands of years (Frõlich, 2002). Protein is probably the most valuable constituent of milk, due to its high nutritional quality and unique physico-chemical and functional properties. These properties are fundamental to the production and characteristics of many dairy products, such as cheese or yogurt (Rattray & Jelen, 1996; Huppertz *et al*., 2006).

Enzymes are another important component of milk, although not from a nutritional point of view. The two enzymes that are regularly utilised from a practical point of view by the dairy industry, are alkaline phosphatase and lactoperoxidase. Alkaline phosphatase (ALP) has a thermal resistance greater than that of most non-endospore-forming microbes commonly found in milk. This enzyme is deactivated

when heated to 71.6°C for 15 s. Therefore, ALP is used universally as an indicator of successful implementation of high temperature short time (HTST) pasteurisation (McKellar *et al*., 1994; Lombardi *et al*., 2000).

Lactoperoxidase in contrast, is used for assessing the effectiveness of an ultra high temperature (UHT) treatment of milk as this enzyme is inactivated by temperatures higher than 80°C (Anon., 2003). Thus, UHT milk after an effective heat treatment would test negatively for lactoperoxidase activity, whilst HTST pasteurised milk remains lactoperoxidase positive (Villamiel *et al*., 1999).

Ultrasonication is one of the non-thermal alternatives to pasteurisation. The formation of cavitating bubbles during ultrasonication is thought to inactivate microbes (Morton *et al*., 1982). An ultrasonic treatment offers several additional benefits, among them, low energy utilisation (Neppiras, 1984; Ciccolini *et al*., 1997), and no sensory losses that have been ascribed to thermal processes. The importance of different milk components when processing milk to produce cheese, yogurt, etc. has lead to extensive studies on the effect of heat on the different milk components. Ultrasonication is a relatively new alternative to pasteurisation, and therefore, the need exists to further evaluate the impact of ultrasonication on the different milk components. The aim of this study was to determine if ultrasound has any detrimental impact on native milk proteins, fats and lactose, and whether ultrasound inactivates the alkaline phosphatase and lactoperoxidase enzymes.

Materials and methods

Milk sources

Commercially pasteurised full cream milk, obtained from a local supermarket, and raw milk collected from the Welgevallen Experimental Farm of the University of Stellenbosch were used during this study.

Ultrasonication

A 20 kHz, 750 W Vibra-Cell High Intensity Ultrasonic Processor VCX 750 (Sonics & Materials, Inc., USA), fitted with an autoclavable 13 mm diameter probe, was used for ultrasonication. A 40 ml sample and a jacketed sample holder were used for all treatments. The sample holder was connected to an ice-waterbath (4° - 6°C) to keep

the sample temperature between 24° and 26°C. Samples were treated at 100% wave amplitude for different times (0 to 10 min).

MilkoScan determinations

All milk samples were preserved with Bronopol Mircotabs (D & F Control Systems, Inc.) and analysed for protein (%), fat (%), lactose (%), total solids (%) and somatic cell counts (SCC) (cells per ml) within 24 h of the applied ultrasonic treatment. Analyses were done at the Dairy Institute of the Agricultural Research Council (ARC) at Elsenburg using a MilkoScan FT 6000 (FOSS, Denmark) and a Fossomatic FC 6000 (FOSS, Denmark). Samples were subjected to ultrasonication for 0, 1, 5, 10 and 15 min and five samples were analysed for each treatment time.

Total protein

Total protein determinations were done using the IDF 20B (1993) standard method with a few modifications. One gram of commercially pasteurised full cream milk was weighed into a Kjeldahl flask, and to this 18 ml H_2SO_4 (98.08% m/v) (Saarchem) and 1 Kjeldahl tablet (Saarchem) were added. A 1 g water sample served as the control. Digestion was carried out for 1.5 h using a Büchi Digestion Unit K-424 (Büchi, Flawil, Switzerland). After digestion was completed, the samples were allowed to cool to room temperature and 45 ml distilled water was added to each flask. The flasks were connected to a Büchi Distillation Unit K-350 (Büchi, Flawil, Switzerland) and 85 ml NaOH (32% m/v) (Merck) were automatically added followed by a 4 min distillation. The distillate was collected in a 20 ml H_3BO_3 (4% m/v) (BDH) solution containing 100 µl indicator. The indicator was a mixture of 0.59 g methyl red (Merck) and 0.29 g methylene blue (Merck) in 500 ml 96% (v/v) ethanol (Merck). This was then titrated with 0.05 N H_2SO_4 to the first trace of pink. The burette reading was recorded and the nitrogen content was determined using the following formula (IDF 20B, 1993):

$$\text{Nitrogen} = \frac{1.4 \times N \times TV}{\text{sample weight (g)}}$$

$$= \text{g nitrogen. } 100\ g^{-1} \text{ milk}$$

where 1.4 = 1.4 mg nitrogen neutralised by 1 ml 0.1 N H_2SO_4

N = normality of H_2SO_4

TV = titration value

The crude protein content, expressed as a percentage by mass, was obtained by multiplying the nitrogen content by 6.38 which is the reciprocate of the % nitrogen in protein for dairy products (IDF 20B, 1993). Four samples were analysed for each treatment time (0, 1, 5, 10 and 15 min).

Casein

The casein fraction of the total protein content was obtained by determining the portion of non-casein nitrogen and subtracting this value from the total nitrogen (Robertson, 1999). For the non-casein nitrogen determination, the samples received a pre-treatment before Kjeldahl nitrogen determinations were done. A ten gram milk sample was weighed into a volumetric flask and 70 - 80 ml distilled water (40°C) and 1 ml of a 10% (v/v) acetic acid (Saarchem) solution added and mixed. After 10 min, 1 ml of a 1 N sodium acetate (Saarchem) solution was added. The sample was allowed to cool to room temperature before the volume was adjusted to 100 ml with distilled water. The mixture was filtered (Whatman no. 40) and 20 ml of the filtrate was poured into a Kjeldahl flask, and a nitrogen determination was done. A 20 ml water sample served as the control.

The non-casein nitrogen (NCN) was determined using the following formula (Robertson, 1999):

$$NCN = \frac{1.4 \times N \times TV}{^1/_5 \text{ of sample weighed (g milk)}}$$

$$= \text{g nitrogen.100 g}^{-1} \text{ milk}$$

where 1.4 = 1.4 mg nitrogen neutralised by 1 ml 0.1 N H_2SO_4

N = normality of H_2SO_4

TV = titration value

The crude protein content, expressed as a percentage by mass, was obtained by multiplying the nitrogen content by 6.38 which is the reciprocate of the % nitrogen in protein for dairy products (IDF 20B, 1993). Four samples were analysed for each treatment time (0, 1, 5, 10 and 15 min).

Alkaline phosphatase

Alkaline phosphatase activity was determined according to the standard method of the International Dairy Federation (IDF 82A, 1987). Five ml of a buffered 4-nitrophenyl disodium orthophosphate solution (BDH) was added to 1 ml milk, and incubated in a waterbath at 37°C for 2 h. After 2 h the samples were visually compared with the control. Commercially pasteurised milk was used as a negative control. All determinations were done in duplicate.

Lactoperoxidase

Lactoperoxidase activity was determined by adding 1 ml of a 0.5% (v/v) guaiacol solution (BDH) to 5 ml milk. One drop of hydrogen peroxide (ACE Chemicals) was added and the mixture left to stand at room temperature for 3 min, after which the samples were visually inspected for colour changes. UHT milk served as a negative control. Duplicate determinations were done for each sample.

Statistical analysis

Statistical analysis (using Statistica 7.1 software) was done on the data obtained from the MilkoScan for both the pasteurised and raw milk, as well as the data obtained from the Kjeldahl protein determinations. One-way ANOVA was used to determine if there were significant differences between average measurements for the different time treatments. The Bonferroni post-hoc test was used to compare pairwise treatments. In cases where violations from the ANOVA assumptions were suspect, non-parametric bootstrap was performed. In all cases however, the non-parametric results were the same as the ANOVA results, and therefore only the ANOVA results were reported. Every point on the graphs for the MilkoScan results indicates the average value calculated from 5 repetitions. For the Kjeldahl results, averages were calculated from 4 repetitions. The error-bars represent the 95% confidence interval.

Results and discussion

The dairy industry routinely uses an infra-red based apparatus (MilkoScan) to analyse and evaluate the quality of each supplier's milk. The MilkoScan was therefore used in this study to determine whether possible changes to the composition of both raw and pasteurised milk after ultrasonication could be detected.

Protein

Milk protein is an important milk component in the production of a variety of dairy products as it is linked to total yield of the final product (Soryal *et al*., 2004). An increase in the protein content of milk leads to a higher yield when, for instance, cheese is manufactured. The protein content of milk is dependant on the breed of cow, individual cows of the same breed, lactation stage as well as the season. The protein content of milk is known to vary between 2.9 - 5.0% (Anon., 2003).

The protein (%) data obtained from the MilkoScan for raw and pasteurised milk after ultrasonication were statistically analysed and are given in Fig. 1. Data obtained from the MilkoScan for raw milk after an ultrasonic treatment are summarised in Table 1, and the data from the MilkoScan for pasteurised milk that had been ultrasonicated are summarised in Table 2.

The data for raw milk showed a statistically significant increase ($p=<0.01$) in the protein content from 0 min (3.030%) to 1 min (3.218%) of the ultrasonic treatment, after which there were no further statistically significant changes noted for the protein content for the remainder of the ultrasonic treatment (Fig. 1A). The increase observed for the protein content of raw milk after ultrasonication is within the fluctuation range for protein (2.9 - 5.0% as given by Anon. (2003)). Therefore, the increase in the protein content as observed with the MilkoScan would be acceptable to the dairy industry (L. van der Westhuizen, Dairy Institute, ARC-Elsenburg, Stellenbosch, South Africa, personal communication, 2006). The slight (0.171%) increase in protein content observed for raw milk after ultrasonication would not have a negative impact on total cheese yield if the milk was intended for the manufacturing of cheese, as an increase in protein content is generally accepted to increase the cheese yield.

A statistically significant decrease ($p=0.01$) in the protein content of pasteurised milk after ultrasonication was observed from a 1 min (3.116%) to a 10 min (3.106%) treatment (Fig. 1B). Although a statistically significant decrease was found, the measurements still fall within the acceptable 0.05% fluctuation for replicates analysed with the MilkoScan (FOSS Integrator IMT software e-manual) (L. van der Westhuizen, Dairy Institute, ARC-Elsenburg, Stellenbosch, South Africa, personal communication, 2006). The protein component of milk is one of the main contributors to total cheese yield (Pulina *et al*., 2006), with an increase in protein content resulting in an increase in total cheese yield. The slight decrease (0.010%) in the protein content noted for pasteurised milk after ultrasonication would have no negative impact on cheese yield.

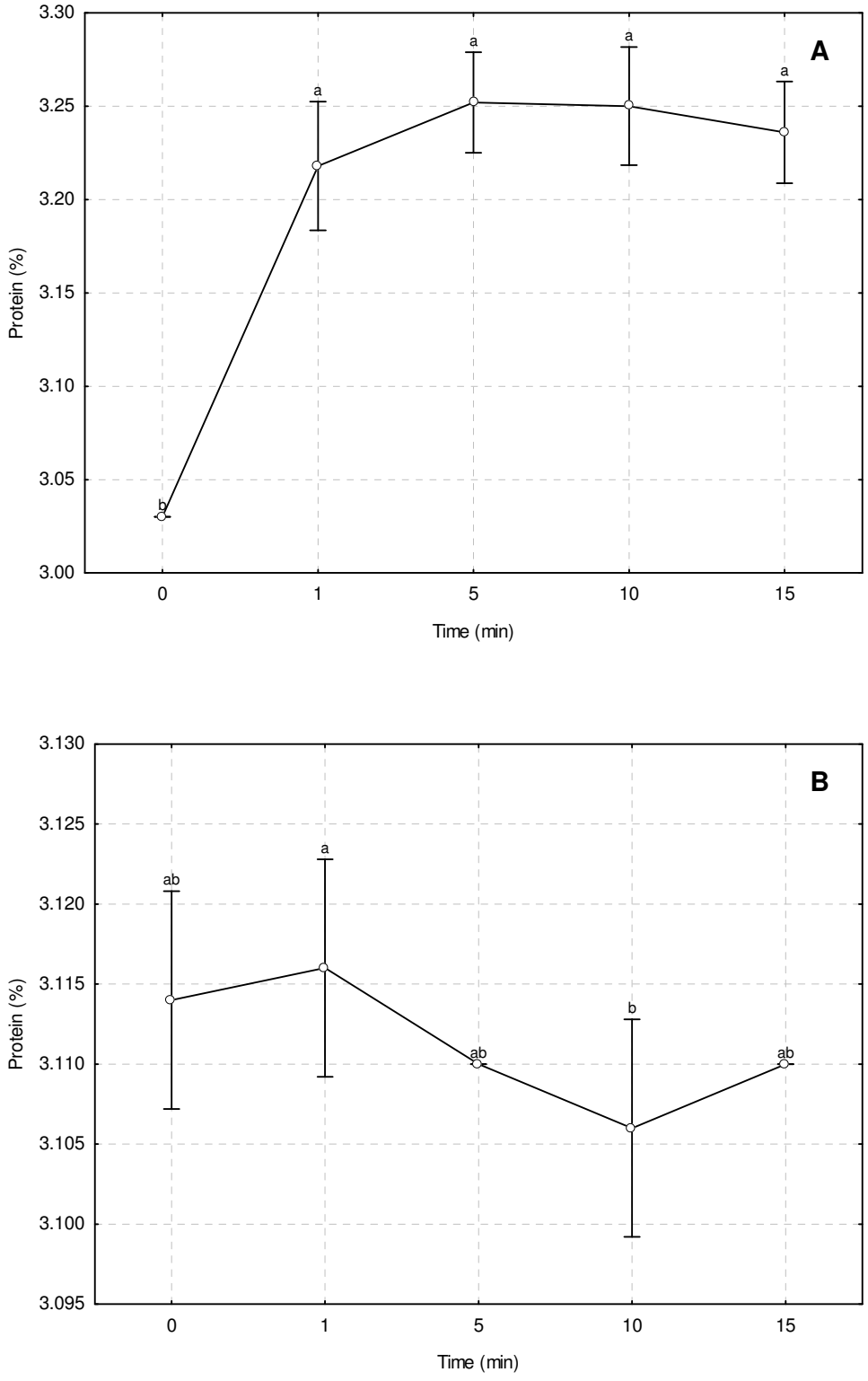

Figure 1 Statistical analysis of the data (MilkoScan) of the protein content (%) of raw (A) and pasteurised (B) milk after ultrasonication (Each data point represents five values. The standard deviation was used as the error-bar. Means with different letters are statistically significantly different, $p \leq 0.05$).

Table 1 MilkoScan results of the different milk components after an ultrasonication treatment of raw milk

Fraction	Treatment time				
	0 min	**1 min**	**5 min**	**10 min**	**15 min**
Protein (%)	3.030^{b} (3.03)	3.218^{a} (3.19-3.25)	3.252^{a} (3.22-3.28)	3.250^{a} (3.22-3.28)	3.236^{a} (3.20-3.25)
Fat (%)	2.544^{c} (2.53-2.56)	2.624^{a} (2.60-2.66)	2.668^{b} (2.66-2.68)	2.668^{b} (2.63-2.69)	2.656^{ab} (2.61-2.67)
Lactose (%)	4.798^{c} (4.79-4.80)	4.812^{a} (4.80-4.83)	4.824^{ab} (4.82-4.83)	4.828^{b} (4.82-4.83)	4.826^{b} (4.82-4.83)
Total solids (%)	11.092^{b} (11.07-11.11)	11.374^{a} (11.31-11.45)	11.404^{a} (11.17-11.50)	11.466^{a} (11.40-11.52)	11.438^{a} (11.36-11.47)
SCC (cells.ml^{-1})	229 400^{b} (216 000-240 000)	12 800^{a} (9 000-15 000)	7 000^{a} (3 000-11 000)	6 800^{a} (6 000-8 000)	8 000^{a} (4 000-15 000)

The values given are averages (n = 5); values in parentheses are the minimum and maximum values of five samples.
SCC = somatic cell count.

Table 2 MilkoScan results of the different milk components after an ultrasonication treatment of pasteurised milk

Fraction	Treatment time				
	0 min	**1 min**	**5 min**	**10 min**	**15 min**
Protein (%)	3.114^{ab} (3.11-3.12)	3.116^{a} (3.11-3.12)	3.110^{ab} (3.11)	3.106^{b} (3.10-3.11)	3.110^{b} (3.11)
Fat (%)	3.478^{a} (3.47-3.48)	3.478^{a} (3.47-3.48)	3.518^{b} (3.51-3.52)	3.518^{b} (3.51-3.52)	3.520^{b} (3.52)
Lactose (%)	4.800^{a} (4.79-4.81)	4.808^{a} (4.80-4.82)	4.818^{a} (4.80-4.83)	4.814^{a} (4.81-4.83)	4.810^{a} (4.80-4.82)
Total solids (%)	12.112^{a} (12.10-12.12)	12.122^{a} (12.10-12.14)	12.166^{b} (12.15-12.17)	12.158^{b} (12.14-12.18)	12.160^{b} (12.15-12.17)
SCC (cells.ml^{-1})	71 200^{b} (66 000-77 000)	27 000^{c} (24 000-32 000)	9 400^{a} (7 000-13 000)	5 800^{a} (3 000-7 000)	4 600^{a} (4 000-6 000)

The values given are averages (n = 5); values in parentheses are the minimum and maximum values of five samples.
SCC = somatic cell count.

