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Ultrasound Inactivation of Bacteria and Yeast in Aqueous Solutions and Skim Milks

Shengpu Gao

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Food Science, The University of Auckland, 2014.
“Three passions, simple but overwhelmingly strong, have governed my life: the longing for love, the search for knowledge, and unbearable pity for the suffering of mankind.”

Bertrand Russell
ABSTRACT

Ultrasonic treatment is considered a potential method to inactivate microorganisms in food. The aims of this study were to determine the effects of both low-frequency (20 kHz) and high-frequency (850 kHz) ultrasound treatment on bacteria and yeast, and to develop a fundamental understanding of the mechanisms involving ultrasonic cavitation. *Enterobacter aerogenes*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *S. epidermidis* SK, *S. pseudintermedius* and a yeast, *Aureobasidium pullulans*, were sonicated at 20 kHz (0.04–0.85 W/ml, 5–60 min) or 850 kHz (0.04–0.25 W/ml, 2.5–60 min) by batch sonication equipment under controlled temperatures (< 30°C). The bacteria and yeast were suspended in aqueous solutions and skim milks.

The relationship between the effects of inactivation and physico-chemical properties of bacteria at different growth phases was investigated by low-frequency ultrasound. Lethal damage of *E. aerogenes* and *B. subtilis* was caused by ultrasonication, while *Staphylococcus* spp. were not affected markedly. This was mainly due to the protection of the capsule layer (extracellular polysaccharides) outside this gram-positive cell against mechanical damage induced by ultrasound cavitation. A theoretical model based on shear forces produced by cavitation was established. This model was validated on further low-frequency sonication experiments performed on *E. aerogenes* and *A. pullulans*.

*E. aerogenes*, *B. subtilis* and *S. epidermidis* were also treated by high-frequency ultrasound. It was found that all the bacteria were inactivated noticeably due to the generation of hydroxyl radicals and hydrogen peroxide during sonication. A post-ultrasonication effect was identified where bacteria were inactivated even after sonication was completed.

*E. aerogenes* was also sonicated in skim milk with varying protein concentrations, and the effects of ultrasonication on the physic-chemical properties of milk were
considered. The bacteria in milk were markedly inactivated in the low-frequency system, while they were not affected by the high-frequency one. It was also found that ultrasonication, under the conditions used in this thesis, did not notably influence the milk proteins or the structure and size of the casein micelles. However, it partially denatured whey proteins and reduced the size of the residual fat globules. Further, the proteins at the surface of the casein micelles might be cleaved.
ACKNOWLEDGEMENTS

First and foremost, I would like to give my sincere gratitude and express my deep appreciation to my supervisor, Professor Yacine Hemar. I thank him for giving me the opportunity to work on this challenging and exciting project, providing me systematic guidance, and training me in this scientific field. Indeed, without his dedication to the equipment setting, manuscripts preparation, paper publications and proofreading, the thesis would not have been possible.

I would like to gratefully acknowledge my co-supervisor Professor Gillian Lewis for all the valuable technical advice and encouraging me to keep moving on the road of science. Without her immense knowledge of microbiology and warm guidance, the project simply would not have run so smoothly and successfully.

I wish to thank Professor Muthupandian Ashokkumar from University of Melbourne for giving me some guidance on high-frequency ultrasound and providing help for manuscripts preparation. I give my warm thanks to Ms. Yimin Dong for training me in the techniques of microbiology and for her great assistance and advice throughout my studies. I also respectfully acknowledge Professor Conrad Perera, Dr. Don Otter and Ms. Sreeni Pathirana for their great support for my studies. I would like to thank many teachers and friends who shared their experiences, especially Dr. Adrian Turner for the help with TEM techniques. Special thanks to Mr. Brian Lythe from the International Office and Mr. Colin Ting from the Scholarships Office. Particular thanks to my good friend Mahmoud Khalifa for being my after-hours lab partner and supporting my study practically and mentally. Great thanks to my good friend Shu Ki (Elisa) Lam who consistently helps and inspires me on the academic road.

I am also grateful to my colleagues and friends, Lu (Ray) Ren, Sara Paturel, Zhi Yang, Wenzhe Bai, Vidya Washington, Zuliiha Yahya, Marlene Pillay, Anne-Marie Perchec, Kelvin Lau, Elizabeth Woo, Anna Lau, Zhao Li, Weibin Qin, Rong Chen, Nor Fazliyana Mohtar, Norliza Binti Julmohammad, Yun Ping Neo, Trang Duong, Meifang Zhou, Kelly Roberts, Mahjoub Ejmal, Rachel Lamb, Lindy Scicot, Yongli
Xie, Wenying Zhao, Hong Liu, Lance Xu, Daying Wen, Shaoping Zhang, Linda Zhang, Martin Middleditch, Ruth Payne, Andrew Dopheide, Jeffery Tang, Augusto Barbosa, Jully Pinheiro, Niha Phukan, Ann-Katrein Baer, Weam Banjar, Tina Tian, Anis Arzami, Yantao Song, John Spires, Terry Gruijters, Sujeewa Hettinewa and other friends for their friendship, motivation and support during my study.

Special thanks to my colleagues Xuewan Xu, Encheng Chen and Shiwen Wang, who came with me in the same flight to Auckland, like comrades in arms, helping and encouraging each other to keep energetic in here. Great thanks to my cousin Huizi Li and Tian Lan for their selfless help in my study and looking after my life in Auckland. Lovely thanks to my host mother Helen Jermyn for the yummy western food. Sincere thanks to Professor Lin Song and Professor Lianqi Du for offering me endless love and supports for me and my family throughout my studies.

I would like to thank my leaders and colleagues from the General Administration of Quality Supervision, Inspection and Quarantine (AQSIQ) and the China National Institute of Standardization, for supporting me throughout my studies. Particular thanks to Mr. Qinghai Wu, Professor Yueming Qiu, Professor Wen Liu, Mr. Huan Li, Associate Professor Jianjun Xu, and Associate Professor Zhenyu Yun. I would like to thank the New Zealand Ministry of Foreign Affairs and Trade for providing me a New Zealand China Food Safety Scholarship under the New Zealand China Free Trade Agreement. Special thanks to Ms. Gabrielle Isaak, Ms. Ursula Egan, Ms. Bridget Nankivell and Ms. Sally Page.

I owe a special note of gratitude to my family. My parents and my wife have given me deep love, encouragement during my studies and helped me through many difficult times. For the past three years, my wife has cared for our son since he was born and taken care of the whole family. My mother is a great woman who raised me and is still looking after my son. My parents-in-law, my sisters and my brother-in-law who always offer their unconditional love to support the family and me. My sincere thanks to you all! Also, I would like to thank my son, Tiantian, for giving me motivation to return home as soon as possible. My sincere thanks to you all!
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# TABLE OF CONTENTS

**ABSTRACT** ................................................................................................................................. III

**ACKNOWLEDGEMENTS** ............................................................................................................. V

**CO-AUTHORSHIP FORMS** ........................................................................................................ VII

**TABLE OF CONTENTS** ................................................................................................................ XI

**LIST OF FIGURES** ..................................................................................................................... XVI

**LIST OF TABLES** ........................................................................................................................ XX

**ABBREVIATIONS** ...................................................................................................................... XXI

**CHAPTER 1 INTRODUCTION** .............................................................................................. 1

1.1 Background ...................................................................................................................... 2

1.2 Research objectives ......................................................................................................... 4

1.3 Targeted bacteria and yeast ............................................................................................ 4

1.3.1 *Enterobacter aerogenes* ......................................................................................... 5

1.3.2 *Bacillus subtilis* ........................................................................................................ 7

1.3.3 *Staphylococcus epidermidis* ................................................................................... 7

1.3.4 *Aureobasidium pullulans* ........................................................................................ 7

1.4 Thesis structure ............................................................................................................... 8

**CHAPTER 2 LITERATURE REVIEW** ....................................................................................... 9

2.1 Introduction ................................................................................................................... 10

2.2 Introduction to microorganisms .................................................................................... 10

2.2.1 Bacteria and other microorganisms in food ............................................................. 10

2.2.2 Microbial growth ..................................................................................................... 11

2.2.3 Bacterial cell wall and surface properties ................................................................. 12

2.2.3.1 Cell wall type ....................................................................................................... 12

2.2.3.2 Hydrophobicity and hydrophilicity ...................................................................... 14

2.2.3.3 Cell surface charge ............................................................................................. 15

2.2.3.4 Capsules and slime (Extracellular polysaccharides) ........................................... 15

2.2.3.5 Cell appendages ............................................................................................... 16

2.3 Ultrasound ..................................................................................................................... 16

2.3.1 Ultrasound waves ..................................................................................................... 16

2.3.2 Ultrasonic cavitation ............................................................................................... 18

2.3.3 Ultrasonic equipment ............................................................................................. 22

2.3.3.1 Laboratory equipment ......................................................................................... 22

2.3.3.2 Industrial equipment .......................................................................................... 25
2.3.4 Factors affecting cavitation threshold ................................................................. 26
  2.3.4.1 Intensity and amplitude ................................................................................. 26
  2.3.4.2 Frequency ...................................................................................................... 27
  2.3.4.3 Temperature .................................................................................................. 28
  2.3.4.4 External pressure .......................................................................................... 29
2.4 Ultrasound inactivation of bacteria and other microorganisms ......................... 29
  2.4.1 Effects of cavitation threshold ......................................................................... 30
    2.4.1.1 Effects of intensity and amplitude ............................................................... 30
    2.4.1.2 Effects of frequency ................................................................................... 31
    2.4.1.3 Effects of temperature .............................................................................. 32
    2.4.1.4 Effects of external pressure ....................................................................... 33
  2.4.2 Media ............................................................................................................... 34
    2.4.2.1 Viscosity and media .................................................................................. 34
    2.4.2.2 Volume ....................................................................................................... 35
    2.4.2.3 pH ............................................................................................................. 35
    2.4.2.4 Initial microbial number ............................................................................ 36
  2.4.3 Properties of microorganisms .......................................................................... 37
    2.4.3.1 The type of cell wall .................................................................................. 37
    2.4.3.2 Size and shape .......................................................................................... 38
    2.4.3.3 Bacteria with spores ................................................................................ 39
    2.4.3.4 Growth phases ........................................................................................ 39
    2.4.3.5 Viruses ..................................................................................................... 40
  2.4.4 Ultrasound as a hurdle technology ................................................................... 40
2.5 Effects of ultrasound treatment on food ............................................................... 43
  2.5.1 Ultrasound processing of food systems ........................................................... 43
  2.5.2 Inactivation of microorganisms in food systems ............................................. 44
2.6 Ultrasonic inactivation in milk and effects on milk properties .............................. 49
  2.6.1 Milk ............................................................................................................... 49
    2.6.1.1 Milk composition ...................................................................................... 49
    2.6.1.2 Proteins .................................................................................................... 50
  2.6.2 Microbial inactivation in milk ......................................................................... 54
  2.6.3 Effects of ultrasound on the physical and chemical properties of milk .......... 55
2.7 Concluding remarks ............................................................................................. 56
5.2 Introduction ................................................................................................................. 103
5.3 Materials and methods ............................................................................................... 106
  5.3.1 Materials ............................................................................................................... 106
  5.3.2 Preparation of microbial suspensions .................................................................. 106
  5.3.3 Ultrasound treatment........................................................................................... 107
  5.3.4 Microbial enumeration ......................................................................................... 107
  5.3.5 Hydrogen peroxide measurement ....................................................................... 108
  5.3.6 TEM ....................................................................................................................... 109
5.4 Results and discussion ................................................................................................. 109
  5.4.1 Generation of OH• and H• radicals and H$_2$O$_2$ ...................................................... 109
  5.4.2 Effect of sampling time on bacteria counts.......................................................... 111
  5.4.3 Ultrasound treatment of bacteria ........................................................................ 113
  5.4.4 Post ultrasonication effects .................................................................................. 118
  5.4.5 Ultrasound treatment of yeast ............................................................................. 122
5.5 Conclusions .................................................................................................................. 125
5.6 Acknowledgements ..................................................................................................... 126