Kjeldahl protein

Kjeldahl nitrogen analysis was used to determine the crude protein as well as the casein fraction of the total protein. Total milk protein contains about 80% casein (Anon., 2003), and the casein is the dominant factor affecting curd firmness, syneresis rate, moisture retention, and ultimately the cheese quality and yield (Guo *et al*., 2001; Zeng *et al*., 2006).

Data obtained for Kjeldahl protein determinations on pasteurised milk are given in Fig. 2 and the data are summarised in Table 3. The results indicated that there was no statistically significant changes in either the total protein content (p=0.93) (Fig. 2A) or the casein fraction of the total protein content (p=0.82) (Fig. 2B) after ultrasonication of pasteurised milk. The large error-bars depicted in Fig. 2 are a result of the small range of the values on the Y-axis. It was decided not to do Kjeldahl protein determinations on raw milk, due to possible interference by the large fat globules of the unhomogenised milk. The results obtained from this study indicate that, based on the fact that there was no decrease in the protein content, the use of ultrasonicated milk for the production of cheese would probably have no negative effect on cheese yield.

Fat

Milk fat is another component of milk that is positively correlated to cheese yield (Soryal *et al*., 2004). A higher milk fat content ultimately leads to a higher yield of the final product. The fat content of milk typically varies between 2.5% and 6.0% depending on the breed of cow, stage of lactation and season (Anon., 2003).

The data obtained from the MilkoScan for the fat content (%) of raw and pasteurised milk that had been ultrasonicated were statistically analysed and are given in Fig. 3, and the fat content data obtained for the raw and pasteurised milk that had been ultrasonicated, are summarised in Tables 1 and 2, respectively.

The fat content of raw milk showed a statistically significant increase (p=<0.01) from 0 min (2.544%) to a 1 min (2.624%) ultrasonic treatment and also from 1 min (2.624%) to 5 min (2.668%) of ultrasonication (Fig. 3A). The total increase in fat content from 0 min to 5 min of ultrasonication was 0.124%. After 5 min of ultrasonication, no further statistical changes in the fat content were observed for the remainder of the treatment time. The homogenisation of fats caused by ultrasonication (Villamiel & de Jong, 2000; Wu *et al*., 2001) leads to a decrease in the fat globule size, with a subsequent increase in the surface area of the milk fat globule membrane (MFGM) (Lopez, 2005). The MilkoScan uses an infra-red light-based method and the increase in

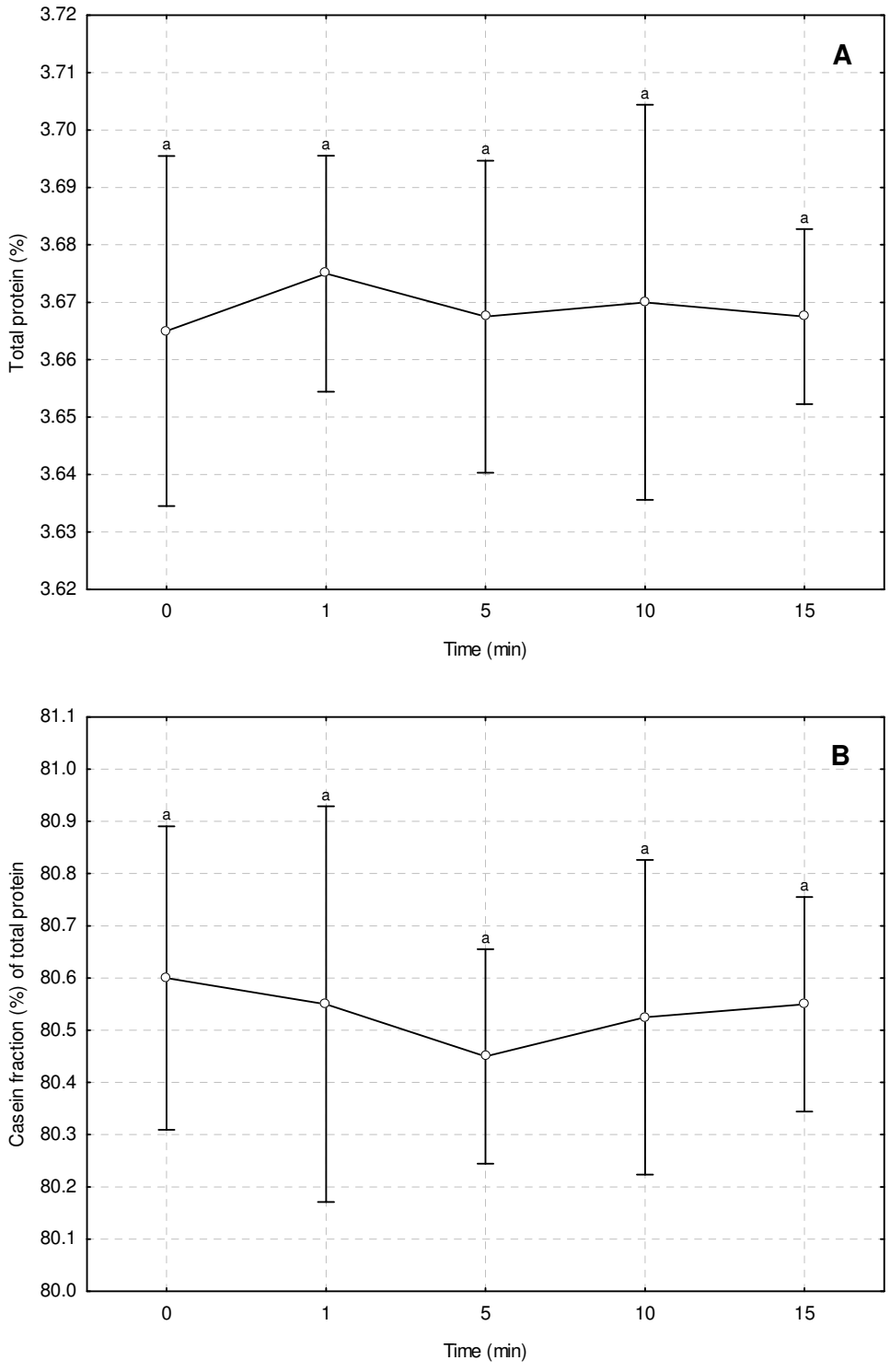

Figure 2 Statistical analysis of the data (Kjeldahl) of the total protein content (%) (A) and casein fraction (%) of total protein content (B) of pasteurised milk after ultrasonication (Each data point represents four values. The standard deviation was used as the error-bar. Means with different letters are statistically significantly different, $p \leq 0.05$).

Table 3 Kjeldahl protein results for pasteurised milk after an ultrasonication treatment

Fraction	Treatment time				
	0 min	**1 min**	**5 min**	**10 min**	**15 min**
Total protein (%)	3.6650 (3.65-3.69)	3.6750 (3.66-3.69)	3.6675 (3.65-3.69)	3.6700 (3.65-3.70)	3.6675 (3.66-3.68)
Casein (%) of total protein	80.600 (80.4-80.8)	80.550 (80.3-80.8)	80.450 (80.3-80.6)	80.525 (80.4-80.8)	80.550 (80.4-80.7)

The values given are averages (n = 4); values in parentheses are the minimum and maximum values of four samples.

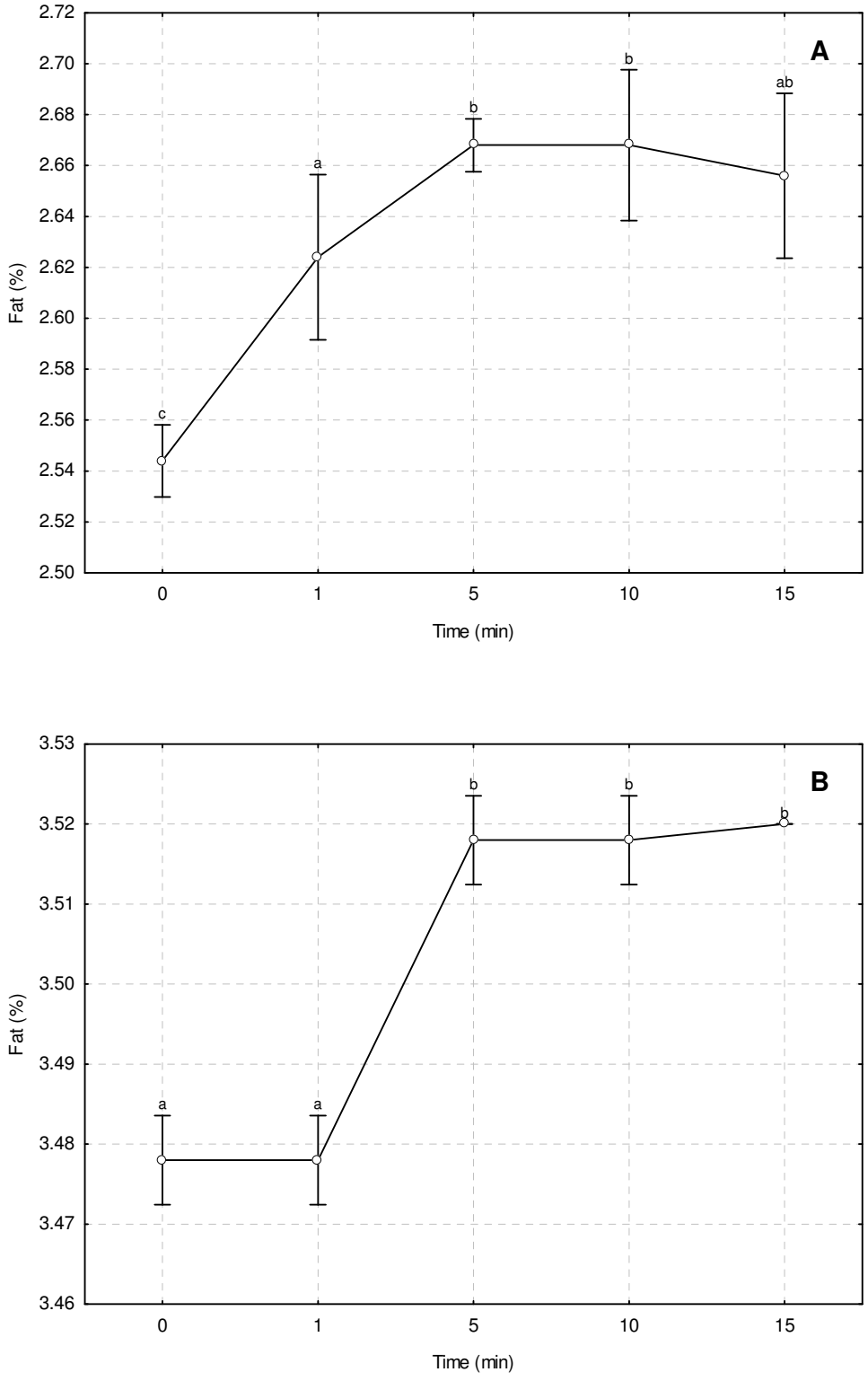

Figure 3 Statistical analysis of the data (MilkoScan) of the fat content (%) of raw (A) and pasteurised (B) milk after ultrasonication (Each data point represents five values. The standard deviation was used as the error-bar. Means with different letters are statistically significantly different, $p \leq 0.05$).

MFGM surface area leads to higher fat content readings. The same trend is observed when raw and pasteurised/homogenised milk is analysed by the MilkoScan (L. van der Westhuizen, Dairy Institute, ARC-Elsenburg, Stellenbosch, South Africa, personal communication, 2006). Homogenisation is employed by the dairy industry to reduce the size of the fat globules, thereby preventing creaming and coalescence during storage (Huppertz *et al*., 2003). The homogenisation effect of ultrasonication is therefore an added benefit, as it might be possible to eliminate the homogenisation step during fresh milk processing. Replacing both thermal pasteurisation and homogenisation with one process, i.e. ultrasonication could probably be cost effective in terms of initial equipment expenses as well as maintenance of the equipment.

The data obtained indicated a statistically significant increase ($p=<0.01$) in fat content for pasteurised milk from a 1 min (3.478%) to a 5 min (3.518%) ultrasonic treatment, after which no statistically significant changes were observed for the remainder of the ultrasonic treatment (Fig. 3B). Although a statistically significant increase was found, the measurements fall within the acceptable 0.05% fluctuation for replicates analysed with the MilkoScan (FOSS Integrator IMT software e-manual) (L. van der Westhuizen, Dairy Institute, ARC-Elsenburg, Stellenbosch, South Africa, personal communication, 2006). The slight increase in the fat content of pasteurised milk after ultrasonication would thus not negatively impact the yield of any processed milk product.

Lactose

Lactose is a carbohydrate found exclusively in milk and is utilised as a carbon source during fermentation processes for the production of yogurt, cheese, etc. (Williams *et al*., 2000; Chammas *et al*., 2006). The lactose content of milk varies between 3.6 and 5.5% (Anon., 2003).

The MilkoScan data obtained for the lactose content (%) of raw and pasteurised milk after an ultrasonic treatment were statistically analysed and are given in Fig. 4, and the data obtained for the raw and pasteurised that had been ultrasonicated are summarised in Tables 1 and 2, respectively.

The results obtained for the lactose content of raw milk (Fig. 4A) indicated a statistically significant increase ($p=<0.01$) in lactose from 0 min (4.798%) to 1 min (4.812%) of ultrasonication, and also from 1 min (4.812%) to 5 min (4.824%) of the ultrasonic treatment. Although a statistically significant increase was found, the measurements fall within the acceptable 0.05% fluctuation for replicates analysed with

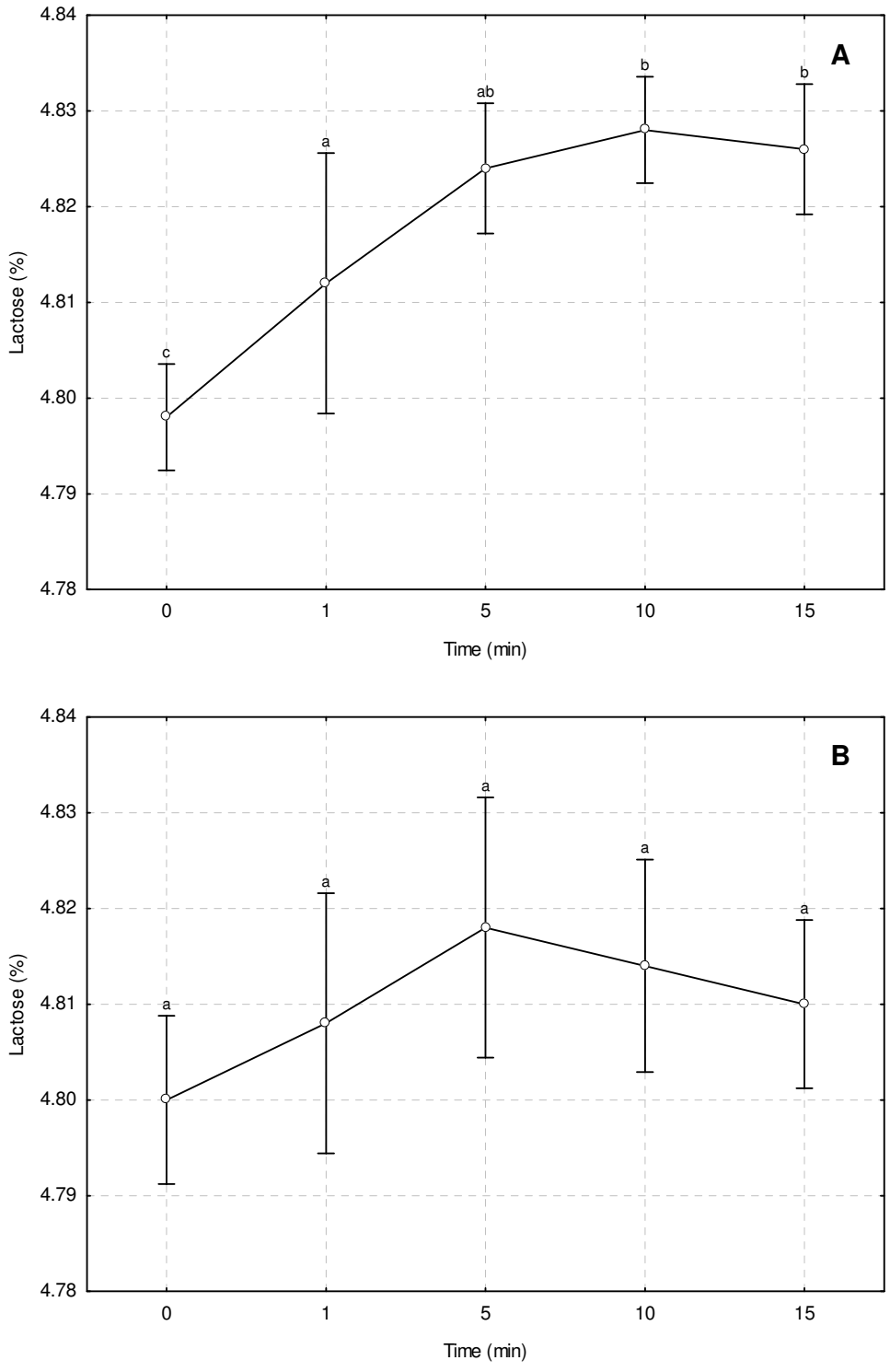

Figure 4 Statistical analysis of the data (MilkoScan) of the lactose content (%) of raw (A) and pasteurised (B) milk after ultrasonication (Each data point represents five values. The standard deviation was used as the error-bar. Means with different letters are statistically significantly different, $p \leq 0.05$).

the MilkoScan (FOSS Integrator IMT software e-manual) (L. van der Westhuizen, Dairy Institute, ARC-Elsenburg, Stellenbosch, South Africa, personal communication, 2006). No further statistically significant increase was observed after 5 min of ultrasonication of the raw milk.

The data obtained showed no statistically significant changes for the lactose content (p=0.06) (Fig. 4B) of pasteurised milk after the ultrasonic treatment. During yogurt processing, lactose is fermented by the lactic acid bacteria (LAB) (Chammas *et al*., 2006), to produce lactic acid, resulting in a lowering of the pH (Sánchez *et al*., 2005). As no statistically significant difference was observed for the lactose content of both pasteurised and raw milk after ultrasonication, it is suggested that it would be safe to use ultrasonicated milk for the manufacturing of yogurt. The availability of carbohydrates for fermentation by the LAB remains unchanged, therefore, the same tempo of lactic acid production during yogurt processing should be achieved.

Total solids

The term total solids (TS) of milk is a collective name given to the main components of milk excluding water. These include proteins, fat, lactose and minerals (Anon., 2003).

The TS (%) data obtained from the MilkoScan for raw and pasteurised milk that had been ultrasonicated were statistically analysed and are given in Fig. 5. Data obtained from the MilkoScan for raw milk after an ultrasonic treatment are summarised in Table 1, and the ultrasonication of pasteurised milk data from the MilkoScan are summarised in Table 2.

The TS content of raw milk showed a statistically significant increase (p=<0.01) from 0 min (11.092%) to a 1 min (11.374%) ultrasonic treatment, after which no further statistically significant changes were observed for the remainder of the ultrasonic treatment (Fig. 5A). The TS content of milk obtained with the MilkoScan is a combination of the data obtained for the protein, fat and lactose content. The increases previously observed for the protein (Fig. 1A) and fat content (Fig. 3A) of raw milk after ultrasonication explain the significant increase in the TS content observed for raw milk after it had been treated ultrasonically. Thus it was concluded that the increases noted for the TS are linked to the increases observed for the protein and fat content, which was ascribed to the homogenisation effect of ultrasonication. Milk with a high TS leads to a higher cheese yield (Soryal *et al*., 2004), therefore, it is suggested that the increase in TS would not negatively impact cheese yield, should ultrasonicated milk be used for cheese production.

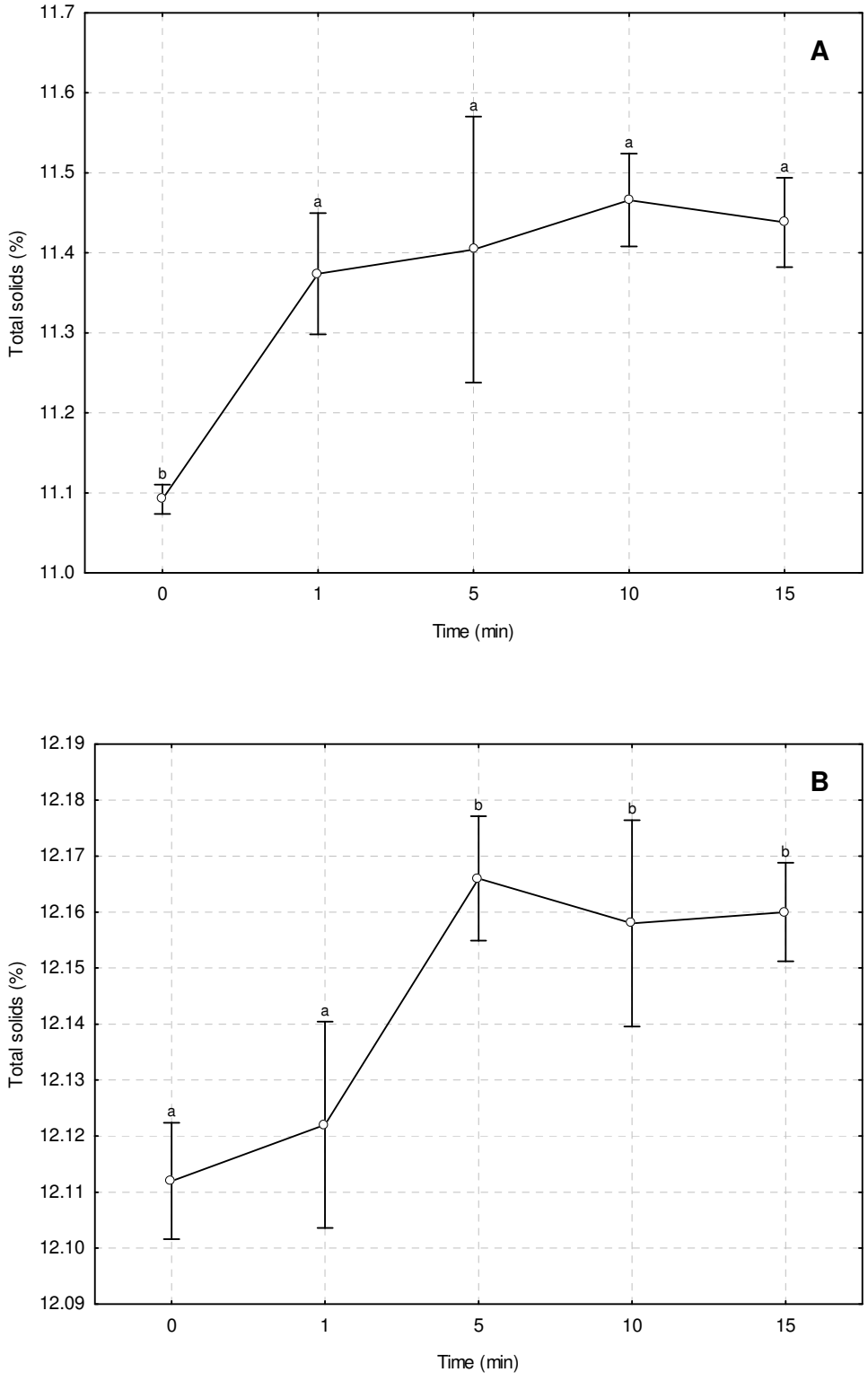

Figure 5 Statistical analysis of the data (MilkoScan) of the total solids (%) of raw (A) and pasteurised (B) milk before and after ultrasonication (Each data point represents five values. The standard deviation was used as the error-bar. Means with different letters are statistically significantly different, $p \leq 0.05$).

A statistically significant increase (p=<0.01) was observed for the TS content of pasteurised milk from a 1 min (12.122%) to a 5 min (12.166%) ultrasonic treatment, after which no statistically significant changes were observed for the remainder of the ultrasonic treatment (Fig. 5B). This increase in the TS content falls well within the 0.05% fluctuation for replicates (FOSS Integrator IMT software e-manual) (L. van der Westhuizen, Dairy Institute, ARC-Elsenburg, Stellenbosch, South Africa, personal communication, 2006). As stated above, the TS of milk, as measured by the MilkoScan is a combination of the proteins, fats and lactose, and therefore, the increase in TS from 1 min to 5 min of ultrasonication could again be linked to the increase in the fat and protein content of pasteurised milk observed from a 1 min to a 5 min ultrasonic treatment (Fig. 3B) (L. van der Westhuizen, Dairy Institute, ARC-Elsenburg, Stellenbosch, South Africa, personal communication, 2006). The slight (0.044%) increase in the TS content of pasteurised milk after an ultrasonic treatment would therefore not have any negative impact on the total yield of dairy products.

Somatic cell count

The somatic cell count (SCC) of milk is commonly used as an indicator of mastitis in dairy cows, and results in reduced milk quality and milk yield (Santos *et al*., 2003).

The data obtained from the MilkoScan for the somatic cell count (SCC) (cells.ml^{-1}) of raw and pasteurised milk after an ultrasonic treatment were statistically analysed and are given in Fig. 6. The data obtained for the raw milk that had been ultrasonicated as well as for pasteurised milk after an ultrasonic treatment are summarised in Tables 1 and 2, respectively.

A statistically significant decrease (p=<0.01) in SCC was also observed when raw milk was given an ultrasonic treatment. Cell counts decreased from 229 400 cells.ml^{-1} (0 min) to 12 800 cells.ml^{-1} after 1 min of ultrasonication (a 94.42% reduction), after which no further statistically significant decreases were observed. The SCC of the raw milk was found to be 8 000 cells.ml^{-1} after a 15 min ultrasonic treatment (Fig. 6A).
The data obtained for the SCC of pasteurised milk after the ultrasonic treatment showed a statistically significant decrease (p=<0.01) in SCC from 0 min (71 200 cells.ml^{-1}) to 1 min (27 000 cells.ml^{-1}) and also from 1 min (27 000 cells.ml^{-1}) to 5 min (9 400 cells.ml^{-1}) of treatment. The SCC of the pasteurised milk was 4 600 cells.ml^{-1} after 15 min of ultrasonication (Fig. 6B). The reduction in SCC after ultrasonication of pasteurised milk would, however, not improve the sensory quality or the shelf-life of the milk. It is well known that milk with a high SCC has a reduced sensory quality (Munro *et*

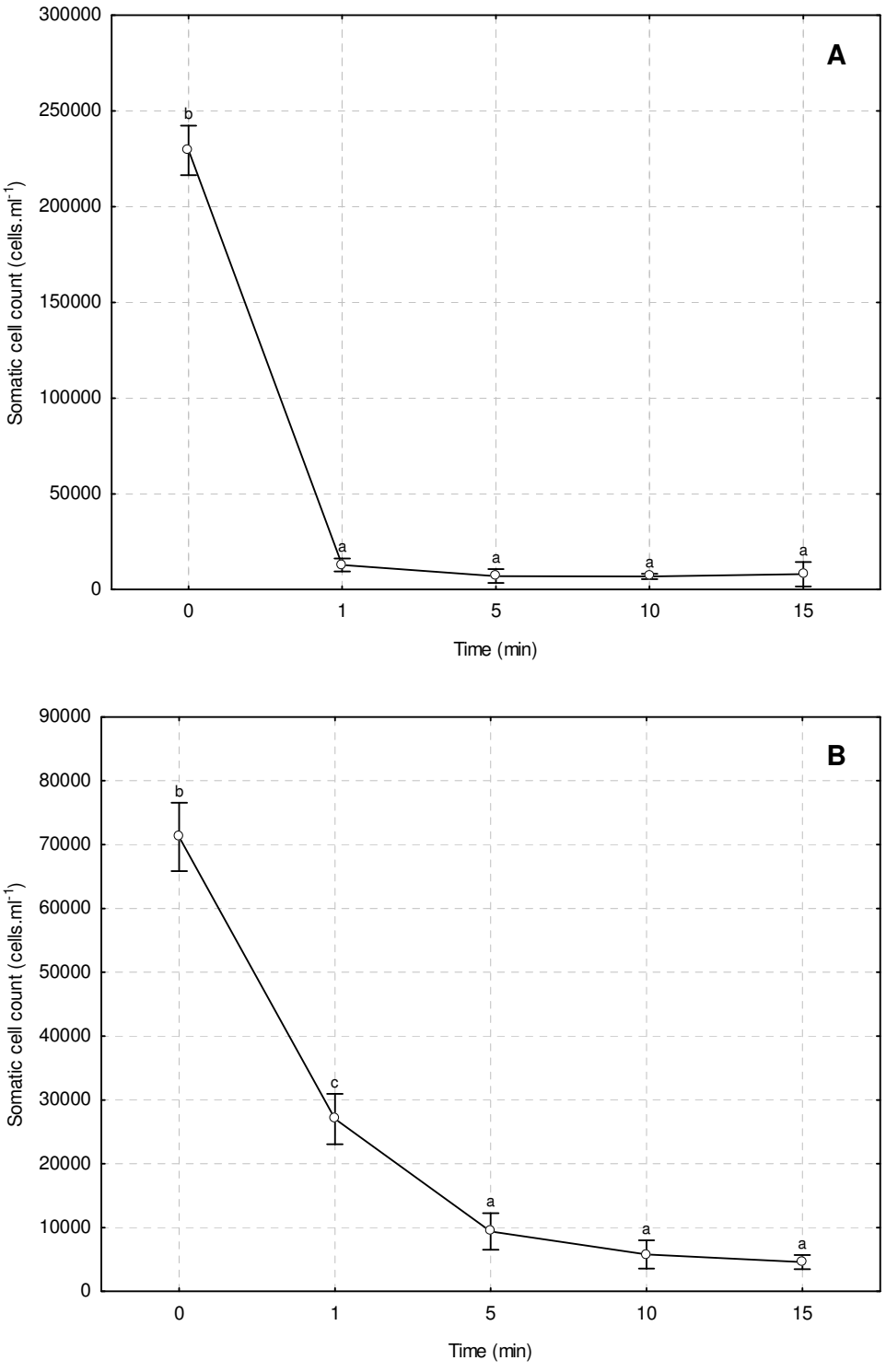

Figure 6 Statistical analysis of the data (MilkoScan) of the somatic cell counts (cells.ml^{-1}) of raw (A) and pasteurised (B) milk after ultrasonication (Each data point represents five values. The standard deviation was used as the error-bar. Means with different letters are statistically significantly different, p≤0.05).

al., 1984) and shelf-life (Ma *et al*., 2000). Although the SCC was reduced by ultrasonication, it would however, not improve the low quality of milk associated with milk with high SCC levels. It is of utmost importance that the quality of raw milk be considered before accepting milk, as no processing method can compensate for milk of a poor quality.

Alkaline phosphatase

Alkaline phosphatase (ALP) is an endogenous enzyme that is always present in raw milk, with 30 - 40% of the enzyme bound to the milk fat globule membranes. The rest of the enzyme is dispersed throughout the skimmed milk fraction, and probably associated with the lipoproteins (Painter & Bradley, 1997). This enzyme splits certain phosphoric acid-esters into phosphoric acid and the corresponding alcohols (Anon., 2003). Alkaline phosphatase is destroyed by pasteurisation at 72℃ for 15 s (Anon., 2003), therefore, the ALP test is commonly used for assessing the effectiveness of pasteurisation and also the safety of dairy products (Scharer, 1938; Griffiths, 1986).

The results for alkaline phosphatase activity of ultrasonicated and non-ultrasonicated milk are given in Fig. 7. In this study, it was found that ultrasonication of raw milk does not decrease ALP activity. It is clear from the data in Fig. 7 that untreated raw milk, raw milk that had been ultrasonicated for 5 min, and raw milk that had been ultrasonicated for 10 min all remained positive for ALP activity. Villamiel & de Jong in 2000 also showed that ultrasonication of milk, without the addition of heat, results in a positive ALP test. As would be expected, the commercially pasteurised milk tested negative for phosphatase activity. It can, therefore, be concluded that the ALP test cannot be used for assessing the effectiveness of ultrasonication as APL enzymes are not inactivated during ultrasonication.

Lactoperoxidase

Lactoperoxidase is an enzyme found mainly in the whey fraction of milk (Harper, 1976) and catalyses the transfer of oxygen from hydrogen peroxide to other substrates (Anon., 2003; Fox & Kelly, 2006). Lactoperoxidase enzymes are used as an indicator of successful ultra high temperature treatments as these enzymes are inactivated by heat treatments above 80℃ (Griffiths, 1986; Anon., 2003). Therefore, HTST pasteurised milk remains peroxidase positive. Ultra high temperature milk tests as peroxidase negative (Villamiel *et al.,* 1999) as UHT milk is heated to temperatures of above 100℃.