CHAPTER 6 INACTIVATION OF ENTEROBACTER AEROGENES IN RECONSTITUTED SKIM MILK
BY HIGH- AND LOW-FREQUENCY ULTRASOUND ................................................................. 127
6.1 Abstract ....................................................................................................................... 128
6.2 Introduction ................................................................................................................. 129
6.3 Material and methods ................................................................................................. 131
  6.3.1 Material, bacterial and milk samples preparation ............................................... 131
  6.3.2 Ultrasonication ..................................................................................................... 132
  6.3.3 RP-HPLC analysis ................................................................................................ 133
  6.3.4 Viscosity measurement ........................................................................................ 135
  6.3.5 TEM and Cryo-TEM observation ........................................................................ 135
  6.3.6 Particle size measurement ................................................................................... 136
6.4 Results and Discussion ................................................................................................. 136
  6.4.1 Inactivation of bacteria in water and skim milk ................................................... 136
  6.4.2 Effects of ultrasonication on viscosity and particle size of skim milk................... 141
  6.4.3 Effect of ultrasound treatment on milk proteins ................................................ 144
6.5 Conclusions .................................................................................................................. 147
6.6 Acknowledgements ..................................................................................................... 148

CHAPTER 7 GENERAL DISCUSSION, OVERALL CONCLUSIONS AND FUTURE WORK .... 149
7. 1 General discussion ...................................................................................................... 150
LIST OF FIGURES

Figure 1.1 Light micrographs of targeted bacteria (Gram staining) and yeast (wet mount). A: Enterobacter aerogenes (stationary phase); Inset a: Enterobacter aerogenes (exponential phase); B: Bacillus subtilis; C: Staphylococcus epidermidis; D: Aureobasidium pullulans. Scale bar is 20 µm. ................................................................. 6

Figure 1.2 Transmission electron micrographs of targeted bacteria. A: Enterobacter aerogenes; B: Bacillus subtilis; C: Staphylococcus epidermidis. Scale bar is 1 µm. .................. 6

Figure 2.1 Microbial growth curve in a closed system. ................................................................. 11

Figure 2.2 The main properties of bacterial cell wall. ................................................................. 12

Figure 2.3 Gram-positive and gram-negative cell walls. A: a gram-positive envelope from Staphylococcus epidermidis; B: a gram-negative envelope of Enterobacter aerogenes. ...... 13

Figure 2.4 Frequency range of sound (Redrawn from Mason et al., 2005) . .......................... 17

Figure 2.5 Schematic of ultrasonic cavitation (Redrawn from Soria and Villamiel, 2010). .... 18

Figure 2.6 Shear forces and micro-jets induced by ultrasonic cavitation. ...................... 20

Figure 2.7 Illustration of the low-frequency equipment used in this thesis. It consisted of (1) a power supply and pulse control panel, (2) a transducer, (3) an upper fixed horn, (4) a detachable horn, (5) samples surrounded by an ice bath (6). Noise during ultrasound treatment was partly attenuated by a sound abatement chamber (7). ................................. 24

Figure 2.8 Illustration of the high-frequency unit used in this thesis. (1) An ultrasound generator, (2) a transducer, (3) a double-walled cylindrical glass vessel connected (4) a water bath, and (5) a test-tube containing samples. ......................................................... 25

Figure 2.9 Dual bonding model proposed for the casein micelle structure (Horne, 1998). 53

Figure 3.1 Growth curves of Enterobacter aerogenes (■), Bacillus subtilis (●) and Staphylococcus epidermidis (▲). Error bars correspond to standard deviations. ..................... 67

Figure 3.2 Particle size distribution for Enterobacter aerogenes (■), Bacillus subtilis (●) and Staphylococcus epidermidis (▲). ................................................................................. 68

Figure 3.3 Log of survival ratio (Log (N/N0)) for sonicated (A) Enterobacter aerogenes, (B) Bacillus subtilis and (C) Staphylococcus epidermidis. Symbols are: Bacteria at stationary phase (■); Washed bacteria at stationary phase (●); Bacteria at exponential phase (▲). The bacteria suspensions were sonicated at different powers for 20 min (20 kHz). Error bars correspond to standard deviations. ................................................................. 71
Figure 3.4 Transmission electron micrographs of (A) *Enterobacter aerogenes*, (B) *Bacillus subtilis* and (C) *Staphylococcus epidermidis* before (1) and after (2 and 3) 20 min ultrasound treatments (20 kHz). Ultrasound powers were: A2: 5.0 W; A3: 12.7 W; B2, B3, C2 and C3: 5.0 W.......................................................... 73

Figure 3.5 Transmission electron micrographs of different species/strains of *Staphylococcus*: (A) *Staphylococcus epidermidis*, (B) *Staphylococcus epidermidis* SK, and (C) *Staphylococcus pseudintermedius*. Scale bars correspond to 0.5 µm. .................................................. 75

Figure 3.6 Log of survival ratio (Log \((N/N_0)\)) for different species/strains of *Staphylococcus*: *Staphylococcus epidermidis* (□); *Staphylococcus epidermidis* SK (■); and *Staphylococcus pseudintermedius* (●). The bacteria suspensions were sonicated at different powers for 20 min (20 kHz). Error bars correspond to standard deviations. ................................. 76

Figure 4.1 Schematic representation of (A) shear forces due to collapsing cavity near a solid surface, and (B) the proposed theoretical model.............................................................. 89

Figure 4.2 (A) Transmission electron and light micrographs of *Enterobacter aerogenes* and (B) light micrographs *Aureobasidium pullulans* before (1) and after sonication (2). *E. aerogenes* was sonicated at 12 W for 20 min (20 kHz), and *A. pullulans* was sonicated at 12 W for 20 min (20 kHz), Scale bars correspond to 1 µm for *E. aerogenes* and 20 µm for *A. pullulans*. Inset A3 is the light micrograph of *Enterobacter aerogenes* before sonication, showing the extent of aggregation....................................................................................................... 92

Figure 4.3 *Enterobacter aerogenes* survival ratio Log \((N/N_0)\) as a function of sonication time for (A) different sonication powers and for (B) different initial cell numbers (20 kHz). In (A) the initial cell number is \(~10^8\) CFU/ml and sonication powers are: 4 W (■), 8 W (●), and 12 W (▲). Inset in (A) reports the theoretical number of cavitations \(\mu\) as a function of the sonication powers. In (B) symbols for initial cell numbers are: \(~1.2 \times 10^8\) CFU/ml (■), \(~1.3 \times 10^6\) CFU/ml (●), and \(~6.5 \times 10^4\) CFU/ml (▲). Both in (A) and (B) error bars correspond to standard deviation and solid lines are the result of the fit obtained using the proposed model.................................................................................................................................... 94

Figure 4.4 *Aureobasidium pullulans* survival ratio Log \((N/N_0)\) as a function of sonication time for (A) different sonication powers and for (B) different initial cell numbers (20 kHz). In (A) the initial cell number is \(~10^7\) CFU/ml and sonication powers are: 4 W (■), 8 W (●), 10 W (▲), and 12 W (▼). Inset in (A) reports the theoretical number of cavitations \(\mu\) as a function of the sonication powers. In (B) symbols for initial cell numbers are: \(~6.0 \times 10^6\) CFU/ml (■), and \(~9.8 \times 10^4\) CFU/ml (●). Both in (A) and (B) error bars correspond to standard deviation and solid lines are the result of the fit obtained using the proposed model.................................................................................................................................... 95
Figure 4.5 Viscosity (mPa·s) at 25°C for *Enterobacter aerogenes* (■, □) and *Aureobasidium pullulans* (●, ○) as a function of ultrasound treatment (20 kHz). Error bars correspond to standard deviations. *E. aerogenes*’ initial cell number is ~10^8 CFU/ml (■) or ~10^6 CFU/ml (□). *A. pullulans*’ initial cell number is ~10^7 CFU/ml (●) or ~10^5 CFU/ml (○). Ultrasound power was 8 W and error bars correspond to standard deviations................. 98

Figure 5.1 Hydrogen peroxide (■) produced by high-frequency sonication of Milli-Q water and pH (●) of PSS, as a function of the ultrasound power. Ultrasound frequency was 850 kHz and sonication time was 20 min. Error bars correspond to standard deviations............. 111

Figure 5.2 Log of survival ratio (Log (N/N₀)) of *Enterobacter aerogenes* as a function of storage time (different sampling time after ultrasonication) for bacterial suspensions sonicated under different conditions (850 kHz). Control (without ultrasound treatment) (■); ultrasonicated at 50 W for 20 min (●); ultrasonicated at 62 W for 10 min (▲); and ultrasonicated at 62 W for 20 min (▼). Error bars correspond to standard deviations..... 113

Figure 5.3 Log of survival ratio (Log (N/N₀)) for ultrasonicated bacteria for 20 min as a function of different ultrasound powers (850 kHz). (A) *Enterobacter aerogenes*, (B) *Bacillus subtilis* and (C) *Staphylococcus epidermidis*. Symbols are: bacteria at stationary phase (■); and bacteria at exponential phase (●). Error bars correspond to standard deviations..... 114

Figure 5.4 Transmission electron micrographs of (A) *Enterobacter aerogenes*, (C) *Bacillus subtilis* and (D) *Staphylococcus epidermidis* before (1) and after (2, 3 and 4) ultrasound treatments (850 kHz, 62 W, 20 min). (B) *Enterobacter aerogenes*, before (1) and after (2, 3 and 4) the addition of H₂O₂ (200 mM)................................................................................. 116

Figure 5.5 Log of survival ratio (Log (N/N₀)) of bacteria as a function of the concentration of t-butanol. *Enterobacter aerogenes* at 50 W (□); *Enterobacter aerogenes* at 62 W (■); *Bacillus subtilis* at 50 W (●); *Bacillus subtilis* at 62 W (●); *Staphylococcus epidermidis* at 50 W (△); *Staphylococcus epidermidis* at 62 W (▲). Ultrasound frequency was 850 kHz and error bars correspond to standard deviations. ............................................................ 118

Figure 5.6 Log reduction of *Enterobacter aerogenes* as a function of reaction time after addition of different H₂O₂ amounts: 0.214 mM (■), 5.88 mM (●), 200 mM (▲). Note that ultrasonication at 62 W for 20 min produces 0.214 mM H₂O₂. Error bars correspond to standard deviations. .......................................................... 121

Figure 5.7 Log of survival ratio (Log (N/N₀)) for ultrasonicated yeast and bacteria as a function of ultrasonication time in different initial numbers at 850 kHz. The initial cell numbers of *Aureobasidium pullulans* are ~4.2×10⁷ CFU/ml (■) and ~3.1×10⁵ CFU/ml (●). The initial cell numbers of *Enterobacter aerogenes* are ~1.5×10⁸ CFU/ml (□), ~1.7×10⁶ CFU/ml (○), and ~1.5×10⁴ CFU/ml (△). Error bars correspond to standard deviation...... 124
Figure 5.8 Light micrographs of *Aureobasidium pullulans* (A) before ultrasound treatment and (B) after ultrasound treatment at 850 kHz and 62 W for 60 min. .......................... 125