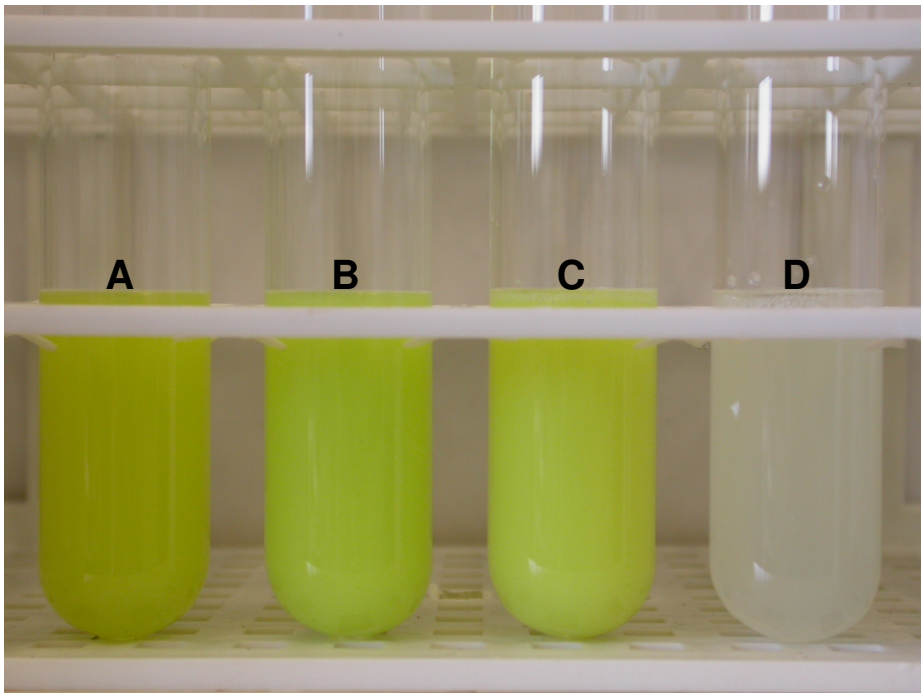

Figure 7 Impact of ultrasonication on the alkaline phosphatase activity in milk (A = raw milk; B = raw milk + 5 min ultrasonication; C = raw milk + 10 min ultrasonication; D = negative control - pasteurised milk).

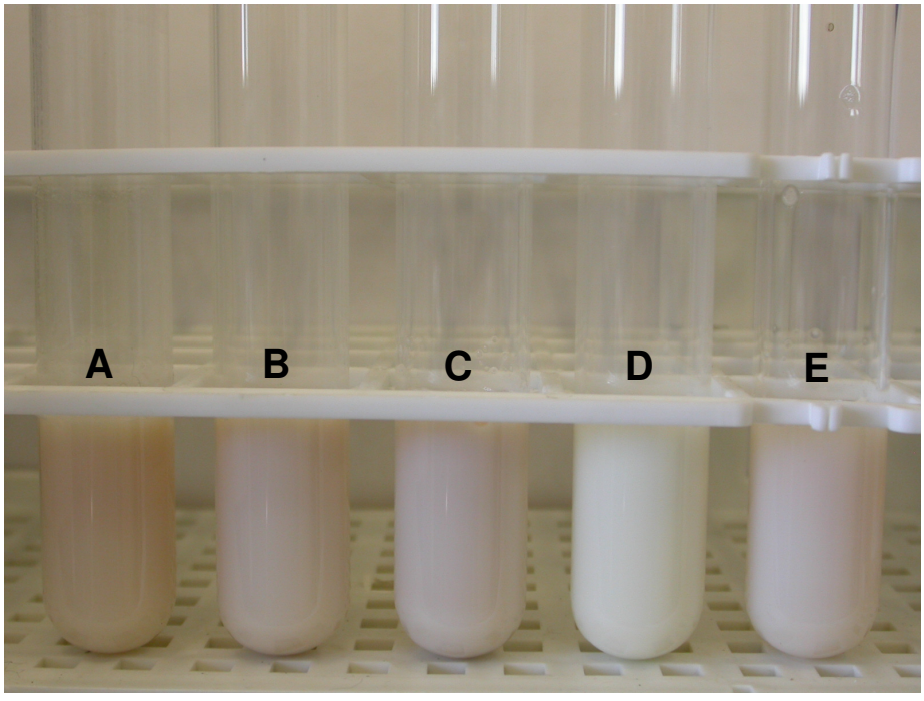

Figure 8 Impact of ultrasonication on the lactoperoxidase activity in milk (A = raw milk; B = raw milk + 5 min ultrasonication; C = raw milk + 10 min ultrasonication; D = negative control - UHT milk; E = pasteurised milk).

The results obtained for the peroxidase test of ultrasonicated and non-ultrasonicated milk are given in Fig. 8. This study showed that ultrasonicating raw milk for either 5 min or 10 min reduces peroxidase activity to a degree/extent comparable with that found in pasteurised milk. However, total inactivation of peroxidase, as was found when UHT milk was tested, could not be achieved with an ultrasonic treatment time of 10 min. It is thus clear that the peroxidase test cannot be used as a fast indicator of an effective ultrasonic treatment.

Conclusions

This study clearly shows that ultrasound does not have a negative impact on the total protein or casein content of milk. Ultrasonication has a homogenising effect on the milk fat and has no negative effect on the lactose content of milk. It is therefore, suggested that ultrasonication may be employed effectively as a means of "pasteurisation" with no adverse effects on e.g. cheese yield.

Unfortunately, ultrasound does not inactivate alkaline phosphatase or lactoperoxidase enzymes. These enzymes can thus not be used to indicate a successful ultrasonic treatment. If ultrasonication is to be used as an alternative to thermal pasteurisation, a need exists to find a quick and efficient method to indicate whether ultrasonication was sufficient in terms of ensuring a microbiologically safe product. The method should be comparable to the phosphatase and peroxidase tests in terms of simplicity and accurateness.

Catalase was among the indicators of pasteurisation investigated. The amount of catalase in milk is relatively high, and therefore it might be feasible to investigate the sensitivity of this enzyme to inactivation by ultrasonication as a possible means of indicating a successful treatment.

If ultrasonication was to be used in combination with a mild heat treatment to target the heat-resistant microbes found in milk, the heat would allow the phosphatase enzymes to be inactivated. In this case, the phosphatase test could still be employed as a quick and efficient method to indicate the elimination of spoilage and possible pathogenic bacteria, and therefore, a successful treatment.

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CHAPTER 7

IMPACT OF ULTRASONICATION, HEAT OR THERMO-ULTRASONICATION ON THE SURVIVAL OF SELECTED DAIRY MICROBES

Abstract

Milk is generally given a heat treatment to extend the shelf-life and to ensure the microbiological safety. Combining heat with other methods could further extend the shelf-life and may potentially reduce the treatment time. The aim of this study was to determine if a combination of ultrasound and heat, rather than each treatment on its own, would be more effective at eliminating *Escherichia coli*, *Bacillus cereus*, *Lactobacillus acidophilus* and *Micrococcus luteus* from milk. Milk samples inoculated with selected microbes were treated with ultrasound only, heat only (63° or 72°C), or simultaneous treatments of ultrasound and heat (thermo-ultrasonication) at 52°, 63° or 72°C. Complete elimination of all viable cells (1 x 10^6 cfu.ml^{-1}) was achieved within 4 min for *E. coli*, *Lb. acidophilus* and *M. luteus* when thermo-ultrasonicated at 72°C, although heat only applied at 72°C for 4 min was also sufficient to ensure total elimination of *Lb. acidophilus*. *Escherichia coli* was not treated at 72°C, due to its known sensitivity to heat. Thermo-ultrasonication at 63°C was also more effective at eliminating viable *E. coli*, *Lb. acidophilus* and *M. luteus* cells than when only ultrasound or heat at 63°C was applied. Neither ultrasound, nor heat, nor thermo-ultrasonication regimes used in this study was enough to ensure total elimination of all viable *B. cereus* cells/endospores. A 78% elimination of *B. cereus* (1 x 10^4 cfu.ml^{-1}) was achieved by 10 min thermo-ultrasonication at 72°C. When only ultrasound was applied for 10 min, 96% of the 1 x 10^5 cfu.ml^{-1} viable *B. cereus* cells/endospores in milk were eliminated.

Introduction

Heat is the most commonly applied pasteurisation or sterilisation method for extending the shelf-life of food products such as milk (Raso *et al*., 1998a). Combining heat with other methods have many advantages in food processing, including reduction of energy costs and the production of safer, more palatable products (Black *et al*., 2005). Any

reduction in the temperature applied or the treatment time would result in lower energy consumption and reduce any detrimental effect of heat on the food. Therefore, combining heat with other physical or chemical methods in order to increase the process efficiency in terms of eliminating microbes continue to be a subject of interest especially for the dairy industry (Ciccolini *et al*., 1997).

The lethal effect of ultrasound has been attributed to cavitation. Cavitation is the result of high power ultrasound, and is the growth, and subsequent collapse of microscopic bubbles as ultrasonic waves travel through a liquid (Scherba *et al*., 1991). The violent collapse of the cavitating bubbles causes the microbial cell walls to shear and rupture, resulting in cell lysis, and ultimately cell death (Hoover, 2000). This method could be used to eliminate microbial cells/endospores from milk, aiding in extension of shelf-life.

In this dissertation (Chapters 3 and 4) it was shown that ultrasonication can be employed for the elimination of microbial cells. All viable *Escherichia coli* cells were eliminated when subjected to a 10 min ultrasonication treatment. A 10 min treatment was shown to be effective at eliminating *Bacillus cereus*, *Lactobacillus acidophilus* and *Micrococcus luteus* cells with a 96%, 87% and 95% elimination achieved, respectively. The demand for all microbial cells to be eliminated, and also to reduce the required treatment time of ultrasonication necessitates further investigations.

The impact of a combination of ultrasound and heat on microbes has been investigated (Ordoñez *et al*., 1984; 1987; Raso *et al*., 1998b; Villamiel & de Jong, 2000). However, only the study of Villamiel & de Jong (2000) was aimed at the elimination of microbes associated with milk, and also at the industrial application of this method by the dairy industry. There is thus a need for more research on the impact of thermo-ultrasonication aimed specifically at dairy microbes.

The aim of this study was to determine if a combination of ultrasound and heat would be more efficient at eliminating microbes from milk than a single treatment of either method. The effect of thermo-ultrasonication on the survival of *E. coli* and the more ultrasound and/or heat resistant *B. cereus*, *Lb. acidophilus* and *M. luteus* cells was evaluated.

Materials and methods

Bacterial cultures

The four "test" microbes (*E. coli*, *B. cereus*, *Lb. acidophilus* and *M. luteus*) used in this study and their specific growth requirements are summarised in Table 1. Sterile growth medium (10 ml) (Table 1) was inoculated with a "test" microbe and incubated at the appropriate temperature for 24 h. Five ml of each 24 h culture was then used to inoculate 90 ml sterile growth medium and this was incubated for a further 24 h prior to the ultrasonic and/or heat treatment. Standard growth curves of each "test" microbe were prepared as described in Chapters 3 and 4 and was used as a reference to standardise cell inoculum sizes.

Ultrasonication

Two ml of each batch culture was centrifuged for 10 min at 6 000 x *g* (Eppendorf Centrifuge 5415D, Hamburg). Bacterial pellets were suspended in sterile saline solution (SSS) and the data from the standard curves used to determine the desired cell concentration for inoculation of the suspension medium. Full cream (3.4% milk fat) UHT (ultra high temperature) milk, was inoculated with a "test" microbe to give a final concentration of as near as possible to 1 x 10^6 colony forming units per ml ($cfu.ml^{-1}$).

For ultrasonication, a 60 ml sample of the inoculated milk was pipetted into a sterile, jacketed glass sample holder connected to a waterbath to maintain a sample temperature of either 52°C, or 63°C or 72°C. Where ultrasound only was used, the jacketed glass sample holder was connected to an ice-waterbath (4° - 6°C) to maintain a sample temperature of 24° - 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 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 (100%, 124 µm). The Vibra-Cell is also able to monitor the energy (in Joules) as well as the temperature of the sample being processed. Samples were treated at 100% wave amplitude for different times (0 - 10 min) at the specified temperatures.

All ultrasonic treatments were done in duplicate 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

Table 1 Growth media, incubation times and temperatures used for the four "test" microbes

Microbe	USFSCC[a]	Medium	Incubation	
			Time (h)	Temperature (°C)
Escherichia coli	11775	NB[b]/PCA[c]	24	37°
Bacillus cereus	1335	NB/PCA	24	35°
Lactobacillus acidophilus	1348	MRS[d]	24	35°
Micrococcus luteus	173	NB/PCA	24	35°

[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).

selected for counting (Anon., 1997). Ultra high temperature milk samples that had not been inoculated with a "test" microbe, served as the controls. The controls showed no microbial growth after incubation.

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. The *D*-value at 20 kHz/750 W was abbreviated as D_{US}, and the *D*-value for the combinations of ultrasound and heat as $D_{US + 52℃}$, $D_{US + 63℃}$ and $D_{US + 72℃}$.

Heat treatment

Three "test" microbes (*B. cereus*, *Lb. acidophilus* and *M. luteus*) were used for the heat only experiment. Ultra high temperature milk was inoculated with each microbe separately and these were treated at two different temperatures (63° and 72℃) using a waterbath to maintain the required treatment temperature. Duplicate samples were taken before the inoculated milk received any heat treatment, as well as duplicate samples every 2 min at each treatment temperature, with 10 min being the maximum treatment time. Ultra high temperature milk samples (for each "test" microbe) that had not been inoculated with a "test" microbe, served as the controls. The controls showed no microbial growth after incubation. It was decided not to include a heat treatment of 52℃ as a separate single heat treatment as 63° and 72℃ are used for low-temperature long-time (LTLT) and high-temperature short-time pasteurisation (HTST), respectively.

The efficacy of heat treatments in terms of eliminating microbes was measured by their decimal reduction time (*D*). The *D*-value at either 63° or 72℃ was abbreviated as either $D_{63℃}$ or $D_{72℃}$.

Results and discussion

The four "test" microbes used in this study were chosen for different reasons. *Escherichia coli* was included due to its importance in the dairy industry as the indicator of faecal contamination, even though it is known to be sensitive to heat treatments as well as ultrasonication (Chapter 3). *Bacillus cereus* is associated with "blowing" of cheese (Hull *et al.*, 1992). In addition, *B. cereus* was included as this microbe forms

heat-resistant endospores. *Lactobacillus acidophilus* and *M. luteus* were previously (Chapter 4) found to be resistant to ultrasonication and were considered suitable candidates for thermo-ultrasonication. Microbes used for this study varied with respect to Gram characteristics, cell morphology and endospore formation.

Escherichia coli

The presence of *E. coli* in dairy products generally serves as an indication of faecal and/or post-pasteurisation contamination (Holsinger *et al*., 1997). *Escherichia coli* also has the ability to readily form biofilms on equipment surfaces (Stopforth *et al*., 2003). The SA "milk law" clearly states that no *E. coli* may be present in 1.0 ml of pasteurised milk (Anon., 1997). It is therefore important to ensure that milk receives the correct treatment to eliminate all *E. coli* cells that may be present.

The actual cell counts (cfu.ml^{-1}) for ultrasonication at four different operating temperatures (no heat, 52°, 63° and 72°C) are given in Fig. 1. The recalculated data are given in the form of log graphs in Fig. 2 and the results are summarised in Table 2.

In this study, ultrasonication of *E. coli* (1 x 10^6 cfu.ml^{-1}) without the addition of heat resulted in a 100% elimination of viable cells (a 5.72 log reduction) after 10 min of treatment (Fig. 2). Thermo-ultrasonication at 52°C for 10 min resulted in a 3.96 log reduction (a 99.99% elimination of viable cells) (Fig. 2), and a 5.99 log reduction (100% elimination) of viable cells was recorded within 4 min for a thermo-ultrasonic treatment at 63°C (Fig. 2). When cells were thermo-ultrasonicated at 72°C, a 100% elimination (a 6.17 log reduction) of viable cells was recorded within 2 min of treatment (Fig. 2). The *D*-values, as calculated for this study, were found to be: D_{US} = 1.93 min; $D_{US + 52°C}$ = 2.14 min; $D_{US + 63°C}$ = 0.75 min and; $D_{US + 72°C}$ = 0.26 min (Table 2).

Thermo-ultrasonication (52°C) was least effective for the elimination of *E. coli* from milk. An ultrasonic treatment without the addition of heat was slightly more efficient at eliminating *E. coli*. Dumalisile *et al*. (2005) reported that *E. coli*, with an initial concentration of 1 x 10^6 cfu.ml^{-1}, was reduced to 9 viable cells after a 20 min heat treatment at 63°C. In this study, thermo-ultrasonication at 63°C gave a 100% elimination within 4 min of treatment. Thermo-ultrasonication at 72°C, as used in this study, proved to be even more efficient than a combination using 63°C with a 100% elimination within 2 min of treatment. *Escherichia coli* was not treated with heat at 52°C as this temperature is not used for either LTLT or HTST pasteurisation, and was considered to be too low to have any impact on viable cells as a single treatment.