Figure 6.1 Log of survival ratio \( \log (N/N_0) \) of *Enterobacter aerogenes* in skim milk and water as a function of sonication time. In water, 20 kHz, 8.0 W (■); In 5% skim milk, 20 kHz, 8.2W (●); In 10% skim milk, 20 kHz, 8.5W (▲); In 15% skim milk, 20 kHz, 9.2W (▼); In 5% skim milk, 850 kHz, 50 W (○); In 10% skim milk, 850 kHz, 50 W (△). Error bars correspond to standard deviation. ........................................................................................................................................ 137

Figure 6.2 Transmission electron micrographs of *Enterobacter aerogenes* in water and 5% skim milk before and after ultrasonication. A1: Before ultrasonication in water; A2: Before ultrasonication in milk, the cells remained stable and intact; B1 and B2: After ultrasonication at 20 kHz for 20 min (8.5W), the bacteria had misshapen structures which were highlighted by circles; C1 and C2: After ultrasonication at 850 kHz for 60 min (50 W), the cells remained stable and intact. .......................................................................................................................... 140

Figure 6.3 Viscosity change of milk and bacteria (*Enterobacter aerogenes*) suspensions. Water in 10% milk, 20 kHz, 8.5 W (■); Bacteria suspension in 10% milk, 20 kHz, 8.5 W (●); Water in 10% milk, 850 kHz, 50 W (□); Error bars correspond to standard deviation...... 141

Figure 6.4 The particle size diameter of milk (10% skim milk) and fat globules samples as a function of sonication time: milk, 20 kHz, 8.5 W (■); milk, 850 kHz, 50 W (●); Fat globules, milk: EDTA=1:100 (v/v), 20 kHz, 8.5 W (▲). Error bars correspond to standard deviation.143

Figure 6.5 (A) Chromatograms of caseins from skim milk by reversed-phase high-performance liquid chromatography (RP-HPLC): Control skim milk (solid line); Ultrasonicated skim milk, 30 min, 20 kHz, 8.5 W (Dotted line). (B) The relative reductions of concentration of caseins from RP-HPLC: κ-casein (■) α-casein (●), and β-casein (▲); Error bars correspond to standard deviation. .......................................................................................................................... 145

Figure 6.6 (A) Chromatograms of whey proteins from skim milk by reversed-phase high-performance liquid chromatography (RP-HPLC): Control skim milk (solid line); Ultrasonicated skim milk, 30 min, 20 kHz, 8.5 W (Dotted line). (B) The relative reductions of concentration of whey proteins from RP-HPLC: α-lactalbumin (■) and β-lactoglobulin (●); Error bars correspond to standard deviation. .......................................................................................................................... 146

Figure A1.1 Gel electrophoresis of DNA. Two lines of L are 1 kb ladders, Line 1 is the control (H₂O), Line 2 is *Staphylococcus* spp. (SK), Line 3 is *Staphylococcus epidermidis*, Line 4 is *Staphylococcus* spp. (A). ............................................................................................................. 202

Figure A1.2 Colonies of *Enterobacter aerogenes* on Nutrient agar (overnight, 37°C). .... 204
LIST OF TABLES

Table 2.1 Examples of the applications of ultrasound in food systems. ............................... 44
Table 2.2 Ultrasound inactivation of microbes in foods. ...................................................... 47
Table 3.1 Some physical and biological characteristics of the bacteria used in this study... 69
Table 5.1 Log reduction of bacteria (Enterobacter aerogenes) in different pH buffer. ...... 119
Table A1.1 Microbial growth media. ................................................................................... 195
Table A1.2 PB36 and PB38 Primers. .................................................................................... 199
Table A1.3 PCR conditions................................................................................................. 199
**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\rho$</td>
<td>Density of the medium</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Viscosity of media</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Shear stress</td>
</tr>
<tr>
<td>$\delta$</td>
<td>Half of the surface-surface distance between two microorganisms</td>
</tr>
<tr>
<td>$\phi$</td>
<td>The probability for the collapse of a cavity to kill a microorganism</td>
</tr>
<tr>
<td>$\phi$</td>
<td>The volume fraction of microorganisms</td>
</tr>
<tr>
<td>$\mu$</td>
<td>The number of cavitation bubbles per unit time</td>
</tr>
<tr>
<td>$d$</td>
<td>Distance between collapsing bubble and microbial surface</td>
</tr>
<tr>
<td>$d_c$</td>
<td>Critical distance for breaking up microorganisms</td>
</tr>
<tr>
<td>$r$</td>
<td>Radius of a spherical microorganism</td>
</tr>
<tr>
<td>$v$</td>
<td>Velocity of collapsing cavitation bubbles</td>
</tr>
<tr>
<td>$P$</td>
<td>Ultrasound power</td>
</tr>
<tr>
<td>$N$</td>
<td>Microbial survival number</td>
</tr>
<tr>
<td>$N_0$</td>
<td>Initial microbial number</td>
</tr>
<tr>
<td>$\alpha$-CN</td>
<td>$\alpha$-casein</td>
</tr>
<tr>
<td>$\alpha$-Lac</td>
<td>$\alpha$-lactalbumin</td>
</tr>
<tr>
<td>$\alpha_{s1}$-CN</td>
<td>$\alpha_{s1}$-casein</td>
</tr>
<tr>
<td>$\alpha_{s2}$-CN</td>
<td>$\alpha_{s2}$-casein</td>
</tr>
<tr>
<td>$\beta$-CN</td>
<td>$\beta$-casein</td>
</tr>
<tr>
<td>$\beta$-Lg</td>
<td>$\beta$-lactoglobulin</td>
</tr>
<tr>
<td>$\kappa$-CN</td>
<td>$\kappa$-casein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCP</td>
<td>Colloidal calcium phosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>HHP</td>
<td>Hydraulic high pressure</td>
</tr>
<tr>
<td>HILP</td>
<td>High intensity light pulses</td>
</tr>
<tr>
<td>HTST</td>
<td>High-temperature short-time</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Igs</td>
<td>Immunoglobulins</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PEF</td>
<td>Pulsed electric fields</td>
</tr>
<tr>
<td>PMF</td>
<td>Pulsed magnetic fields</td>
</tr>
<tr>
<td>PSS</td>
<td>Physiological salt solution</td>
</tr>
<tr>
<td>LTLT</td>
<td>Low-temperature long-time</td>
</tr>
<tr>
<td>MS</td>
<td>Manosonication</td>
</tr>
<tr>
<td>MTS</td>
<td>Manothermosonication</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reversed-phase high-performance liquid chromatography</td>
</tr>
<tr>
<td>SC-CO$_2$</td>
<td>Supercritical carbon dioxide</td>
</tr>
<tr>
<td>SMP</td>
<td>Skim milk powder</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>TS</td>
<td>Thermosonication</td>
</tr>
<tr>
<td>UHT</td>
<td>Ultra-high temperature treatment</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasound treatment</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WCA</td>
<td>Water contact angle</td>
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CHAPTER 1

Introduction
1.1 Background

Food safety problems have become a global issue affecting both developed and developing countries. Bacteria, viruses, and yeasts contaminate food and with moulds are the most common causes of food spoilage and food poisoning. Thermal treatments including pasteurisation and sterilisation are the most common techniques used for inactivating microorganisms in food industries for illness prevention and food preservation (Piyasena et al., 2003; Rahman, 2007; Awad et al., 2012).

Thermal treatments can be divided into two categories: mild heat treatment and severe heat treatment, which is determined by the amount of heat applied during processing. Mild heat treatment such as pasteurisation and blanching, can destroy pathogens, reduce bacterial count, and inactivate enzymes. There are several methods of pasteurisation for milk, such as low-temperature long-time (LTLT) pasteurisation (e.g. 63°C for 30 min or more) and high-temperature short-time (HTST) pasteurisation (e.g. 72°C for 15 s). Severe heat treatment includes canning and ultra-high temperature treatment (UHT) (Vaclavik and Christian, 2008). Heating duration and temperature vary; normally temperatures are in the range of 116°C–121°C for canning. However, these traditional microbial control methods can cause the loss of important nutritional components and affect the physical and sensorial characteristics of food including flavour, texture and colour (Kadkhodaee and Povey, 2008). As a result, non-thermal bacterial inactivation and sterilisation technologies are considered novel methods to ensure the safety and quality of food products, and these technologies include ultrasound (US), hydraulic high pressure (HHP), chlorination, ozonation, high-voltage pulsed electric field (PEF) and pulsed magnetic field treatment (PMF).

A variety of applications for ultrasonic processing, include food processing such as preservation and extraction, with reduced impact on nutritional content and on the overall food quality under controlled sonication conditions, has recently been described (Chemat et al., 2011). Ultrasound can disrupt cell walls and release cellular
contents while avoiding further destroy of the cellular components such as proteins and nucleic acid, compared to heating or oxidation processes, which denature them easily (Mason and Lorimer, 1988). Ultrasonic treatment has now being considered as an alternative method for pasteurisation, sterilisation and inactivation of microorganisms (Knorr et al., 2002; Chemat et al., 2011).

Bacteria inactivation using ultrasound treatment was first reported in the 1920s (Harvey and Loomis, 1929), and it was shown that luminous bacteria were inactivated by using 406,000 frequency ultrasound\(^1\). During the 1950s to 1960s, the mechanism of microbial inactivation began to be investigated (Davies, 1959; Earnshaw et al., 1995). High-power ultrasound treatment of bacteria has shown to cause physical damage and disintegration of bacterial cells, release of intracellular compound and results in solubilisation of particulate matter (Foladori et al., 2007); it is accepted that the main causes of these affects are mechanical damages which are the result of acoustic cavitation. However, in most cases, the exact mechanism and effects of this damage are not exhaustive and are still in disagreement, and also there is a lack understanding of how this general effect manifests for different microbial species and in different media. Specifically, the exact mechanism of the inactivation of microorganisms related to their physico-chemical properties is still not well understood. Establishing a theoretical model will help understand the mechanism of bacterial inactivation by ultrasound cavitation. In addition, most of the research has focused on low-frequency ultrasonic inactivation rather than high-frequency treatment and the treatment was targeted only on one or two species of bacteria. Thus, a paralleled study of both low- and high-frequency systems for the same species of microorganisms is necessary. Furthermore, it is worth to apply ultrasound inactivation in different media. The physical and functional property changes of milk by ultrasonication are also worth examining.

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\(^1\) No frequency units were given in Harvey and Loomis’ paper.
1.2 Research objectives

The main objective of this study is to systematically investigate the effects and mechanisms of ultrasound treatment on the inactivation of bacteria and yeast in different matrices. The conditions of ultrasound processing are various; the number of microorganisms that strongly related to food safety issues is vast; the matrices for applying ultrasound are numerous. Therefore the following factors were considered to achieve the objective. Firstly, low-frequency (20 kHz) and high-frequency (850 kHz) ultrasound were used. The low-frequency ultrasound system was used at high power (0.04–0.85 W/ml, 0.7–12.7 W) where the mechanical effects are generated by ultrasonic cavitation. In addition to mechanical effects, sonochemical reactions are also generated under high-frequency ultrasound, and these include hydroxyl free radicals which recombine to form hydrogen peroxide. Secondly, microorganisms were chosen based on different cell properties such as shape, size, and the type of cell wall. They include bacteria *Enterobacter aerogenes*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *S. epidermidis* SK, *S. pseudintermedius* and a species of yeast, *Aureobasidium pullulans*. Thirdly, various matrices for applying ultrasound were considered, including tap water, physiological salt solution, broth and skim milks at different protein concentrations.