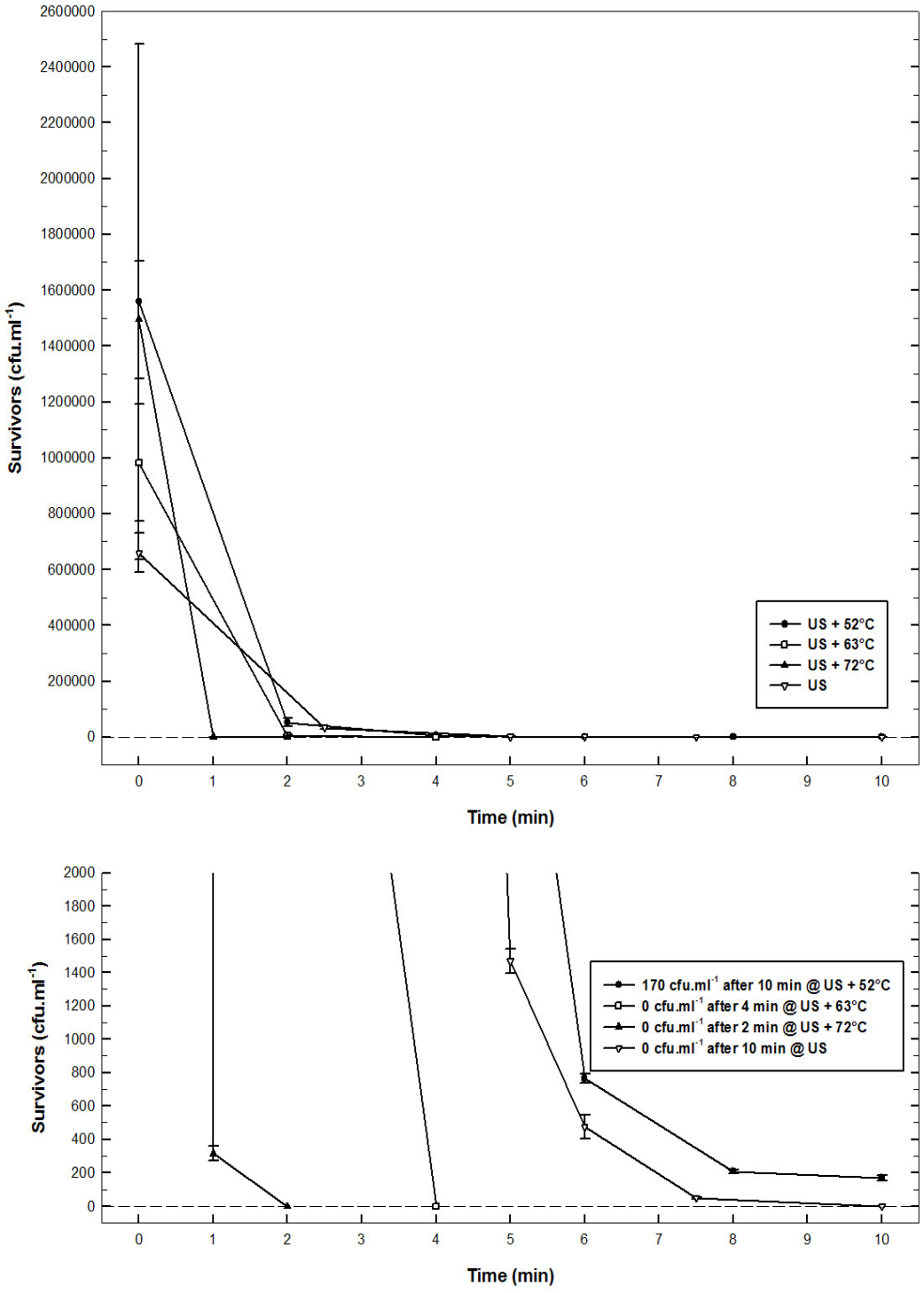

Figure 1 Impact of thermo-ultrasonication on *Escherichia coli* in milk. The lower graph represents an expanded Y-axis (US = ultrasound. Each data point represents quadruple values. The standard deviation was used as the error-bar).

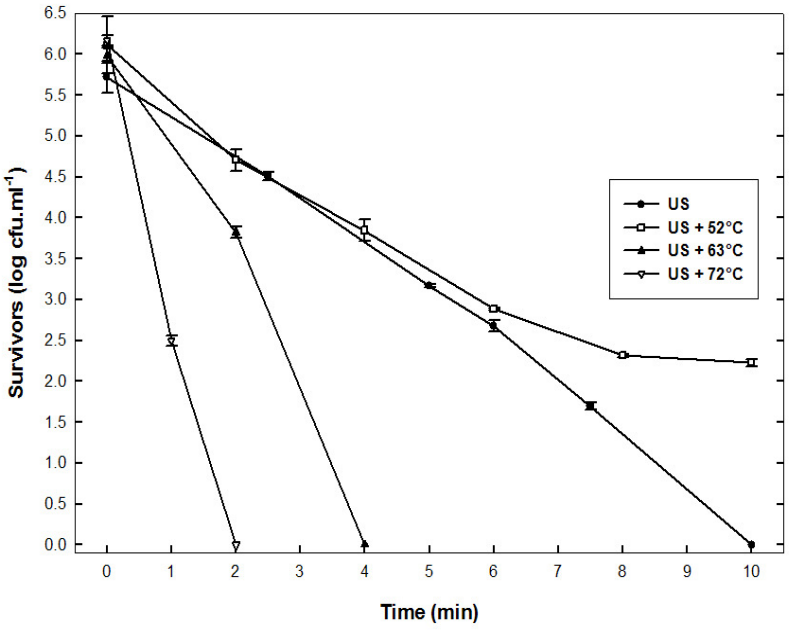

Figure 2 Impact of ultrasonication and thermo-ultrasonication at 52°, 63° and 72°C on *Escherichia coli* in milk (US = ultrasound. Each data point represents quadruple values. The standard deviation was used as the error-bar).

Table 2 Summary of the *D*-values, log reductions and % reductions of *Escherichia coli* in milk obtained over 10 min ultrasound and/or heat treatments

Treatment	**Time (min)**	***D*-value (min)**	**log reduction**	**% reduction**
US	10	1.93	5.72	100
US + 52℃	10	2.14	3.96	99.99
*63℃	10	4.61	1.44	96.41
US + 63℃	4	0.75	5.99	100
US + 72℃	2	0.26	6.17	100

US = ultrasonication.
* Data adapted from Dumalisile (2004).

Due to the known heat sensitivity of *E. coli* (Holsinger *et al*., 1997), this "test" microbe was not given singular heat treatments at 63° and 72°C. This study clearly indicates that thermo-ultrasonication at either 63° or 72°C reduces the required treatment time when compared to only ultrasonication to ensure the total elimination of the *E. coli* strain used in this study. Processing at lower temperatures is more cost-effective and by reducing the treatment time, a dairy processing plant may also increase the volumes processed per day by using thermo-ultrasonication as opposed to a heat treatment only. *Escherichia coli* is known to be heat sensitive, and is destroyed by HTST pasteurisation (Holsinger *et al*., 1997), however, a thermo-ultrasonic treatment applied to milk may be effective in eliminating the more heat resistant spoilage and potentially pathogenic microbes.

The high initial inoculum used in this investigation (1 x 10^6 cfu.ml^{-1}) must be taken into consideration when evaluating the results. According to the SA “milk law” only raw milk with a microbial load of less than 200 000 cfu.ml^{-1} may be used for further processing. Although the initial microbial load in this study was five times higher than the permitted legal limits, a 100% elimination of viable *E. coli* cells was still achieved. It is thus evident that thermo-ultrasonication (63° or 72°C) can be implemented for the production of milk surpassing the quality control requirements for pasteurised milk.

Bacillus cereus

Bacillus cereus is a potential foodborne pathogen and is renowned for being one of the leading causes of bacterial food poisoning in several countries, with milk frequently being implicated as one of the carriers (Notermans *et al*., 1997; Beattie & Williams, 1999). It is thus important for the dairy industry to ensure that fresh milk sold to the public should not contain any viable *B. cereus* cells.

The actual cell counts (cfu.ml^{-1}) for ultrasonication at four different operating temperatures (no heat, 52°, 63° and 72°C) and heat treatments at two different temperatures (63° and 72°C) are given in Figs. 3 and 4, respectively. The recalculated data are given in the form of log graphs in Fig. 5 and the results are summarised in Table 3. It should be noted that a microscopic investigation revealed that the *B. cereus* inoculum used in this study consisted of a mixture of vegetative cells and endospores.

A technical problem that arose during this study was that a 1 x 10^6 cfu.ml^{-1} inoculum could not be reached within the prescribed incubation time, and it was, therefore, decided to use an inoculum size of as close to 1 x 10^4 cfu.ml^{-1} as possible.

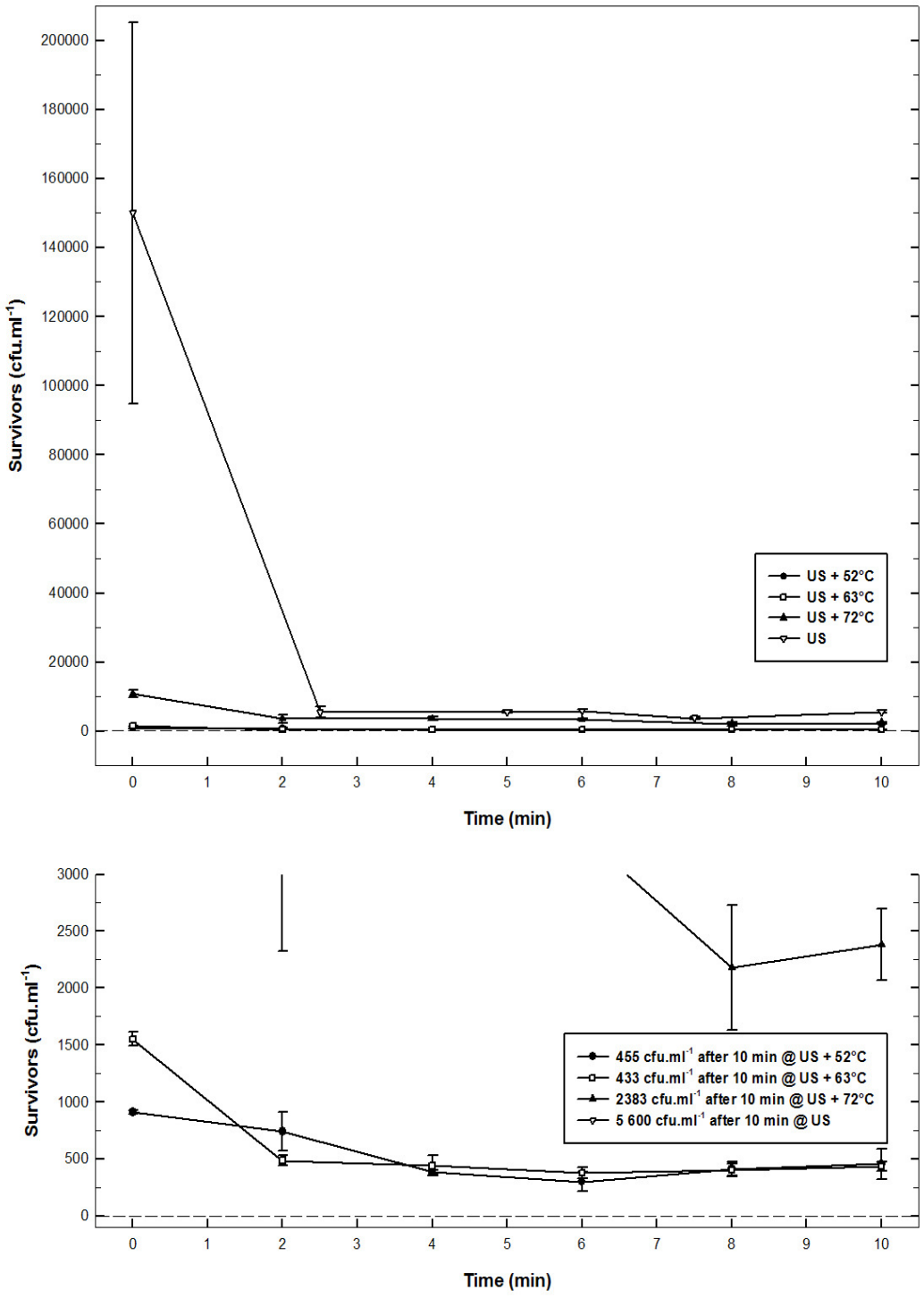

Figure 3 Impact of thermo-ultrasonication on *Bacillus cereus* in milk. The lower graph represents an expanded Y-axis (US = ultrasound. Each data point represents quadruple values. The standard deviation was used as the error-bar).

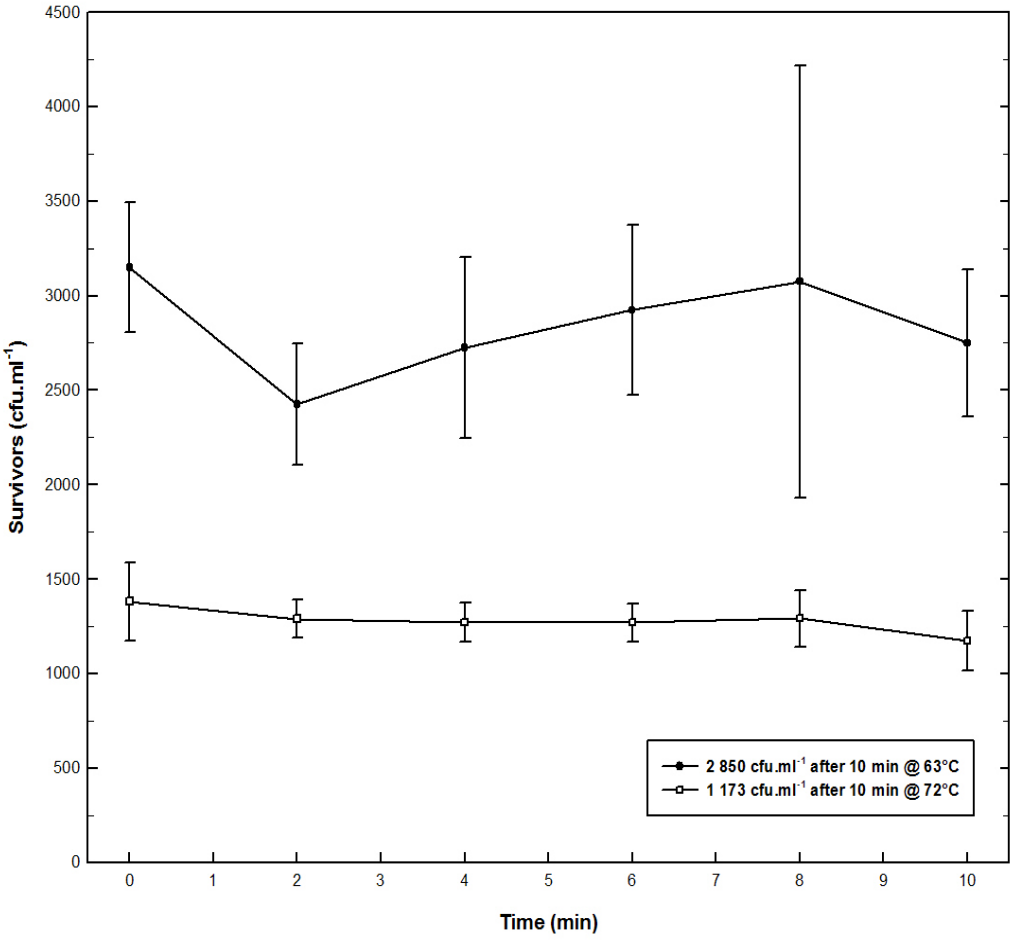

Figure 4 Impact of heat on *Bacillus cereus* in milk (Each data point represents quadruple values. The standard deviation was used as the error-bar).

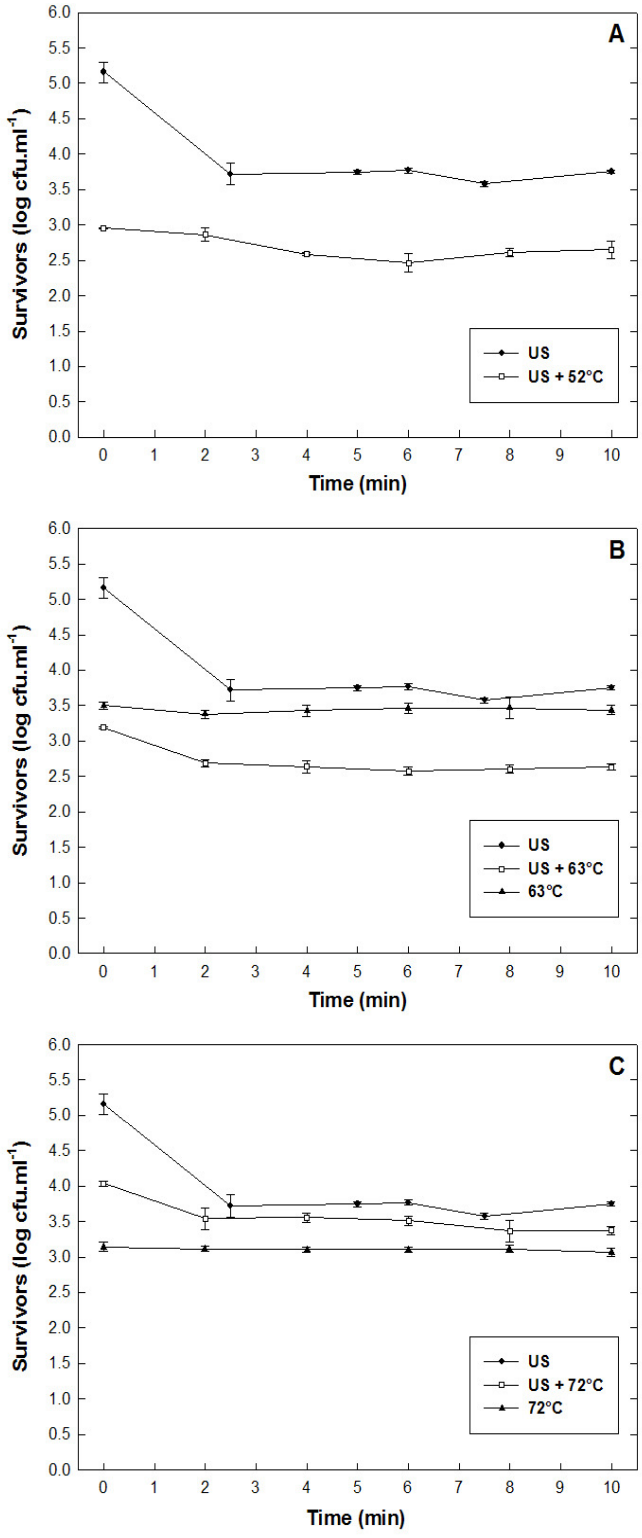

Figure 5 Impact of ultrasound, heat or thermo-ultrasonication at 52°C (A), 63°C (B) and 72°C (C) on *Bacillus cereus* in milk (US = ultrasound. Each data point represents quadruple values. The standard deviation was used as the error-bar).

Table 3 Summary of the *D*-values, log reductions and % reductions of *Bacillus cereus* in milk obtained over 10 min ultrasound and/or heat treatments

Treatment	Time (min)	*D*-value (min)	log reduction	% reduction
US	10	nc	1.41	96.27
US + 52℃	10	nc	0.31	50.00
63℃	10	nc	0.06	12.70
US + 63℃	10	nc	0.56	72.06
72℃	10	nc	0.07	15.18
US + 72℃	10	nc	0.65	78.04

US = ultrasonication.
nc = not calcutated (*D*-value could not be calculated as a single log reduction was not reached).

In this study, *Bacillus cereus*, at an initial concentration of 1 x 10^4 cfu.ml^{-1} showed some resistance to the effect of ultrasonication, heat and thermo-ultrasonication. A 10 min ultrasonic treatment of a *B. cereus* inoculum consisting of both vegetative cells and endospores eliminated 96.27% of the inoculum (a 1.41 log reduction) (Figs. 5A-C). Thermo-ultrasonication (52°C) did not improve the efficiency of ultrasound, with only a 0.31 log reduction (50% elimination) recorded (Fig. 5A). Heat at 52°C was not used as a single treatment as 52°C is not used as a pasteurisation temperature, contrary to 63°C (LTLT) and 72°C (HTST).

When the inoculum was subjected to 63°C for 10 min, a 12.70% elimination (a 0.06 log reduction) of viable cfu's was recorded (Fig. 5B). Thermo-ultrasonication at 63°C increased the efficiency of the process when compared to US + 52°C and resulted in a 72.06% elimination of this microbe (a 0.56 log reduction) after 10 min of treatment (Fig. 5B).

A single heat treatment at 72°C improved the elimination of *B. cereus* when compared to a heat treatment at 63°C with a 15.18% elimination, or a 0.07 log reduction achieved after 10 min at 72°C (Fig. 5C). Thermo-ultrasonication at 72°C resulted in a 78.04% elimination (a 0.65 log reduction) after 10 min of treatment (Fig. 5C). Due to the non-linear response of *B. cereus* to ultrasonication, it was decided that a D_{US}-value would not give a true reflection of the results. The *D*-values for *B. cereus* treated with heat, or thermo-ultrasonication could also not be determined as a single log reduction was not reached (Table 3).

The data obtained from this study showed that thermo-ultrasonication is not more effective in the elimination of *B. cereus* cfu's when compared to a singular ultrasonic treatment. The higher initial concentration of viable cells used when no heat was added to ultrasonication (1 x 10^5 cfu.ml^{-1}) could explain the higher efficiency in terms of % elimination of that treatment when compared to the different combinations of thermo-ultrasonication (1 x 10^4 cfu.ml^{-1}). It is also difficult to determine the ratio of vegetative cells to endospores of the inoculum, and a higher concentration of *B. cereus* vegetative cells will result in a seemingly more efficient process.

Data showed that when a *B. cereus* inoculum was ultrasonicated without the addition of heat, a single log reduction was achieved within the first 2.5 min of ultrasonication whereafter no further reduction occurred (Fig. 5). It was hypothesised that all the vegetative *B. cereus* cells were eliminated within the first 2.5 min of ultrasonication, with only the resistant endospores (Berger & Marr, 1960) remaining. It was, therefore, concluded that *B. cereus* is sensitive to the damaging effect of

ultrasound if the cells are in a vegetative phase. Thus, it is recommended to treat raw milk as soon as possible to try and combat the development of heat and ultrasound resistant microbial endospores.

This study indicates that thermo-ultrasonication is more effective at eliminating *B. cereus* from milk when compared to a singular heat treatment. The addition of ultrasound to an existing thermal processing plant would, therefore, increase the efficiency of a heat treatment in eliminating *B. cereus* from milk. Unfortunately, a combination of these two microbial inactivation methods failed to ensure the total elimination of this endospore-former.

Lactobacillus acidophilus

Lactobacillus acidophilus is a lactic acid producing microbe with a thick Gram-positive cell wall (Kleerebezem & Hugenholtz, 2003). The dairy industry classifies *Lb. acidophilus* as a spoilage microbe in terms of fresh milk as it produces lactic acid.

The actual cell counts (cfu.ml^{-1}) after ultrasonication at four different operating temperatures (no heat, 52°, 63° and 72°C) and of heat treatments at two different temperatures (63° and 72°C) are given in Figs. 6 and 7, respectively. The recalculated data are given in the form of log graphs in Fig. 8 and the results are summarised in Table 4.

In this study, ultrasonication of *Lb. acidophilus* at an initial concentration of 1 x 10^{6} cfu.ml^{-1} without the addition of heat, resulted in an 87.48% elimination of viable cells (a 0.91 log reduction) after a 10 min treatment (Figs. 8A-C). Thermo-ultrasonication at 52°C resulted in a 1.58 log reduction (97.26% elimination) in viable cells after 10 min of treatment (Fig. 8A). *Lactobacillus acidophilus* was not treated with heat at 52°C as this temperature is not generally used for either LTLT or HTST pasteurisation, and additionally it was thus considered to be too low to have any significant impact on viable cells as a single treatment.

A 10 min heat treatment at 63°C eliminated 97.88% of viable *Lb. acidophilus* cells (a 1.68 log reduction) (Fig. 8B), and thermo-ultrasonication (63°C) resulted in a 4.68 log reduction (a 99.99% elimination) of viable cells after 10 min of treatment (Fig. 8B).

Heat at 72°C, as well as thermo-ultrasonication at 72°C eliminated 100% of the viable cells (a 6.24 and 6.48 log reduction, respectively) within 4 min of treatment (Fig. 8C). Due to the heat sensitivity of *Lb. acidophilus* at 72°C, the enhanced efficiency of

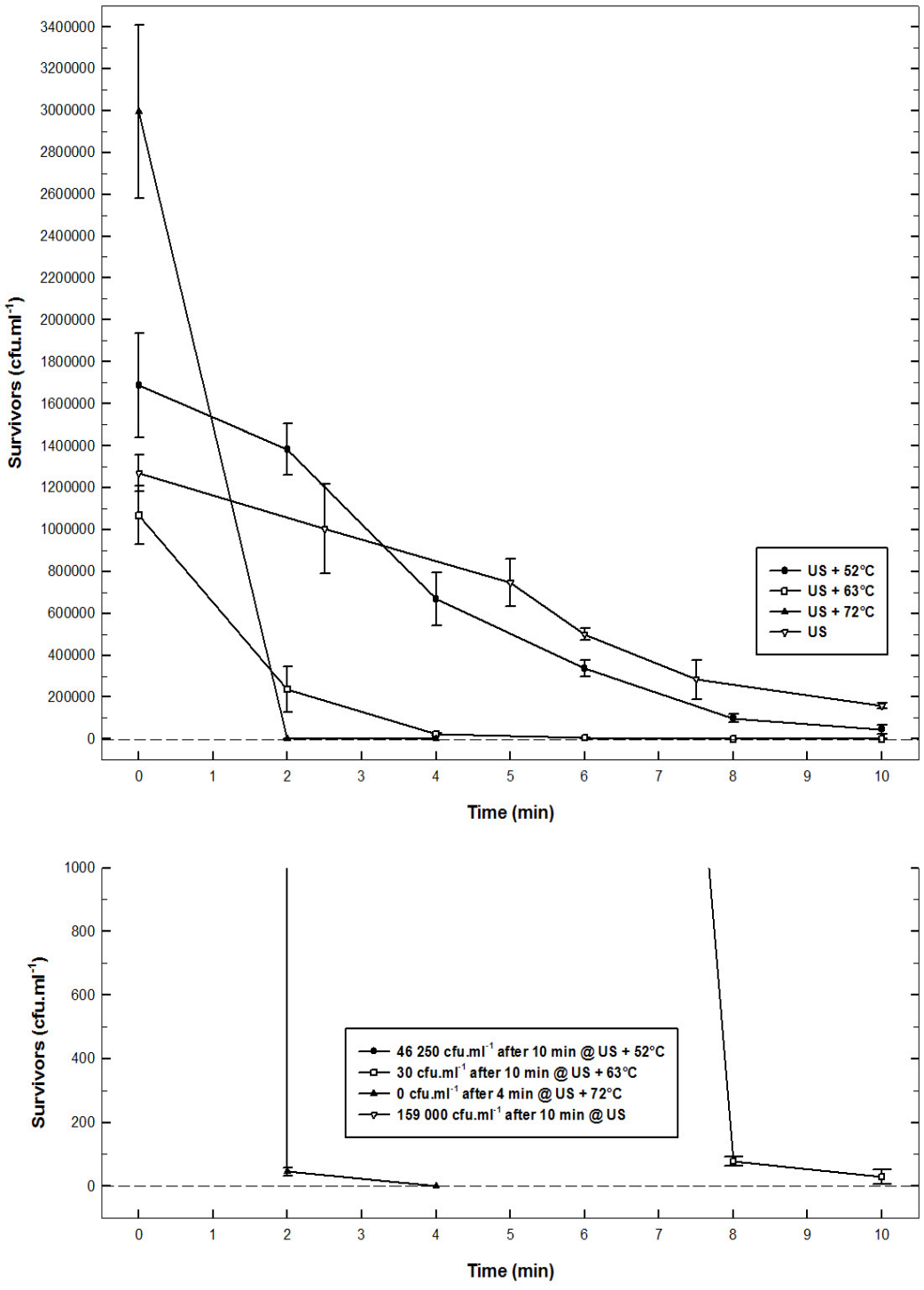

Figure 6 Impact of thermo-ultrasonication on *Lactobacillus acidophilus* in milk. The lower graph represents an expanded Y-axis (US = ultrasound. Each data point represents quadruple values. The standard deviation was used as the error-bar).

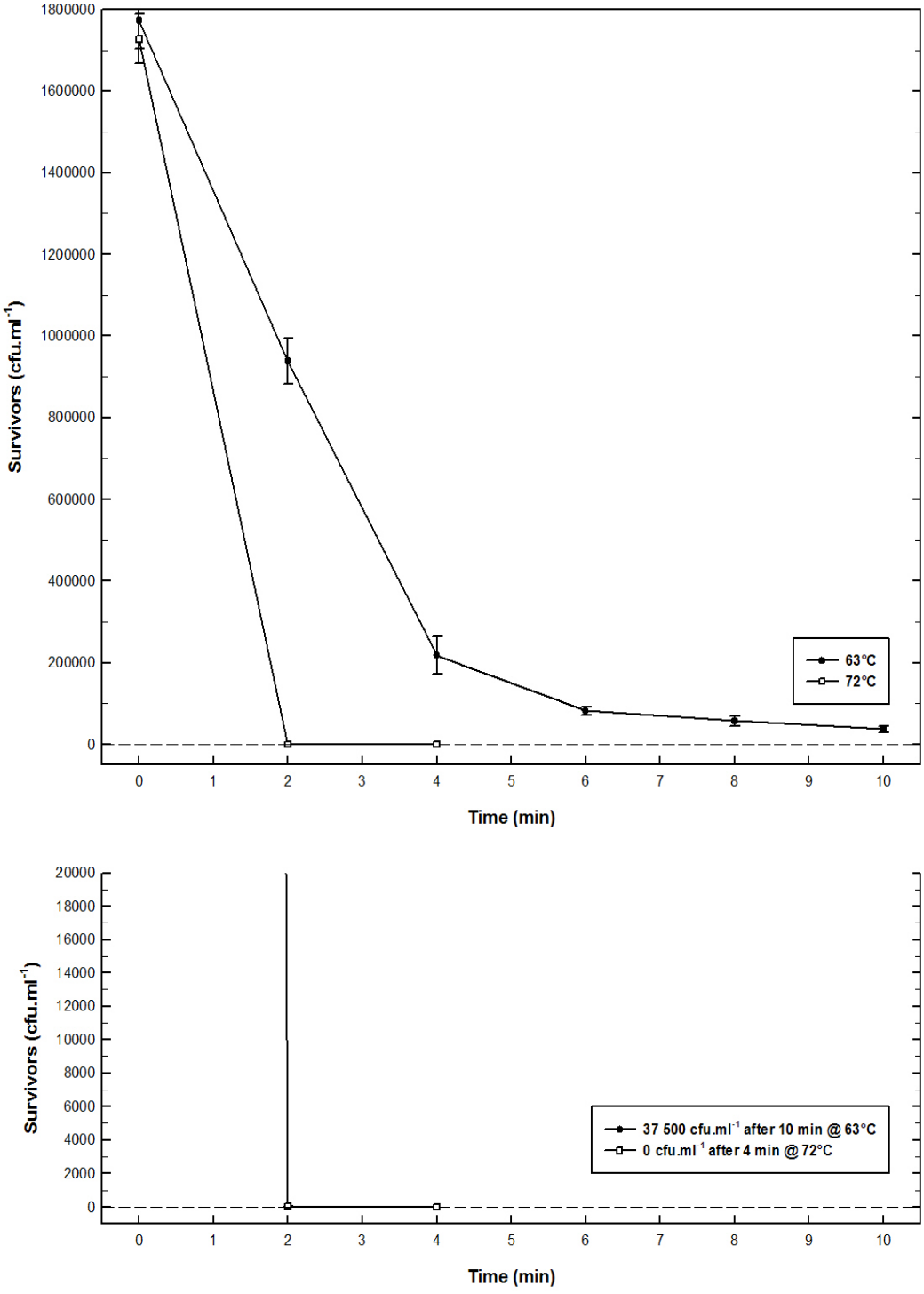

Figure 7 Impact of heat on *Lactobacillus acidophilus* in milk. The lower graph represents an expanded Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

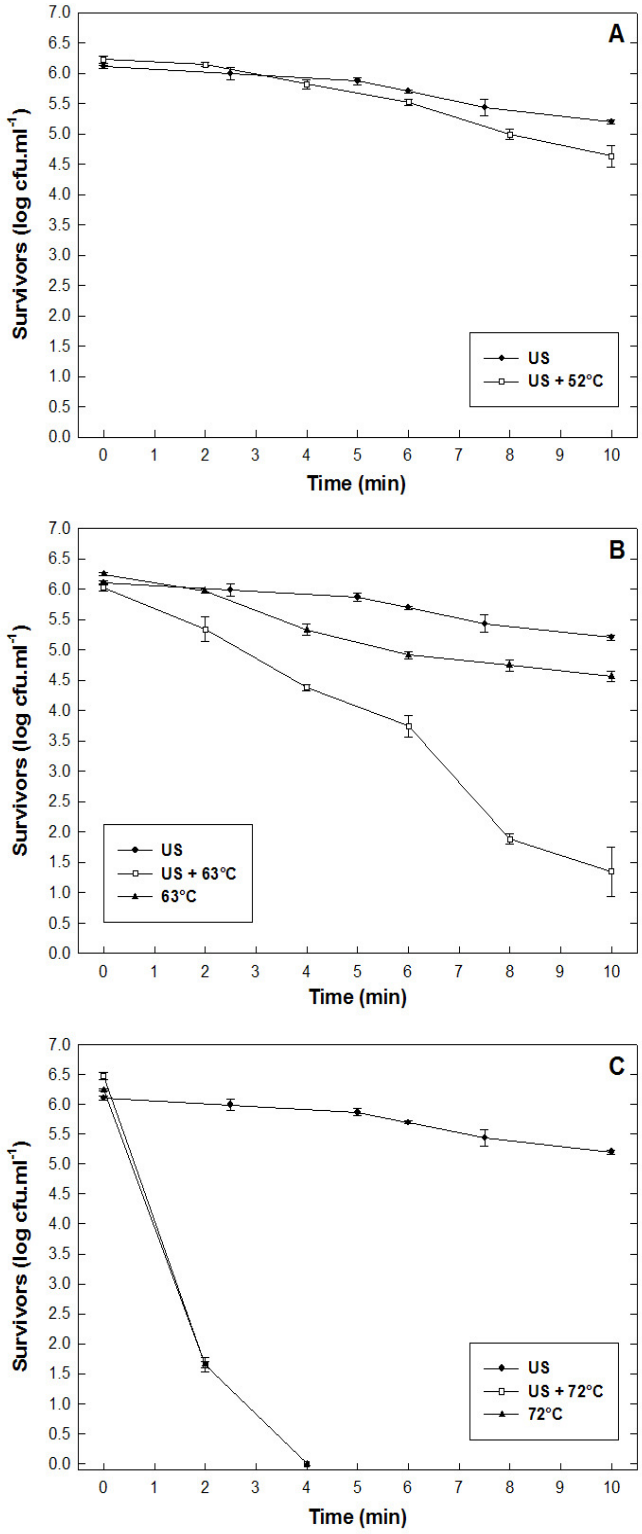

Figure 8 Impact of ultrasound, heat or thermo-ultrasonication at 52°C (A), 63°C (B) and 72°C (C) on *Lactobacillus acidophilus* in milk (US = ultrasound. Each data point represents quadruple values. The standard deviation was used as the error-bar).