Overall, the main aims of this thesis are to determine the effects of both low- and high-frequency ultrasound treatment on bacteria and yeast, and to develop a fundamental understanding of the mechanisms involved in the inactivation of microorganisms by ultrasound cavitation.

1.3 Targeted bacteria and yeast

In order to choose the appropriate bacteria and yeast for ultrasonication, 18 strains were cultured and studied\(^2,^3\), namely, the bacteria *Escherichia coli* 3000, *Enterobacter*  

\(^2\) The general methods for preparing media and solutions, as well as microbial culture, isolation and storage are shown in Appendix A1.1 and A1.2.
aerogenes, Photobacterium phosphoreum, Bacillus subtilis, Enterococcus faecalis (two strains), Enterococcus faecalis 1239, Enterococcus faecalis Goat TDS, Enterococcus faecalis Ex FT, Staphylococcus epidermidis, Staphylococcus epidermidis SK, Staphylococcus pseudintermedius, Micrococcus luteus, and the yeasts Saccharomyces cerevisiae, Saccharomyces cerevisiae M2, Candida albicans, Rhodotorula rubra and Aureobasidium pullulans. After incubation and identification, the basic characteristics of these microorganisms were studied, mainly including the shape, size, axis and ends of cells, arrangement, and Gram staining nature. Owing to their different sizes and types of cell walls, five strains of bacteria and one species of yeast were chosen for this study. The main targeted microorganisms for ultrasound treatment were E. aerogenes, B. subtilis, S. epidermidis, S. epidermidis SK, Staphylococcus pseudintermedius and A. pullulans. Particularly, E. aerogenes, B. subtilis, S. epidermidis and A. pullulans were focused. Images of the bacteria and yeast through a light microscope and transmission electron microscope (TEM) are shown in Figure 1.1 and Figure 1.2 and a detailed description follows.

1.3.1 Enterobacter aerogenes

E. aerogenes is a gram-negative rod-shaped bacterium that tends to be coccus-shaped in the stationary phase (Figure 1.1A and Figure 1.2A). The image of E. aerogenes at the exponential phase is shown in inset Figure 1.1a. E. aerogenes belongs to the family of Enterobacteriaceae, and they have a size range of 0.3–1.0 µm × 1.0–6.0 µm (Imhoff, 2005). The size of the E. aerogenes strain used in this study was approximately 0.84 × 1.0 µm in the stationary phase. E. aerogenes exists widely in water, soil, dairy products, as well as human and animal faeces (Grimont and Grimont, 2006). Although E. aerogenes is a non-pathogenic bacterium, it may still spoil food (Rahman and Kang, 2009).

---

3 Photobacterium phosphoreum was cultured in/on Marine broth/agar at room temperature, and the other bacteria were cultured in/on Nutrient broth/agar at 37°C. The yeasts were cultured in/on Nutrient broth/agar or YPD (agar). The components of these media are shown in Appendix A1.3.

4 The procedure of Gram staining, spore staining (for Bacillus subtilis) and yeast staining are shown in Appendix A1.4. No spores were found from B. subtilis (20 h growth).
Figure 1.1 Light micrographs of targeted bacteria (Gram staining) and yeast (wet mount). A: Enterobacter aerogenes (stationary phase); Inset a: Enterobacter aerogenes (exponential phase); B: Bacillus subtilis; C: Staphylococcus epidermidis; D: Aureobasidium pullulans. Scale bar is 20 µm.

Figure 1.2 Transmission electron micrographs of targeted bacteria. A: Enterobacter aerogenes; B: Bacillus subtilis; C: Staphylococcus epidermidis. Scale bar is 1 µm.
1.3.2 *Bacillus subtilis*

*B. subtilis* is a gram-positive rod-shaped bacterium and normally has a size range of 0.7–0.8 µm × 2.0–3.0 µm (Schleifer, 2009). The strain of *B. subtilis* used in this study has size of 0.6–1 µm × 2–5 µm, and images of the bacteria are shown in Figure 1.1B and Figure 1.2B. *B. subtilis* contamination is a common cause of food spoilage, and its spores have a high resistance to thermal treatment, which makes it a serious food quality and safety problem (Jagannath et al., 2005).

1.3.3 *Staphylococcus epidermidis*

*S. epidermidis* is a gram-positive coccus and it is 0.8–1.0 µm in diameter (Schleifer, 2009); images of the bacteria are shown in Figure 1.1C and Figure 1.2C. *S. epidermidis* is a common skin microorganism (Schleifer, 2009) that easily adheres to material surfaces to form biofilms (Katsikogianni et al., 2006). If biofilms are formed on food, they will result in serious microbiological contamination (Wingender and Flemming, 2004). *S. epidermidis* is catalase-positive\(^5\) (Götz et al., 2006), which can be tested by hydrogen peroxide (Serra et al., 2008).

1.3.4 *Aureobasidium pullulans*

*A. pullulans* is a yeast-like fungus and is well known as black yeast (Cooke, 1959; Hoog, 1993). Images of the yeast are shown in Figure 1.1D. *A. pullulans* is found in various environments such as soil, water, wood, rocks and plant materials especially with high humidity (Urzi et al., 1999; Chi et al., 2009). The size of the yeast was about 2–5 µm × 4–10 µm when it was cultured in Nutrient broth at 28°C for 72 h.

---

\(^5\) Catalase-positive bacteria possess catalase that is a common enzyme. Hydrogen peroxide is degraded into water and oxygen by the catalysed reaction of catalase.
1.4 Thesis structure

Chapter 1 gives a brief background on ultrasonic inactivation of microorganisms and the objectives of the thesis.

Chapter 2 reviews the literature on the properties of microorganisms, the fundamental theories of ultrasound, different ultrasonic inactivation of microorganisms and the effects of ultrasound on milk.

Chapter 3 investigates the effects of low-frequency ultrasound treatment on different bacteria (\textit{E. aerogenes}, \textit{B. subtilis}, \textit{S. epidermidis}, \textit{S. epidermidis} SK, \textit{S. pseudintermedius}). The growth phase of the bacteria was considered, and the relationship between the inactivation efficiency and the physical and biological properties of the bacteria were investigated.

Chapter 4 is devoted to develop a fundamental understanding of the physical mechanism involving ultrasound cavitation in the inactivation of microorganisms. A theoretical model was established based on shear forces resulting from the cavitation collapse near a surface. This model was validated on low-frequency ultrasound treatment of the bacteria \textit{E. aerogenes} and the yeast \textit{A. pullulans}.

Chapter 5 investigates the effects of high-frequency ultrasound treatment on different bacteria and yeast at different growth phases. In addition post-ultrasonication effects (after ultrasonication ceased) were considered.

Chapter 6 focuses on ultrasonic inactivation of \textit{E. aerogenes} in skim milks at different protein concentrations. Both low- and high-frequency ultrasound treatments were used. The effects of ultrasound on the physico-chemical properties of milk were investigated.

Chapter 7 discusses the overall experiments, concludes the main findings of this study and proposes the future work directions.
CHAPTER 2

Literature review
2.1 Introduction

This study was focused to investigate the effects and mechanisms of both low- and high-frequency ultrasound treatment on bacteria and yeast, then apply the ultrasound treatments into skim milk. Therefore, this chapter reviews the properties of the microorganism cells, the fundamental theories of ultrasound, ultrasonic inactivation of microorganisms in varying aqueous solutions and foods especially milks, and the resultant effects of ultrasound on the physico-chemical properties of milk.

2.2 Introduction to microorganisms

2.2.1 Bacteria and other microorganisms in food

Four main groups of microorganisms—bacteria, yeasts, moulds, and viruses—are strongly related to food safety, as many of them can potentially cause foodborne diseases (Ray, 2004). Bacteria, yeasts and moulds are the major causes for food spoilage.

Bacteria are single-celled, prokaryote microorganisms that show a broad range of specific morphologies including spherical (cocci), rod (bacilli), and curved (comma) shapes (Prescott et al., 2002; Ray, 2004). The size of most bacteria is about 0.5–1.0 × 2.0–10 μm, and they can be arranged in clusters, chains or tetrads (Ray, 2004). A bacterium mainly includes a plasma membrane, cytoplasm, nucleoid with chromosomes, ribosomes and inclusion bodies (Tortora et al., 2013). Some significant bacterial foodborne pathogens include Campylobacter jejuni, Salmonella species, Escherichia coli, Listeria monocytogenes, Bacillus cereus, Clostridium botulinum, Clostridium perfringens, Shigella, Yersinia enterocolitica, Vibrio parahaemolyticus, Vibrio vulnificus, Staphylococcus aureus, and Enterobacter sakazakii (Suzuki, 1999; Gurtler et al., 2005; Carlin et al., 2010; Velusamy et al., 2010).

Yeasts and moulds are single-celled eukaryotes and multicellular eukaryotic
microorganisms. *Saccharomyces cerevisiae*, as a typical yeast in foods, can cause food spoilage and generate alcohol and CO₂ (Loureiro, 2000; Ray, 2004). Viruses are regarded as non-cellular microorganisms. Some viruses can cause foodborne diseases and some bacteriophages can lead to fermentation failure which indirectly effects the food qualities (Ray, 2004). Yeast, moulds and viruses all have specific structure and sizes which imply different potential effects from ultrasound.

2.2.2 Microbial growth

There are normally four stages in the growth of microorganisms: the lag phase, exponential phase (log phase), stationary phase, and death phase (Figure 2.1) (Monod, 1949; Novick, 1955). During the lag phase, there is no instantaneous increase of the cell number when microorganisms are transferred into fresh media. Microbes reproduce markedly with a maximal division rate in the exponential phase, and the cells number remain stable and growth ceases in the stationary phase (Monod, 1949; Novick, 1955; Prosser and Tough, 1991; Kolter et al., 1993; Russell and Cook, 1995). During rapid log growth phase, bacteria and other microorganisms are more sensitive to inactivation including radiation exposure and antimicrobial drugs (Twentyman and Bleehen, 1973; Abedi-moghaddam et al., 2004). In the death phase, the cell number decreases logarithmically (Prescott et al., 2002).

![Diagram of Microbial Growth Curve](image)

*Figure 2.1* Microbial growth curve in a closed system.
2.2.3 Bacterial cell wall and surface properties

The main properties of bacterial cell walls that may influence the effects of ultrasound are summarised in Figure 2.2 and described in the sections below.

![Figure 2.2 The main properties of bacterial cell wall.](image)

2.2.3.1 Cell wall type

Bacteria can be considered either gram-positive or gram-negative based on their response to gram staining, a system which was developed by Christian Gram in 1884 (Prescott et al., 2002). Gram positive and negative bacteria are stained in different colours due to their difference in cell wall structures. The structures of gram-positive and gram-negative cell walls are shown in Figure 2.3. Gram-positive bacteria have a thicker and more robust cell wall than gram-negative bacteria (Drakopoulou et al., 2009). Due to the different thickness of cell wall which results various degrees of resistance to mechanical effects, the cell wall type is widely considered one of the main factors for influencing the ultrasound inactivation of bacteria.

A gram-positive cell wall is composed of many layers of homogeneous peptidoglycan or murein, which is about 20–80 nm thick and lies outside the plasma membrane. Whereas, gram-negative cells have a 2–7 nm peptidoglycan layer and a 7–8 nm-thick additional structure called the outer membrane. Therefore, the cell wall of gram-positive cells is stronger than that of gram-negative bacteria because of the extra thickness of the peptidoglycan layer (Schleifer and Kandler, 1972; Zuber et al., 2006;
Drakopoulou et al., 2009). Also, gram-positive bacteria have a tighter adherent peptidoglycan layer than gram-negative bacteria (Drakopoulou et al., 2009).

The peptidoglycan structure is different between gram-positive cells and gram-negative cells. Peptidoglycan or murein is a complicated polymer that consists of numerous similar subunits, it contains sugar derivatives and several different amino acids. One type of amino acids called D-amino acids can protect cells from attacks by peptidases. Normally, the carboxyl group from the terminal D-alanine is linked to the amino group of diaminopimelic acid directly; however gram-positive cell walls generally contain a peptide interbridge as a link between the above two components. In contrast, there is no peptide interbridge for most gram-negative cell wall peptidoglycan. As a result, the peptidoglycan sac, linked and formed by the cross-links between peptides and peptide interbridges, is stronger for gram-positive bacteria than that of gram-negative (Perkins, 1963; Glauert and Thornley, 1969; Schleifer and Kandler, 1972; Shockman and Barrett, 1983; Wilson et al., 2002; Jørgensen et al., 2003).

Figure 2.3 Gram-positive and gram-negative cell walls. A: a gram-positive envelope from *Staphylococcus epidermidis*; B: a gram-negative envelope of *Enterobacter aerogenes*.
The structure of bacterial endospore mainly contains exosporium, coat, outer membrane, cortex, spore cell wall, inner membrane and central core (Moir and Smith, 1990; Prescott et al., 2002). The cortex occupies about half volume of the spore, it is composed of peptidoglycans that are less cross-linked in spores than in vegetative cells (Prescott et al., 2002).

2.2.3.2 Hydrophobicity and hydrophilicity

Hydrophobicity means that a particle or molecule is non-polar, usually repels water, the molecule is unable to initiate hydrogen bonding and the surface energy is normally low (Mozes and Rouxhet, 1987); whereas, a hydrophilic molecule is polarised, thus is capable of hydrogen bonding, and can dissolve easily in water or other polar substances. Water is less structured when it surrounds a hydrophobic surface because of the function of intermolecular hydrogen bonding; in contrast, water is more structured when it is near a hydrophilic surface (An and Friedman, 2000). Surface hydrophobicity plays an important role in ultrasonication. A hydrophobic surface will attract a cavitation bubble since this has hydrophobic property. In which case, damage of the surface may be enhanced by ultrasound treatment.

Water contact angle (WCA) measurement is a significant method of examining bacterial cell surface hydrophobicity (Mozes and Rouxhet, 1987). A high WCA indicates hydrophobicity and a low WCA is a reflection of hydrophilicity (An and Friedman, 2000). Microorganisms can be divided into three groups depending on the value of their WCA: hydrophobic (WCA>90°); moderately hydrophobic (WCA=50°–60°); and hydrophilic (WCA<40°) (Mozes and Rouxhet, 1987). For example, it was found that Enterobacter aerogenes was moderately hydrophobic (WCA=62°) and S. cerevisiae was hydrophilic (WCA=26°) (Mozes and Rouxhet, 1987). Whereas, the water contact angle of E. aerogenes MBLA-0400 was 43° (Mozes et al., 1988). It was reported that Staphylococcus epidermidis was hydrophilic judging by the water contact angle measurement of 18.25° (Dickson and Koohmaraie, 1989). S. epidermidis ATCC 35983, S. epidermidis ATCC 35984 and S. epidermidis
ATCC 12228 were hydrophilic, and their contact angles were 22°, 19° and 10°, respectively, at pH 7 (Gallardo-Moreno et al., 2011). The hydrophobicities of seven strains of *Bacillus subtilis* were measured, and the study found that the hydrophobicities varied among different strains (Ahimou et al., 2000). The water contact angles of six strains, including ATCC 7058, ATCC 12432, ATCC 12695, ATCC 15129, ATCC 15476 and ATCC 15561, were less than 40°, and only ATCC 15811 was about 48°. Some researchers have characterised hydrophobic status for 27 species of bacteria based on adhesion, and found that 44% of the hydrophobic bacteria and 17% of the hydrophilic bacteria were gram-positive (McNamara et al., 1997). Moreover, the results varied with different methods, and the hydrophobicity status was also affected by the growth medium.

### 2.2.3.3 Cell surface charge

Bacteria in aqueous suspension are considered to be negatively charged (Kohnen and Jansen, 2000). Therefore, it is hard to distinguish the ultrasound inactivation effect by the property of cell surface charge. After testing *S. epidermidis, B. subtilis,* and *E. coli,* it was reported that bacteria had greater negative charges than positive charges (Dickson and Koohmaraie, 1989).

### 2.2.3.4 Capsules and slime (Extracellular polysaccharides)

There is a large structural layer that lies outside the cell wall of some bacteria, usually composed of polysaccharides called capsules or slime. They can potentially change the cell properties and thus influence the effects of ultrasound inactivation on bacteria. If it is well organised and difficult to wash off, the layer is considered to be a capsule; if the layer is unorganised and can be easily removed, it is called slime (Hussain et al., 1993; An et al., 2000; Kiers et al., 2001; Prescott et al., 2002). There are several functions of capsules, including protecting bacteria against desiccation, resisting phagocytosis, expelling bacterial viruses and toxic materials, and protecting the host’s defence mechanisms (Yamada et al., 1988; Prescott et al., 2002; Franz et al., 2003). Capsules can be identified by India ink staining method (Yamada et al., 1988).
reported that capsules were found at both the stationary growth phase and exponential phase for *S. epidermidis* (Hogt et al., 1985). *S. epidermidis* RP62A (ATCC35984) was also a slime producer (Wang et al., 1993).

### 2.2.3.5 Cell appendages

Cell appendages are the external features of bacterial cells including flagella, fimbriae (pili), and they are not considered components of the cell wall. Flagella are threadlike locomotor appendages for motile bacteria that protrude from the plasma membrane and cell wall; they are about 20 nm in diameter and 15–20 μm long (Prescott et al., 2002). Fimbriae are short, hair-like appendages, composed of helically arranged protein subunits, which are on the surface of some bacteria and are thinner than flagella (Prescott et al., 2002). The bacteria with fimbriae may affect the ultrasound inactivation, since they may dampen the mechanical effects induced by cavitation.

### 2.3 Ultrasound

#### 2.3.1 Ultrasound waves

Ultrasound is a sound beyond human hearing range, and the unit of sound frequency is Hertz [Hz], which represents cycles per second. The range for human hearing is about 20 Hz to 20 kHz (Mason and Lorimer, 2002). Ultrasound is a kind of vibrational energy that generally has a frequency of 20 kHz or more (Mason, 1990; Piyasena et al., 2003). For ultrasound, the frequency range of 20–100 kHz is considered as low frequency, while it is called high frequency when the frequency is greater than 100 kHz (Mason, 1990; Butz and Tauscher, 2002; Piyasena et al., 2003; Patist and Bates, 2008). The ranges of frequencies for sound are shown in Figure 2.4.
The velocity of ultrasound wave ($c$) passing through a medium is given by the equation:

$$c = \lambda f$$  \hspace{1cm} (2.1)

where $\lambda$ is wave length and $f$ is the frequency.

Ultrasound can transmit through different media including gas, liquids and solids as a sound wave (Mason and Lorimer, 1988). Ultrasound produces transverse waves in solid media, whereas longitudinal waves are produced when it passes though gas or liquid media (Mason and Lorimer, 1988). A good example of the production of a transverse wave is the ripples generated when a small stone is dropped into a still pool. The movement of longitudinal ultrasound waves can be demonstrated as the stretching of a coiled spring when it is twitched at one end. A vibrational energy, which is considered a compression wave, is transmitted through the spring. Sound contains a series of the consecutive compression waves and refraction waves, and they are separated in between each other (Figure 2.5). Negative (rarefaction) and positive (compression) acoustic pressures are generated during the motion of ultrasound waves.

**Figure 2.4** Frequency range of sound (Redrawn from Mason et al., 2005).
2.3.2 Ultrasonic cavitation

Ultrasound effects on microbial inactivation are mainly related to the phenomenon of acoustic cavitation (Joyce et al., 2003a; Mason et al., 2003; Mason et al., 2005; O’Donnell et al., 2010; Soria and Villamiel, 2010). Cavitation means “the formation and the subsequent dynamic life of bubbles in liquids”, which includes hydrodynamic, thermal and acoustic cavitation (Suslick, 1988). With the propagation of the ultrasound in liquid media, cavitation microbubbles are generated because of the change in pressure (Suslick, 1988). The processing of ultrasonic cavitation can be described by the following schematic diagram (Figure 2.5) (Soria and Villamiel, 2010).

![Figure 2.5 Schematic of ultrasonic cavitation (Redrawn from Soria and Villamiel, 2010).](image)

When ultrasound passes through liquid media, acoustic streaming and mechanical vibration within the liquid will be generated. The molecules of the liquid are pulled...
apart by the negative pressure during the rarefaction cycles of the ultrasound waves. The rarefaction cycles will produce cavities (microbubbles, cavitation bubbles) if sufficient intensity is generated from the ultrasound wave. In order to overcome the cohesive force in water, a negative pressure of about 1500 atm is required. However, a much lower pressure (less than 20 atm) is sufficient for initiating cavitation because the tensile strength of the liquid is lowered with the presence of a nucleation site such as gas and dust.

Normally the innumerable microbubbles contain dissolved gas nuclei. Each bubble will be affected by surrounding bubbles because of the successive compression cycles from ultrasonic waves. During rarefaction cycles, microbubbles grow, during compression cycles, the microbubbles compress. The bubbles grow and after reaching their resonating sizes, they collapse as a result of the continuous ultrasound propagation. Consequently, these bubbles collapse intensely and violently and generate huge amounts of energy. The phenomenon of growth and collapse of microbubbles by ultrasonication is called “acoustic cavitation” (Mason and Lorimer, 2002; Ashokkumar, 2011). The “hot spot” theory was proposed to explain the energy release during ultrasonic cavitation (Suslick, 1990). The compression of cavities implode rapidly, therefore little heat will escape during the collapse, while the heated cavity will be quenched by the surrounding cold liquid simultaneously. The collapse of acoustic cavitation bubbles can produce localised temperature increase up to 5000 K and pressures in the order of 1000 atm (Suslick and Price, 1999).

Cavitation can be divided into two types, one is transient or inertial cavitation, which causes large-scale variations for the size of the bubble and produces a violent bubble collapse, whereas the second type stable cavitation generates small amplitude, weak and symmetrically oscillating bubbles (Suslick, 1988; Scherba et al., 1991; Barnett et al., 1994; Ashokkumar, 2011). Stable cavitation can be produced at the intensity of 1–3 W/cm² (Feng and Yang, 2010).
There are two main effects induced by ultrasound cavitation including mechanical effects and sonochemical reactions. The mechanical effects induced by ultrasound treatment mainly include shear forces and micro-jets (Figure 2.6). In the fluid between cavitation bubbles and a solid surface, a velocity gradient perpendicular to the surface is generated, which causes a shear force to act on the surface. The shear stress ($\sigma$) induced by cavitation can be approximated by equation (2.2) (Maisonhaute et al., 2002b):

$$\sigma = \frac{\eta v}{x} \quad (2.2)$$

where $\eta$ is the viscosity of media, $v$ is the velocity of collapse, $x$ is the distance between a solid surface and the cavitation bubble.