Table 4 Summary of the *D*-values, log reductions and % reductions of *Lactobacillus acidophilus* in milk obtained over 10 min ultrasound and/or heat treatments

Treatment	**Time (min)**	***D*-value (min)**	**log reduction**	**% reduction**
US	10	nc	0.91	87.48
US + 52℃	10	5.53	1.58	97.26
63℃	10	3.68	1.68	97.88
US + 63℃	10	2.20	4.68	99.99
72℃	4	0.45	6.24	100
US + 72℃	4	0.42	6.48	100

US = ultrasonication.
nc = not calcutated (*D*-value could not be calculated as a single log reduction was not reached).

thermo-ultrasonication at 72°C was not as prominent. However, thermo-ultrasonication was found to be more efficient than only an ultrasonic treatment.

The *D*-values as calculated for *Lb. acidophilus* after exposure to the different treatments were: D_{US} = could not be calculated as a single log reduction was not reached within 10 min of treatment; $D_{US + 52°C}$ = 5.53 min; $D_{63°C}$ = 3.68 min; $D_{US + 63°C}$ = 2.20 min; $D_{72°C}$ = 0.45 min and; $D_{US + 72°C}$ = 0.42 min (Table 4).

This study indicated that for *Lb. acidophilus* a thermo-ultrasonic treatment (52° or 63°C) was more effective at eliminating *Lb. acidophilus* than only an ultrasonic or heat treatment (63°C). The *D*-values (Table 4) indicated that thermo-ultrasonication 72°C was found to be only slightly more effective than a 72°C heat only treatment. Thermo-ultrasonication at 72°C was not found to improve the efficiency of a heat treatment at 72°C, however, at lower temperatures such as 63°C the efficiency of the process, in terms of microbial elimination, was improved by including ultrasonication as part of the treatment. It seems plausible to speculate that milk may be processed at lower temperatures if used in combination with ultrasound.

Micrococcus luteus

Micrococcus spp. have been isolated from both raw (Sablé *et al*., 1997) and pasteurised milk (Aaku *et al*., 2004). This thermoduric psychrotroph can grow at refrigeration temperatures and produce enzymes, toxins and other metabolites and is considered a fresh milk spoilage microbe (Aaku *et al*., 2004).

The actual cell counts (cfu.ml^{-1}) for ultrasonication at four different operating temperatures (no heat, 52°, 63° and 72°C) and heat treatments at two different temperatures (63° and 72°C) are given in Figs. 9 and 10, respectively. The recalculated data are given in the form of log graphs in Fig. 11 and the results are summarised in Table 5.

A 95.23% elimination in viable cells (a 1.32 log reduction) was achieved when *M. luteus,* at an initial concentration of 1 x 10 ^{6} cfu.ml^{-1}, was ultrasonicated for 10 min without the addition of heat (Figs. 11A-C). When *M. luteus* was thermo-ultrasonicated at 52°C for 10 min, 85.47% of the viable cells were eliminated (a 0.84 log reduction) (Fig. 11A). A single heat treatment at 52°C was not included as this temperature is not used for pasteurisation, and was considered too low to have any impact on cell survival.

A 10 min heat treatment at 63°C resulted in a 0.02 log reduction (2.84% elimination), whilst thermo-ultrasonication at 63°C resulted in a 97.34% elimination of viable cells (a 1.59 log reduction) after a 10 min treatment (Fig. 11B).

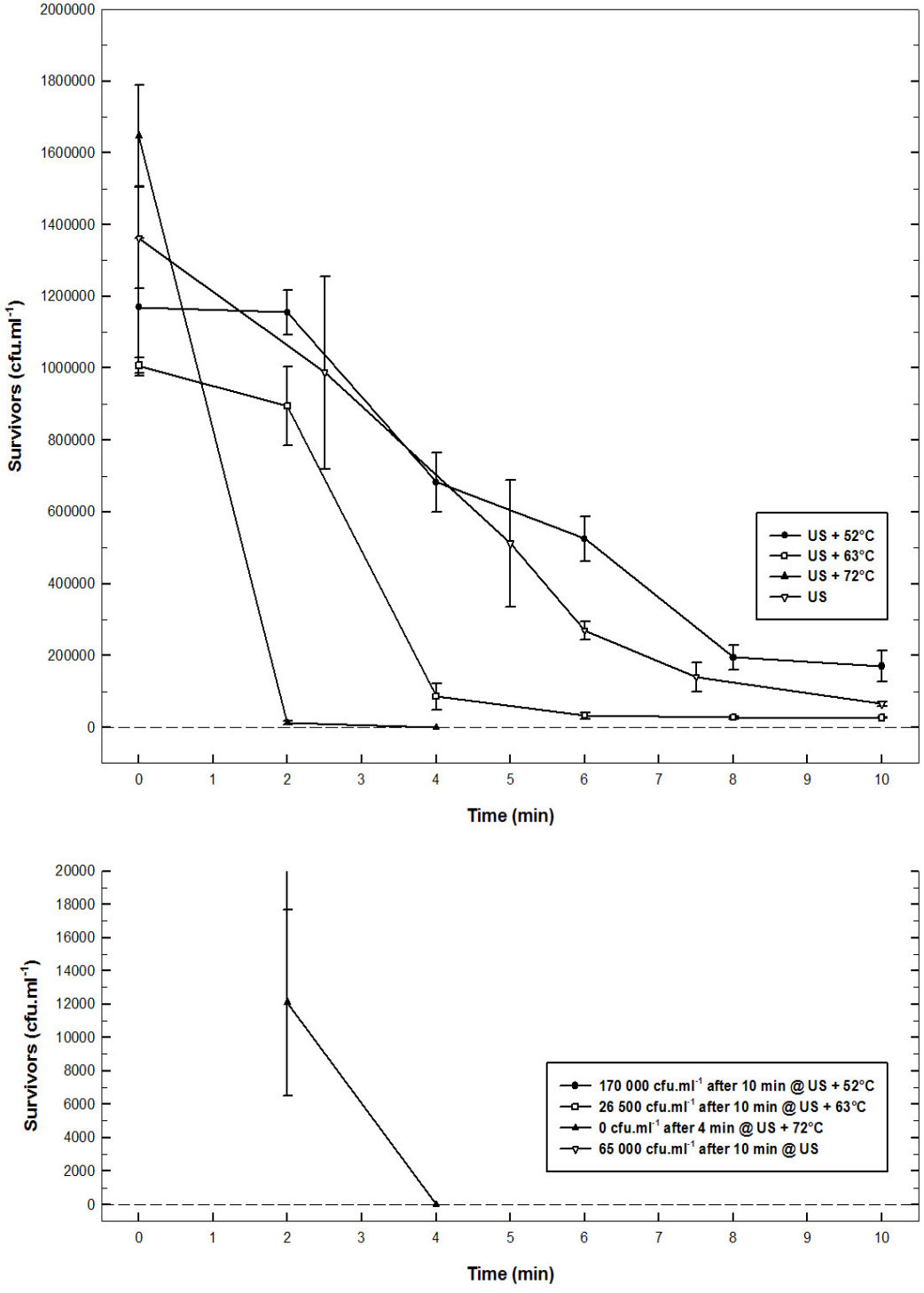

Figure 9 Impact of thermo-ultrasonication on *Micrococcus luteus* in milk. The lower graph represents an expanded Y-axis (US = ultrasound. Each data point represents quadruple values. The standard deviation was used as the error-bar).

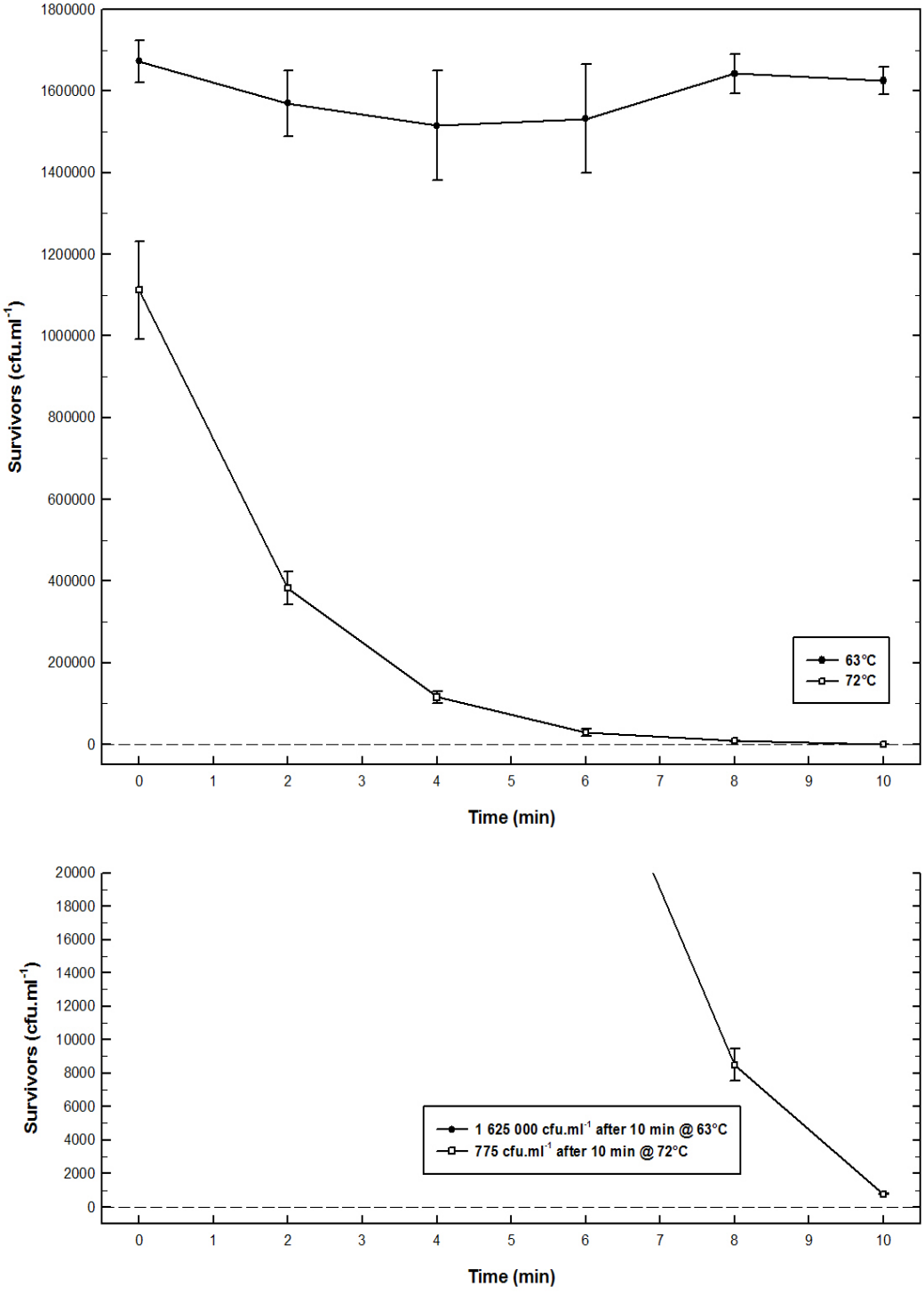

Figure 10 Impact of heat on *Micrococcus luteus* in milk. The lower graph represents an expanded Y-axis (Each data point represents quadruple values. The standard deviation was used as the error-bar).

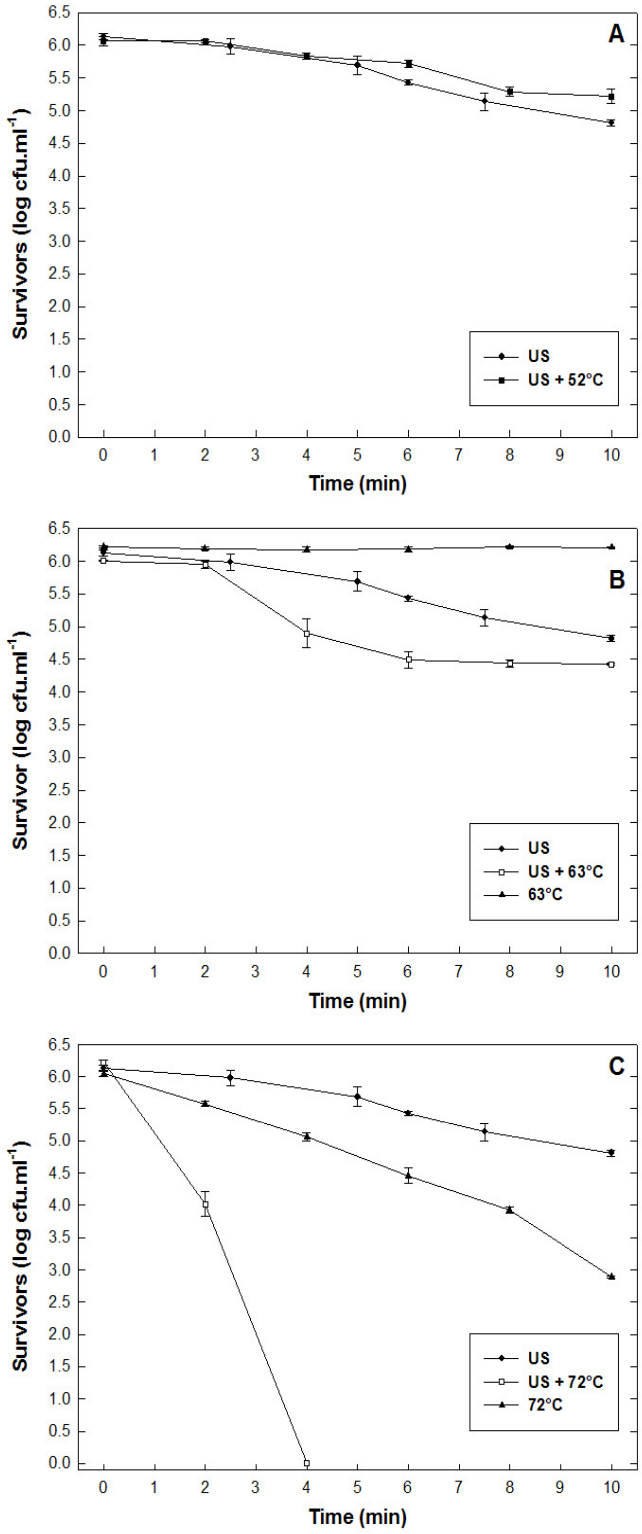

Figure 11 Impact of ultrasound, heat or thermo-ultrasonication at 52°C (A), 63°C (B) and 72°C (C) on *Micrococcus luteus* in milk (US = ultrasound. Each data point represents quadruple values. The standard deviation was used as the error-bar).

Table 5 Summary of the *D*-values, log reductions and % reductions of *Micrococcus luteus* in milk obtained over 10 min ultrasound and/or heat treatments

Treatment	**Time (min)**	***D*-value (min)**	**log reduction**	**% reduction**
US	10	5.85	1.32	95.23
US + 52℃	10	nc	0.84	85.47
63℃	10	nc	0.02	2.84
US + 63℃	10	2.93	1.59	97.34
72℃	10	3.14	3.16	99.93
US + 72℃	4	0.76	6.22	100

US = ultrasonication.
nc = not calcutated (*D*-value could not be calculated as a single log reduction was not reached).