The fluid velocity of the shear stress is approximately 200 m/s at 20 kHz (Maisonhaute et al., 2002a; Maisonhaute et al., 2002b). If the distance between the cavitation bubble and the surface is 1 μm, then a shear stress of $2\times10^5$ Pa is produced (Zuo et al., 2012).

![Figure 2.6 Shear forces and micro-jets induced by ultrasonic cavitation.](image)

When cavitation bubbles collapse near a surface, micro-jets are formed when the collapses are not symmetrical (Lamminen, 2004). Since the solid surface blocks the liquid movement, the liquid flow will accelerate from the opposite side of the solid
surface, and pass through the microbubble (Zuo et al., 2012). The speed of a liquid micro-jet acting on a solid surface can be higher than 100 m/s (Zuo et al., 2012). Shock waves can also be produced during the collapse of cavitation bubbles (Virot et al., 2010), and pressures of 40–60 kbar can be induced at 20 kHz (Pecha and Gompf, 2000).

Studies have shown that bacterial inactivation was mainly caused by mechanical effects induced by cavitation during low-frequency ultrasound treatment and was caused by free radicals at high frequencies (Mark et al., 1998; Al Bsoul et al., 2010). The mechanical effects mainly include shear forces and micro-jets as discussed above. For high-frequency ultrasound in liquid, free radicals are produced (Earnshaw et al., 1995; Hua and Thompson, 2000; Joyce et al., 2011) in addition to mechanical effects, because of the dissociation of water molecules by the collapsing bubbles (Sochard et al., 1997). These free radicals mainly include hydroxyl radicals, which attack the chemical structure of the cell wall and may cause DNA damage, destroy enzymatic activity, and damage liposomes and membrane bacteria (Riesz and Kondo, 1992; Andreassi, 2004; Hunter et al., 2008; Drakopoulou et al., 2009; Cabiscol et al., 2010; Liu et al., 2011). Formation of free radicals was experimentally proven using electron spin resonance spectroscopy and spin trapping techniques (Riesz et al., 1985).

The sonochemical reactions of ultrasonication mainly take place as the following reactions (Dai et al., 2006; Olvera et al., 2008; Thangavadivel et al., 2009):

\[
\text{H}_2\text{O} + )))) \rightarrow \text{OH}^\cdot + \text{H}^\cdot \quad (2.3)
\]

\[
2 \text{OH}^\cdot \rightarrow \text{H}_2\text{O}_2 \quad (2.4)
\]

where )))) stands for ultrasound irradiation.

It was reported that the production of H\textsubscript{2}O\textsubscript{2} was ten times higher at high frequencies than at low frequencies, especially at frequencies between 200 and 1000 kHz (Mark et
al., 1998; Al Bsoul et al., 2010). In some cases, the release of peroxidise enzymes from bacterial cell rupture can further aid oxygen radical production by reacting with H₂O₂, and these oxygen radicals continue to attack to the cell membranes (Jyoti and Pandit, 2003), which suggests free radicals are hardly produced at low frequency.

It was indicated that pH fluctuated when Cryptosporidium parvum was ultrasonicated at 1 MHz, and this was mainly because cavitation produced H’, OH’ and HOO’ (Olvera et al., 2008). The rarefaction and compression cycles are significantly shorter at high frequencies than at low frequencies (Mason and Lorimer, 1988), which means that the collapse of microbubbles occurs very quickly. Therefore the quantity of OH’ and H’ free radicals are high before they recombine (He et al., 2006). However, the H₂O₂ formation was not found below an amplitude⁶ of 3 µm at 27.5 kHz when E. coli XL1-Blue was ultrasonicated (Furuta et al., 2004).

2.3.3 Ultrasonic equipment

There are two categories of ultrasound instruments based on power and frequency: power ultrasound equipment and analytical (including imaging) ultrasound equipment. The later will not be discussed here. Power ultrasound with frequency equal or higher than 20 kHz includes high-power low-frequency ultrasound (20–100 kHz) and low-power high-frequency ultrasound (>100 kHz).

2.3.3.1 Laboratory equipment

There are two principle components for setting up and apply power ultrasound equipment: a working liquid medium, and a source of vibrational energy (Mason and Lorimer, 2002). Samples are needed to be suspended in the liquid medium in order to utilise the effects of ultrasonic cavitation. A transducer is the source of the vibrations that converts the form of energy.

⁶ Note that in the published literature, the amplitude is sometimes given instead of the power.
Electrical or mechanical energy is converted into high frequency sound by the transducer (Mason and Lorimer, 2002). There are mainly three classes of transducers, mechanically-driven, piezoelectrics, and magnetostrictive transducer (Gallego-Juarez, 1989; Mason and Lorimer, 2002). In a mechanically-driven transducer such as the whistle reactor, the ultrasound wave is generated by mechanical effect, whereas the wave is produced by the piezoelectric effect for piezoelectric-based transducers. The piezoelectrics are materials which change their physical dimensions when an electric field is applied to them; and vice versa they produce an electric current if their physical dimension is manipulated (e.g. through deformation) (Shung and Zippuro, 1996). Magnetostrictive transducer is made of materials with magnetostrictive properties such as nickel and iron, which can convert magnetic energy into mechanical energy in a magnetic field.

At laboratory-bench scale, the most common transducer is piezoelectrics (Gallego-Juarez, 1989; Mason and Lorimer, 2002). Several materials are used in the manufacture of piezoelectrics and these include quartz, barium titanate, lead metaniobate and lithium niobate (Shung and Zippuro, 1996; Mason and Lorimer, 2002). The resonance frequency of the piezoelectric depends on the thickness of the material used (Gallego-Juarez, 1989). There are mainly three types of ultrasonic instruments with piezoelectric transducer, probe or horn, bath and cup-horn system (Gallego-Juarez, 1989; Mason and Lorimer, 2002; De La Calle et al., 2009). Probe and bath type are the most widely used which were also used in the study. Probe (or horn) system generally produces low-frequency ultrasound while the bath system is normally used for generating high frequency.

In the probe/horn system, a metal probe/horn directly immerses into samples and it is operated by a transducer, while the transducer is driven by a power supply. The ultrasound waves are generated by the transducer and then propagate into the working media through the probe/horn (Mason and Lorimer, 2002). The probe/horn plays a
role as an amplifier, since the vibrational amplitude of the piezoelectrics is very small (Mason and Lorimer, 2002). The probe/horn is normally made of titanium alloy.

The illustrations of the two common laboratory equipment are shown in Figure 2.7 and 2.8, and they were used in this study. A low-frequency high-power system, the Sonic Ruptor 250 (Omni International, USA) refers to Figure 2.7. This equipment operates at a constant frequency of 20 kHz and its power supply can deliver up to 250 W. This equipment was mainly operated using the horn probe with processing tip of (diameter 12.7 mm of processing tip). The sample (15 ml) was contained in a glass vial (internal diameter 16 mm and height of 55 mm) sitting inside an ice bath to ensure that the sample temperature is always maintained below 30°C.

![Figure 2.7 Illustration of the low-frequency equipment used in this thesis. It consisted of (1) a power supply and pulse control panel, (2) a transducer, (3) an upper fixed horn, (4) a detachable horn, (5) samples surrounded by an ice bath (6). Noise during ultrasound treatment was partly attenuated by a sound abatement chamber (7).](image)

The second equipment used in this thesis is a high-frequency unit (Meinhardt Ultraschalltechnik, Germany (Figure 2.8). This unit operates at a frequency of 850 kHz using an E/805/T cylindrical transducer. Because the operating surface of the
transducer is very large (diameter 75 mm), the unit was used like an ultrasound bath. Water was contained in a double-walled glass cylinder mounted in the transducer. The double-walled glass vessel, containing 250 ml water, was thermostated by circulating chilled water from a water bath (PolyScience SD07R-20-A12E, USA). A cover was used to hold a test tube containing the bacteria sample at the centre of the double-walled glass vessel.

**Figure 2.8** Illustration of the high-frequency unit used in this thesis. (1) An ultrasound generator, (2) a transducer, (3) a double-walled cylindrical glass vessel connected (4) a water bath, and (5) a test-tube containing samples.

### 2.3.3.2 Industrial equipment

There are a wide range of applications for power ultrasound in various industries, such as cleaning, soldering, extraction from plants, emulsification and deburring (Suslick, 1988). Large-scale equipment is needed for applying ultrasound treatment in industrial applications, because the treated volumes are much larger than in a laboratory scale environment. While small scale ultrasound equipment has been widely used in medical and dental cleaning applications, capacity of a large industrial ultrasonic cleaner can vary between 5 to 150 litres (Vijayendran et al., 2008). There are mainly two types of ultrasonic industrial equipment working in either batch or continuous modes.
For example, a large cleaning bath is a typical component, acting as a reacting tank for batch ultrasound system (Mason and Lorimer, 2002). Stainless steel is an ideal material for the manufacture of the tank. Plastic tanks can also be used, but they need to be well bound to the transducer made of stainless or a titanium plate (Mason and Lorimer, 2002). An array of transducers are usually utilised for large tanks in order to supply high power and make the system more efficient (Suslick, 1988). Additional overhead stirring equipment is also needed to enhance the homogenisation of the treated medium. In the case of continuous ultrasound equipment, bath, horn and whistle reactors can be employed (Mason and Lorimer, 2002). In the ultrasound bath configuration, the liquid samples to be treated are pumped into the tank continuously. However, recycling treatments may be needed to enhance the ultrasound effects. Alternatively, series of horn reactors can be implemented on the walls of a working container (Mason and Lorimer, 2002). One of the most successful industrial applications of ultrasound is the liquid whistle, which has been manufactured for homogenisation and emulsification. This equipment works in the continuous mode its treatment output can reach up to 12,000 L/h when processing fruit juices, tomato sauce or mayonnaise (Mason et al., 1996).

### 2.3.4 Factors affecting cavitation threshold

There are many parameters that affect the cavitation threshold, mainly including intensity and amplitude, frequency, temperature and external pressure.

#### 2.3.4.1 Intensity and amplitude

The ultrasound power level is a parameter that can be expressed as power (W, joule/sec), intensity (W/cm², W/ml), or energy (joule) (Hendee and Ritenour, 2003). Ultrasound intensity is proportional to the amplitude of ultrasonic vibration (Santos et al., 2009), and more cavitation is created at higher amplitudes (Salleh-Mack and Roberts, 2007). Power input directly relates to amplitude; power will increase with higher amplitude (Al Bsoul et al., 2010).
The intensity ($I$) of ultrasound can be expressed by (Mason and Lorimer, 1988):

$$I = \frac{P_A^2}{2\rho c}$$

where $P_A$ is the maximum acoustic pressure amplitude of the ultrasound wave, $\rho$ is the density of the medium, and $c$ is the velocity of ultrasound.

$P_A$ is related to the acoustic pressure ($P_a$) created by ultrasound, which can be given by (Mason and Lorimer, 1988):

$$P_a = P_A \sin 2\pi f$$

where $f$ is frequency.

Thus, the intensity of the ultrasound increases when the collapse pressure increases, which results in a strong collapse of microbubbles. However, there must be a high ultrasonic intensity in order to inactivate microbes (Joyce et al., 2003b).

### 2.3.4.2 Frequency

The resonance frequency ($\omega$) of ultrasound is highly related to the resonance size of the acoustic bubble, which can be given by (Minnaert, 1933; Leighton, 1994):

$$\omega = \frac{1}{R} \sqrt{\frac{3\gamma P_0}{\rho}}$$

where $R$ is the mean radius of the microbubble, $\gamma$ is the polytropic coefficient, $P_0$ is the hydrostatic liquid pressure outside the bubble, and $\rho$ is the density of the liquid medium.

Equation (2.7) shows that the size of the bubbles decreases with increasing ultrasound frequency. Also, for high-frequency ultrasound, it is difficult to produce microbubbles during the rarefaction cycle because this cycle is very short (Mason and Lorimer, 1988; Salleh-Mack and Roberts, 2007). Therefore, there is not enough time for
bubbles to collapse completely, hence the energy released during bubble collapse is less violent for high-frequency ultrasounds than for low-frequency ones. The duration of rarefaction cycle can last 25 µs at 20 kHz while it only lasts 0.025 µs at 20 MHz (Mason and Lorimer, 1988). Although there is less energy released in high-frequency ultrasound, it produces more cavitation events (Crum, 1995), and it is sufficient to break water molecules into hydrogen atoms and hydroxyl radicals (OH⁻) at the moment when the cavitation bubble collapses (Leighton, 1994). As a result, the production of hydroxyl radicals before recombination is markedly improved at higher frequencies (He et al., 2006). For example, it was reported that the yield of H₂O₂ at 358 kHz is much greater than at 20 kHz, while it decreases at a frequency of 1062 kHz due to the restriction of the amount of water vapour at very high frequencies (0.90 W/cm²) (Ashokkumar et al., 2008).

2.3.4.3 Temperature

The maximum temperature $T_{\text{max}}$ at the moment of bubble collapse is generally given by (Mason and Lorimer, 1988; Mason, 1990):

$$T_{\text{max}} = T_0 P_m (\gamma - 1) / P_s$$  \hspace{1cm} (2.8)

$$P_{\text{max}} = P_s \left( \frac{P_m (\gamma - 1)}{P_s} \right)^{1/(\gamma - 1)}$$  \hspace{1cm} (2.9)

$$P_s = P_v + P_g$$  \hspace{1cm} (2.10)

$$P_m = P_a + P_h$$  \hspace{1cm} (2.11)

where $T_0$ is the ambient or experimental temperature, $P_{\text{max}}$ is the maximum pressure at the moment of bubble collapse, $P_m$ is the pressure at the moment of collapse, $\gamma$ is the polytropic index of gas or gas vapour mixture and $P_s$ is the pressure at the bubble’s maximum size. $P_v$ is the vapour pressure, $P_g$ is the gas pressure inside of the bubble, $P_a$ is the acoustic pressure and $P_h$ is the hydrostatic pressure.
Cavitation can be generated at lower intensities by increasing the temperature of the ultrasound system (Mason and Lorimer, 1988). Increase of temperature causes the increasing of vapour pressure, and the pressure inside of cavitation bubble will be enhanced. It should be noted that increasing the temperature of the medium reduces its viscosity, which in turns results in cavities produced more easily.

2.3.4.4 External pressure

Both the cavitation threshold and the intensity of microbubble collapse are enhanced by increasing external pressure (Mason and Lorimer, 1988). From equation 2.11, the pressure at the moment of bubble collapse ($P_m$) is determined by the hydrostatic pressure ($P_h$) and the acoustic pressure ($P_a$). Therefore, the increase of $P_h$ results in a very rapid and violent collapse of microbubbles (Equation 2.8 and 2.9).

2.4 Ultrasound inactivation of bacteria and other microorganisms

While the published literature on bacteria is not very exhaustive, the variations in the ultrasound treatment conditions are different from one study to the other. These conditions such as ultrasound equipment, frequencies, ultrasound power and intensity, samples volume, temperature and initial bacteria number were not fully cited. Some of them were not expressed in same manner, and some parameters in many publications were not even reported. This makes quantitative comparison of the published results very difficult.

There are mainly three groups of factors that affect the results of ultrasound treatment on the inactivation of bacteria and other microbes: first, the parameters affecting the cavitation threshold since bacterial inactivation is driven by cavitation effects; second, the properties of the media; and last, the microbes themselves. The factors affecting the cavitation threshold mainly include intensity and amplitude, frequency, temperature and external pressure. The proprieties of media cover viscosity, volume, pH and the initial number of bacteria. The characteristics of bacteria basically include
gram staining status, size and shape, bacterial capsules, bacteria species, spores and growth phases.

2.4.1 Effects of cavitation threshold

Larger intensity causes more violent collapse of microbubbles than smaller intensity (Mason, 1990). Thus, the inactivation rate of microorganisms generally increases with the increasing intensity of ultrasound. Microbes are more resistant to ultrasound at high frequencies than at low frequencies, which is mainly because in a short rarefaction and compression cycle, it is difficult for microbubbles to be produced. Further, because the vapour pressure increases quickly and cavitation bubbles are generated rapidly, bacteria and other microbes such as yeasts are more sensitive to ultrasonication at high temperature. Under high pressures, microbubbles can also collapse more rapidly and violently.

2.4.1.1 Effects of intensity and amplitude

Normally, the higher intensity amplitude of ultrasound applied, the more bacteria are inactivated, since greater energy is released in higher intensity. It was found that the inactivation rate of Mycobacterium sp. 6PY1 increased with increased ultrasound power (Al Bsoul et al., 2010). *E. coli* has been publicised widely for numerous outbreaks that have caused deaths (Piyasena et al., 2003). Ultrasonic inactivation of *E. coli* XL1-Blue was investigated by using a horn type sonicator utilising the squeeze-film\(^7\) effect at 27.5 kHz with high power intensity (Furuta et al., 2004). The results showed that the inactivation rate of bacteria increased with increase in the amplitude of the vibrating face, which was more than 99% in 180 s at an amplitude of 3 μm (p–p) and 2 mm squeeze film (Furuta et al., 2004). *E. coli* ATCC 25922 and NCTC 12900 suspensions also were examined by using ultrasound at different amplitude levels (0.4, 7.5 and 37.5 μm) (Patil et al., 2009). It was found that there was a greater than 5-log reduction of *E. coli* by using ultrasound treatment for 15 min at

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\(^{7}\) The sample was hold in a gap between two transducers.
37.5 μm or less at 7.5 μm amplitude (Patil et al., 2009). The disruption rate and protein release of *S. cerevisiae* was also found to increase with the increase in power from 120 to 600 W, when sonicated at 20 kHz (Liu et al., 2013).

A group of aerobic mesophile microorganisms was ultrasonicated in date syrup at both 10% and 25% of total power\(^8\), and the number of total microorganisms was significantly decreased at higher intensities than lower intensities (Entezari et al., 2004). However, it was reported that at low frequency, the inactivation rate of *Mycobacterium* sp. 6PY1 reached a constant level when the power increased to a certain value. And this was explained by stipulating that the number of cavitation bubbles becomes equal to the bacteria number (Jyoti and Pandit, 2003; Al Bsoul et al., 2010).

### 2.4.1.2 Effects of frequency

It is difficult to determine whether low-frequency or high-frequency ultrasound is more efficient for the inactivation of bacteria because of the reasons stated above. Although shear forces produced in high-frequency ultrasound are less violent than those produced in low-frequency ultrasound, there are more hydroxyl radicals generated at low frequency. It was found that the kill rate at low-frequency high-power ultrasound (20 and 38 kHz) was significantly higher than at high-frequency low-power one (512 and 850 kHz) for the inactivation of *B. subtilis* (Joyce et al., 2003b). The inactivation percentage of *Mycobacterium* sp. 6PY1 was lower at a high frequency (612 kHz) than at a low frequency (60 kHz) at the same power density (114 W/L), and the percentage was 43% and 93%, respectively (Al Bsoul et al., 2010). It was also reported that *E. coli* and *Klebsiella pneumonia* had a continuous and significant decrease in numbers when treated by ultrasound with an intensity of 0.012W/cm\(^2\) for 15 min. At a frequency of 20 kHz, 6.06% of live *E. coli* cells remained, while 18.9% were still live when ultrasonicated at 40 kHz (Joyce et al., 2003b).

\(^8\) Unfortunately, in some publications the exact power or intensity used was not reported.
2011). At a higher frequency (580 kHz), 57.2% of *E. coli* cells were left and 75.5% of *K. pneumonia* were left after 15 min, which was significantly more than at low frequency (Joyce et al., 2011). However, it was reported that the rate of inactivation of *E. coli* IAM 12058 was slightly higher at 500 kHz than at 20 kHz, under at the same sonication power (1.7–12.4 W) (Koda et al., 2009).

### 2.4.1.3 Effects of temperature

Normally, bacteria are more sensitive to ultrasound treatment in an environment with high temperature. Increase in temperature results in an increase in vapour pressure (Mason and Lorimer, 1988), rapid generation of cavitation bubbles (Earnshaw et al., 1995) and the viscosity of the media decreases, thus it lowers the ultrasound power threshold for the generation of cavitation.

Results from studies showed that ultrasonication at a mild temperature (50–60°C) was more effective for microbial inactivation than ultrasonication under ambient temperature (Ciccolini et al., 1997; Russell, 2002). The results on ultrasonication of *E. coli* ATCC 25922 showed that temperature had a significant effect on bacterial inactivation, where a 5-log reduction of *E. coli* was achieved in 3 min with no temperature control, while the optimum ultrasonication time was 10 min when the temperature was maintained at below 30°C (Salleh-Mack and Roberts, 2007). *S. epidermidis* suspensions (10⁵ CFU/ml) were ultrasonicated by low-frequency ultrasound (20 kHz) in different suspension volumes (2, 5 and 10 ml), intensities (4%, 10%, 20% and 30% of maximal energy output of 400 W) and ultrasonication times of 1 or 2 min (Singer et al., 1999). Temperature was the main factor that affected the reduction of live bacteria, and *S. epidermidis* growth was minimal when temperatures exceeded 45°C while there was slight effect when temperatures were between 45 and 50°C. In addition, it was found that ultrasound combined with temperature was significantly effective for reducing *Cronobacter sakazakii* (Adekunte et al., 2010b).

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9 If not controlled by circulating chilled water or by immersion in an ice bath for example, the temperature of the medium increases noticeably during sonication.
For yeast inactivation, it was found that ultrasonication could achieve a desired 5-log reduction in yeast cells *Pichia fermentans* at a moderate temperature (45°C) (Adekunte et al., 2010c). *S. cerevisiae* cells were ultrasonicated in a Sabouraud broth at 35, 45 and 55°C (20 kHz, wave amplitude in the range of 71–107 μm) and it was found that inactivation at 55°C and 45°C increased significantly compared to 35°C (Guerrero et al., 2001). However, the increase in bacteria inactivation did not increase linearly with the increase in temperature (D'Amico et al., 2006; Lee et al., 2009).