When cells were heat treated at 72°C, a 99.93% elimination was achieved after a 10 min treatment (a 3.16 log reduction) (Fig. 11C). Thermo-ultrasonication at 72°C was found to be very effective against *M. luteus* with a 100% elimination of viable cells (a 6.22 log reduction) achieved within 4 min of treatment (Fig. 11C).

The *D*-values calculated for *M. luteus* under the different treatment conditions used in this study were: D_{US} = 5.85 min; $D_{US + 52°C}$ = could not be calculated as a single log reduction was not reached within 10 min of treatment; $D_{63°C}$ could not be calculated as a single log reduction was not reached within 10 min of treatment; $D_{US + 63°C}$ = 2.93 min; $D_{72°C}$ = 3.14 min and; $D_{US + 72°C}$ = 0.76 min (Table 5).

The legally required temperature time combination for HTST pasteurisation (72°C for 15 s) (Anon., 1997) was found, in this study, to be ineffective at achieving total elimination of *M. luteus* cells. Not all viable *M. luteus* cells were eliminated even when the heat treatment time was extended to 10 min. In contrast, when combining ultrasound and heat at 72°C, the required treatment time for total elimination of all viable *M. luteus* cells was reduced to less than 4 min. Reduced processing times would result in an increase in the volume of milk that can be treated in a given time. Processing for a shorter time and increasing the production volume per day holds obvious economic rewards for the dairy industry, in terms of less processing expenditure and a higher sales volume.

Conclusions

In South Africa, the legal definition of pasteurisation (HTST and LTLT) requires a 75% reduction in the standard plate count of the permitted 200 000 cfu.ml^{-1} in raw milk (Anon., 1997). In this study, HTST pasteurisation was found to be ineffective in reducing *B. cereus* counts by at least 75%. Thermo-ultrasonication at 72°C, however, satisfied pasteurisation requirements according to the SA “milk law” (Anon., 1997) for all the microbes evaluated in this study.

The results from this study indicate that thermo-ultrasonication can be employed successfully as an alternative to conventional pasteurisation. Alkaline phosphatase would be inactivated by thermo-ultrasonication at 72°C, resulting in a final product that complies with the current SA "milk law" in terms of the phosphatase test requirements. Thermo-ultrasonication, as a method of eliminating microbes from milk also has other advantages including reduced treatment times, reduced energy costs and increased production volumes, which will in turn be associated with economic rewards.

Furthermore, the complete elimination of three of the "test" microbes, and therefore sterilisation, suggests that thermo-ultrasonication may have some potential as a possible treatment option for future production of extended shelf-life milk.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

Background

Consumers are increasingly demanding minimally processed foods that are fresher, more natural (Mertens & Knorr, 1992) and with a better nutritional content and higher overall quality. There is also a growing demand for alternatives to replace thermal pasteurisation of milk and this has led to investigations on the use of ultrasonication to eliminate microbes. Ultrasonication is a non-thermal, physical method that works by directly eliminating microbes. In addition, ultrasonication has been shown to be less energy-intensive and therefore more cost-effective and environmentally friendly than conventional pasteurisation (Piyasena *et al*., 2003). Ultrasonication has one major advantage in that it does not result in adverse side-effects (such as nutrient and flavour loss) on milk that are associated with heat treatments.

The main objective of this study was to evaluate the impact of ultrasound (20 kHz, 750 W) on a selection of microbes commonly associated with milk and on the native milk components.

Impact of ultrasound on dairy microbes

There are only a few older reports available on the effect of ultrasonication as a treatment option to eliminate or reduce the microbial load of raw milk (Jacobs & Thornley, 1954; Utsunomiya & Kosaka, 1979). Recent advances in ultrasound technology thus necessitate a re-investigation into the potential of this promising non-thermal pasteurisation alternative. The first aim of this dissertation was to evaluate the impact of ultrasound at non-lethal temperatures (24° - 26°C) on nine spoilage and potentially pathogenic microbes generally associated with milk.

The data obtained showed that ultrasonication can successfully be employed for reducing viable cell numbers of a range of microbes from milk including, Gram-positive's and negative's, rods and cocci, an endospore-former and a yeast. Viable cells of all the "test" microbes were reduced by 78% or more (100% elimination for *Escherichia coli* and *Pseudomonas fluorescens*) after a 10 min treatment. The D_{US}-values obtained for *E. coli* and *P. fluorescens* were 2.0 min and 1.2 min, respectively.

The SA "milk law" (Anon., 1997) allows a maximum contamination load of 200 000 cfu.ml^{-1} for raw milk, and requires pasteurisation to reduce viable counts to less than 50 000 cfu.ml^{-1} (a 75% reduction in viable cell counts). The *Grade "A" Pasteurized Milk Ordinance* of America stipulates that commingled raw milk may contain 300 000 cfu.ml^{-1} (100 000 cfu.ml^{-1} for individual suppliers) and pasteurisation must reduce counts to less than 20 000 cfu.ml^{-1} (a 93% reduction in viable counts for milk from multiple suppliers) (Anon., 2003). In addition, British law requires raw milk intended for heat treatment to contain less than 100 000 cfu.ml^{-1}. Pasteurised milk must contain less than 50 000 cfu.ml^{-1} (Anon., 1995). With the above mandatory requirements for milk, ultrasound, as used in this study, was found to effectively eliminate all the "test" microbes from milk to levels acceptable by both the SA and British milk legislation although initial inoculum loads of at least five times higher than that permitted by both countries' laws were used as initial concentration. Furthermore, five of the nine "test" microbes (*E. coli*, *P. fluorescens*, *Saccharomyces cerevisiae*, *Listeria monocytogenes* and *Lactococcus lactis*) were reduced after ultrasonication to less than the 20 000 cfu.ml^{-1} maximum level stipulated by USA regulations. It is however important to note that in this study, inocula exceeding 1 000 000 cfu.ml^{-1} were used as the initial microbial load and thus it is evident that ultrasonication can effectively eliminate microbes from milk to give a final product that complies with the regulations of different countries.

From the data obtained in this study it is recommended that ultrasound is rather applied as a continuous, as opposed to a pulsed-ultrasonic treatment. The continuous ultrasonic treatment of microbes in milk was shown to enhance the efficacy of ultrasound. One further aspect is that it is advisable to treat raw milk as soon as possible as ultrasound was found to be more effective in eliminating cells when low initial levels were treated. This, of course, would reduce the required treatment time. The maximum wave amplitude possible was found to render ultrasonication more efficient in terms of the number of viable cells eliminated. From the basic data it was clear that sensitivity to ultrasonication did not vary between three *E. coli* strains that were tested, suggesting that different strains of the same species would probably respond similarly to the "killing effect" of ultrasound.

This study also revealed that ultrasound is generally ineffective against *Bacillus cereus* endospores. This poses a problem for the dairy industry as it is vital to remove *B. cereus* endospores from raw milk, especially for milk to be used in the manufacturing

of cheese where the presence of *B. cereus* endospores are known to cause blowing defects.

It was also found in this study that the microbial elimination achieved after ultrasonication is constant for different batches (1 x 10^4 cfu.ml^{-1} *E. coli* reduced to 0 (n = 29)) and that ultrasonication can be used to effectively eliminate faecal microbes from milk, thereby leading to an extended shelf-life of fresh milk.

Although ultrasonication was shown to be effective and implementable, it is recommended that the required batch-scale treatment time as used in this study be shortened before any constructive implementation suggestions of this technology is made to the dairy industry. The rather extended treatment times (10 min) used in this study are impractical as it would reduce the volumes of milk that can be treated per day and also increase the cost of treatment for each "batch". A decrease in production would require larger milk storage facilities and would ultimately result in huge economic losses for any processing plant.

Damage mechanisms

Results showing that ultrasonication can effectively eliminate most microbial contaminants from milk are not sufficient to conclude that ultrasonication is a suitable alternative for the treatment of milk. Visual data of the physical damage of the microbial cells as caused by ultrasonication could also probably be used to give an indication as to the possibility of microbial cell repairs. Scanning (SEM) and transmission electron microscopy (TEM) were used to gain visual confirmation of the physical damage that occurs in microbial cells after an ultrasonic treatment. Scanning electron microscopy micrographs indicated extensive surface damage to viable *E. coli* cells. Cell damage to *E. coli*, *Lactobacillus acidophilus* and *S. cerevisiae*, both internally and externally, was additionally visualised with TEM micrographs. Ultrasonication was shown to damage both the cell wall and cell membrane of the bacteria and the cell wall of the yeast investigated, thereby explaining the destruction of the viable cells. Cell lysis, and therefore, cell death as caused by the ultrasonication makes this technology a lucrative alternative to conventional pasteurisation and could serve as proof to the dairy industry that this technology can successfully be used to eliminate microbial contaminants from milk.

The release of microbial proteins and DNA into the environment was also measured. The original hypothesis was to use the release of internal microbial components (indicative of cell lysis) as a simpler method to correlate the presence of

intracellular microbial components to decreases in viable cell numbers. Even though it was proved that microbial proteins and DNA were released after ultrasonication, it was, however, not possible to correlate the concentration of released proteins and DNA to the number of viable cells being eliminated and it was concluded that released proteins or DNA concentrations in solution would not be an effective measurement of "microbial quality" for the dairy industry.

The data from this section of the study did not "appear" to suggest that the ultrasonic treatment have any detrimental impact on the chemical composition of the released intracellular proteins and DNA. However, from a food processing standpoint and before this method can be recommended for the treatment of milk, especially milk intended for further processing (cheese, yogurt, etc.) it is important to know what, if any, impact ultrasound would have on native milk components. A significant decrease in e.g. the protein or fat content of milk would result in yield losses during follow-up cheese or yogurt manufacturing. Thus, any decrease in milk components would render this method a non-viable option to heat treatments.

Impact of ultrasound on milk components

In this part of the study the impact of ultrasonication on the main milk components of both raw and pasteurised milk was assessed. The results confirmed that no statistically significant changes could be detected in either the total protein or the casein contents of milk. Similarly, there were no statistically significant changes in the lactose content of either raw or pasteurised milk after ultrasonication. An increase observed in the fat content of raw milk after ultrasonication was explained to be as a result of the breaking of the milk fat globule membranes by the ultrasound. It is possible that ultrasound acts as a homogeniser. In contrast, ultrasonication was found to have little or no impact on the milk fat of pasteurised (and homogenised) milk. Similarly, ultrasonication was found to statistically significantly reduce the somatic cell count (SCC) of both raw and pasteurised milk. A decrease in the SCC after ultrasonication will of course not improve the quality of milk, associated with high SCC levels. It is thus important for the dairy industry to always source the best possible quality raw milk as no processing technology can compensate for raw milk with a sub-standard quality.

The fact that ultrasonication did not lead to decreases in the native milk components indicates that this method could be employed as a suitable alternative for pasteurisation of milk intended for e.g. cheese manufacturing. As no decreases in milk

components was found, ultrasonication should not have any negative influence on the yield of dairy products that utilise milk protein and milk fat.

In terms of food safety control, an equivalent to the phosphatase test that is used to determine the effectiveness of pasteurisation, must be identified. This "control parameter" must be able to indicate effective ultrasonication in terms of the elimination of microbes. In this study alkaline phosphatase and lactoperoxidase enzymes were found not to be inactivated by the ultrasonic treatment of raw milk. For the implementation of ultrasonication as a single treatment method for milk, it is essential to have a fast and reliable "test" to indicate a microbiologically safe product. The answer might not be enzymatic of nature, however, it should still be simple, accurate and require the minimum in specialised equipment for it to be successful in routine dairy laboratories.

Hurdle technology

Ultrasonication, as a single treatment, was not always found to eliminate 100% of the viable cells of the different microbial strains used throughout this dissertation. In addition the treatment times were found to be too long, and therefore, less implementable. It was thus deemed necessary to combine ultrasound with another existing method in order to provide the dairy industry with a practical and effective alternative to traditional heat pasteurisation. Most milk processing plants have a heat treating system in place, and the addition of an ultrasonic system could lead to an enhancement of the overall quality of heat treated milk. This would require a minimum in capital expenditure as well as "new" running costs with huge advantages in terms of a better and safer end-product. As heat is frequently utilised by the dairy industry, this was the method of choice to complement ultrasonication.

A heat treatment is generally given to milk to extend the shelf-life. A combination of ultrasound and heat should result in a final product with an even longer shelf-life and the combination might also reduce the required treatment time leading to operational savings. In an effort to reduce the treatment time, the impact of a combined thermo-ultrasonication scenario was investigated. The impact of three different treatment methods (ultrasonication, heat and thermo-ultrasonication) on four "test" microbes in milk was thus evaluated. Of the four "test" microbes only the D_{US}-value of *Lb. acidophilus* was reduced by combining ultrasound with heat at 52℃. A temperature of 52℃, in combination with ultrasound is regarded to be too low to enhance the

efficacy of ultrasound, and it was decided to evaluate the two pasteurisation temperature options (63°C - LTLT and 72°C - HTST) in combination with ultrasound.

Although thermo-ultrasonication at 63°C was found not to eliminate all viable cells for all four "test" microbes, the data obtained showed that, with the exception of *B. cereus*, a combination of ultrasound and heat at 63°C could produce a final product that complied to the SA "milk law" (Anon., 1997). According to the SA "milk law" only raw milk with a standard plate count of less than 200 000 cfu.ml^{-1} is acceptable for pasteurisation, and only pasteurised milk with a standard plate count of less than 50 000 cfu.ml^{-1} may be sold. The results from this study clearly showed that it would be possible to process milk for a considerably shorter time by adding ultrasound to an existing treatment process operating at 63°C. Shortening the treatment time would be economically profitable as it would be possible to process larger volumes of milk and thus would reduce treatment costs.

When the third treatment combination, thermo-ultrasonication at 72°C, was applied it was found to be the most effective thermo-ultrasonic combination against all the "test" microbes used in this section of the study. The treatment time for *E. coli* was reduced from 10 min (ultrasonication) (1 x 10^6 cfu.ml^{-1} inoculum, Chapter 3) to 4 min (thermo-ultrasonication at 72°C). Similar results were achieved for *Lb. acidophilus* and *Micrococcus luteus* where total elimination was achieved by a 2 and 4 min thermo-ultrasonic (72°C) treatment, respectively. In Chapter 4 of this dissertation ultrasound was shown to be incapable of eliminating all viable cells of these two microbes after a 10 min ultrasonic treatment. Thus it was concluded that thermo-ultrasonication (72°C) is more efficient at eliminating these microbes than ultrasonication at non-lethal temperatures. High temperature short time (HTST) pasteurisation requirements (72°C for 15 s) (Anon., 1997) were found to be ineffective in ensuring a final product that complied to the legal requirements for milk that was inoculated with *B. cereus.* When the treatment time was extended to 10 min using HTST pasteurisation temperatures only 15% of viable *B. cereus* cells/endospores were eliminated. Thermo-ultrasonication (72°C), however, reduced viable *B. cereus* counts by 78%. Thus, a combination of ultrasound and heat at 72°C was found to more effectively eliminate microbes, especially the more heat resistant *M. luteus* and *B. cereus*.

The successes achieved when using the thermo-ultrasonication at 72°C combination confirms the potential of this hurdle technology. By implementing this hurdle combination it would be possible to process milk at the same temperature as normal HTST pasteurisation, but with the exception that the milk would be free of all

vegetative cells. Such milk will have the advantage of having a longer shelf-life, whilst still being a minimally processed product. Ultra-high temperature (UHT) processing is generally used to eliminate all viable cells from milk, however, UHT milk is known to have a "cooked" flavour. Thermo-ultrasonication would thus eliminate the disadvantages of UHT processing (flavour and cost) and produce a product similar in terms of microbial specifications.

Concluding remarks

The limited successes achieved in this study with ultrasonication led to the evaluation of a thermo-ultrasonication option. The overwhelmingly positive results of this hurdle combination support the recommendation of this hurdle technology for implementation by the dairy industry. With minor modifications, an existing HTST pasteurisation plant would be able to produce a final product comparable to UHT milk.

In this study, microbial endospores were shown to be a treatment problem for batch ultrasonication and HTST pasteurisation as well as for the thermo-ultrasonication combinations. In future studies it might be of value to investigate the physiology of endospore germination as impacted by ultrasound to better understand the mechanism. From such information it may be possible to develop other treatment scenario's as to the simultaneous application of heat and ultrasound, or if milk should rather receive the two treatments successively.

A lack of suitable equipment and scale-up strategies has prevented the application of thermo-ultrasound for industrial processing. But even with the recent advances in ultrasound technology, future research is still required to ensure the industrial implementation of thermo-ultrasonication. One aspect that needs further research is that the system design must allow for maximum contact between the milk and the "cavitation" zone. It may also be feasible to investigate the use of multiple ultrasonic probes with different driving frequencies. By processing milk with a range of ultrasonic frequencies within the "power ultrasound" range of 20 - 100 kHz, it may be found to increase the scope of microbes "sensitive" to ultrasonication. In addition, a closed, continuous flow design, as opposed to the open-system used throughout this study, would be more ideal for the dairy industry, especially where re-contamination is a high risk.

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