### 2.4.1.4 Effects of external pressure

An increase of rapid and violent collapses of microbubbles occur when external pressure increases (Mason and Lorimer, 1988). For instance, *D*-values\(^{10}\) for *Y. enterocolitica* decreased from 1.52 to 0.20 min when this bacterium was ultrasonicated under increases from atmospheric to 600 kPa (Raso et al., 1998a). However, other authors reported that increase of the pressure applied during sonication above a certain threshold (400 kPa) did not further improve bacterial inactivation (Raso et al., 1998a; Condón et al., 2004). This was explained by the fact that ultrasonic field cannot overcome the cohesive forces due to high hydrostatic pressure applied, resulting in the decrease of the number of cavities generated (Raso et al., 1998a; Condón et al., 2004). It was found that the upper limit was 300 kPa (Lee et al., 2009). Inactivation of *E. coli* cells with ultrasonication, manosonication (MS), thermosonication (TS), and manothermosonication (MTS)\(^{11}\) was reported (Lee et al., 2009). They found that the combination of ultrasonication with heat and/or pressurisation significantly shortened the treatment time to obtain a 5-log reduction of bacteria. The inactivation of *B. subtilis* spores was tested using MS and combined MS/heat treatment (Raso et al., 1998b). It was found that approximately 99% of the *B. subtilis* spores were inactivated at 500 kPa, with 117 μm of amplitude for 12 min by MS treatment, and there was a synergistic effect on spore inactivation by using heat.

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\(^{10}\) *D*-value is the time it takes to inactivate 99% of bacteria under well-defined processing conditions.

\(^{11}\) Manosonication (MS), thermosonication (TS), and manothermosonication (MTS) are the use of ultrasound treatment combined with pressure, thermal treatment, both pressure and thermal, respectively.
treatment at 70–90°C with MS at 20 kHz, 300 kPa and 117 μm for 6 min (Raso et al., 1998b). The $D$-value decreased progressively during the ultrasonic inactivation of $C. sakazakii$ with the increasing pressure—it had a 62% decrease when pressure rose from 0 Pa to 100 kPa and a further 33% decrease when the pressure changed from 200 to 300 kPa (Arroyo et al., 2011b).

2.4.2 Media

Cavities cannot be generated easily in highly viscous media because the vibration of ultrasound waves are impeded (Salleh-Mack and Roberts, 2007). There are still some disagreements about the effects of the sonicated volume, the pH of the medium and the initial number of bacteria, on the inactivation of bacteria. However, many researchers indicated that bacteria and yeasts were more sensitive to ultrasound treatment when they are in low numbers.

2.4.2.1 Viscosity and media

The effects of ultrasound inactivation on microorganisms are different when they are present in a food product compared to microbiological aqueous media. For instance it was found that, after ultrasonication for 10 min, a 0.78-log reduction was achieved on $Salmonella$ in milk, compared to a 4-log reduction when they were suspended in peptone water (160 kHz, 100W) (Lee et al., 1989). Cameron and co-workers isolated three species of microbes from pasteurised milk, including $E. coli$, $S. cerevisiae$ and $Lactobacillus acidophilus$. After ultrasound treatment (20 kHz, 750 W), they reported that the reductions were more than 99% for $E. coli$ and $S. cerevisiae$, and 72% and 84% for $L. acidophilus$ when sonicated in saline solution and UHT milk, respectively (Cameron et al., 2008). The resistance to MS treatment for $C. sakazakii$ was found to increase with decreasing in water activity (Arroyo et al., 2011a). In this case water activity was tuned by the addition of sucrose (44.4% (w/v) of sucrose to achieve a water activity to 0.94). Sucrose is known to protect bacterial viability in a number of adverse conditions. In contrast, there was no significant difference in the inactivation
rate of *E. coli* by ultrasound when using 0.1 M phosphate buffered saline (PBS, 0.58%) or 0.9% (w/v) sodium chloride (saline) (Monsen et al., 2009). This is again likely due to the fact that the viscosities of these two media are very similar.

### 2.4.2.2 Volume

Volume of bacteria used in different studies varies vastly from few millilitres to more than 1 litre (Lee et al., 2003; Valero et al., 2007). The volumes used depend on the ultrasound equipment and the objective of the experiments. Normally the inactivation rate decreases with the increasing volume when the same ultrasound equipment and conditions are used. This is due to the fact that the actual ultrasonic energy delivered per millilitre of media (i.e. W/ml) is greater for a small volume of working suspensions than for a large one (Huang et al., 2006a). For instance it was found that about 70% and 30% of *B. subtilis* were inactivated in volumes of 100 ml and 150 ml respectively, while almost no bacteria were inactivated in a volume of 200 ml, when sonicated at 20 kHz for 15 min (Joyce et al., 2003b). In another study, the effectiveness of ultrasound treatment of *Salmonella enteritidis* decreased when the volume of whole egg liquid was increased from 12.5 ml to 25 ml; a log reduction of 2.30 and 1.62, respectively, was reported (Huang et al., 2006a). The removal percentage of *E. coli* was 90, 86 and 85% when treated in an ultrasonic bath at 42 kHz with a power of 70 W for 30 min, in volumes of 200, 400 and 600 ml respectively (Dehghani, 2005). These observations clearly suggested that the microbial inactivation is volume dependent, in order to achieve same effectiveness, higher power is required.

### 2.4.2.3 pH

The use of pH to assist ultrasound inactivation of bacteria has also been investigated. However, there are some discrepancies in the case of *E. coli* ATCC 25922 treated at pH 2.5 and 4.0 using both citric acid and malic acid for pH adjustment (Salleh-Mack and Roberts, 2007). The study found the bacteria were slightly resistant to ultrasound
at higher pH (5.1-log reduction) than in lower pH (5.4-log reduction), while the type of acid used did not affect the results significantly. However, it was reported that there were no significant differences in $D$-values when $S.\ cerevisiae$ KE 162 was sonicated (20 kHz, 71.4 $\mu$m, 35$^\circ$C) at pH 5.6 and 3.0, and the $D$-values were 29.1 and 30.9 min respectively (Guerrero et al., 2001). It was also reported that the pH, ranging from 4.0 to 7.0, did not influence the effects of MS treatment (20 kHz, 117 $\mu$m, 200 kPa) on $C.\ sakazakii$ (Arroyo et al., 2011b).

Note that most studies focused on low-frequency ultrasound treatment, and that at the best of our knowledge, there are no reports of pH on the effect of high-frequency ultrasound treatment of bacteria under different pH conditions. This fundamental knowledge is important, since pH may affect sonochemical reactions in high-frequency system.

2.4.2.4 Initial microbial number

The majority of publications reported that the ultrasound inactivation of bacteria is more efficient on low initial concentrations of microorganisms compared to high initial microorganisms concentrations. It was reported that 99.9% of $E.\ coli$ K12 were inactivated within 3 min at a concentration of $4 \times 10^6$ CFU/ml, while 4 min were needed to achieve the same effect on a concentration of $2 \times 10^9$ CFU/ml (20 kHz, 12.57 W/cm$^3$) (Hunter et al., 2008). $S.\ cerevisiae$ A50 was ultrasonicated by a horn-type sonicator at 27.5 kHz at a range of initial numbers ($10^2$ to $10^5$ cell/ml), and low initial concentrations of cells were more sensitive to ultrasound (about 1.8-log reduction for $10^2$ cell/ml) than high concentrations (about 0.3-log reduction for $10^5$ cell/ml) (Tsukamoto, 2004). However, it was also reported that there were no significant effects of the initial concentrations on the inactivation process. For instance, $Mycobacterium$ sp. 6PY1 suspensions were inactivated by ultrasound at 20 and 612 kHz for 70 min, at concentrations of $2.15 \times 10^{-3}$ to $1.4 \times 10^{-2}$ mg protein/L$^{12}$. The

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$^{12}$ In this case, the concentration was given as the amount of protein per liter.
results showed that independently of the concentration, a removal of approximately 93% and 35.5% at 20 and 612 kHz, respectively, were achieved (Al Bsoul et al., 2010).

Intuitively one would expect that the increase in number of microorganism will decrease the rate of inactivation, due to the increase in viscosity and the clumping of the microorganisms. However, other parameters, such as the bacteria characteristics and ultrasound energy, might also affect the relationship between the initial number and the extent of bacteria inactivation.

2.4.3 Properties of microorganisms

The mechanisms of microorganism inactivation are normally due to the damage of cell structure including cell envelope disruption or physiological dysfunctions such as function loss of key enzymes (Manas and Pagan, 2005). Different species of microbes have different sensitivities to ultrasonication. The idea that gram-positive bacteria are more resistant to ultrasonication than gram-negative bacteria has dominated in the literature for some time. However, recently some publications have shown that there is no significant difference in the inactivation of both types of bacteria. Disagreements were also reported when considering the shape and size of the microorganisms.

2.4.3.1 The type of cell wall

Some reports showed that gram-negative bacteria were more sensitive to ultrasonic inactivation than gram-positive bacteria, due to the cell wall of gram-negative bacteria being thinner and weaker compared to that of gram-positive bacteria. This was reported as early as 1975 (Ahmed and Russell, 1975; Alliger, 1975). More recently, gram-positive streptococci were reported to be more resistant to ultrasound than gram-negative bacteria group coliforms (Blume, 2004). Ultrasound treatment (at 24 kHz and 1500 W/L for 60 min) resulted in average inactivation rates of 99.5%, 99.2% and 99.7% for of the gram-negative bacteria coliforms, faecal coliforms and
Pseudomonas spp., respectively, while only 66% and 84% inactivation was reported for gram-positive bacteria C. perfringens and faecal streptococci, respectively under the same ultrasound treatment conditions (Drakopoulou et al., 2009). Gram-negative E. coli and gram-positive L. acidophilus isolated from pasteurised milk were ultrasonicated at 20 kHz and 750 W for 10 min. The results indicated that more than 99% of E. coli was inactivated, but only 72% and 84% of L. acidophilus were inactivated in saline solution and UHT milk (Cameron et al., 2008). Ultrasonication of gram-negative bacteria including E. coli, Pseudomonas aeruginosa and Haemophilus influenzae, as well as gram-positive bacteria Enterococcus faecalis, S. aureus, and S. epidermidis was carried out by using an ultrasound generator at different temperatures (6–42°C, 20 kHz) (Monsen et al., 2009). The results also showed that the gram-positive bacteria were more resistant to inactivation by ultrasound than the gram-negative bacteria. In another study, gram-positive bacterium Streptococcus thermophilus was found to be more resistant than the gram-negative Pseudomonas fluorescens (sonicated at 20 kHz) (Villamiel and de Jong, 2000a).

However, other researchers indicated that ultrasonication (26 kHz) of gram-positive S. aureus and B. subtilis and gram-negative E. coli and P. aeruginosa did not show any major differences (Scherba et al., 1991). Ultrasound experiments at 20 kHz performed on a mixture of gram-negative and gram-positive bacteria, including E. coli, P. fluorescens, Chryseobacterium meningosepticum, L. acidophilus, Lactococcus lactis, B. cereus and L. monocytogenes also did not show any relationship between gram status and the effects of ultrasonication (Cameron, 2007). These authors explained their observation by the fact that cytoplasmic membrane of these microorganisms consists of a lipoprotein bilayer, rather than cell wall, and thus very sensible to ultrasound inactivation.

2.4.3.2 Size and shape

Larger cells were reported to be more susceptible to ultrasound than small cells (Alliger, 1975; Nesaratnam et al., 1982). This is mainly because they have larger
surface area, which results, under ultrasonication, in higher tensile forces than in the case of microorganisms with a smaller surface area (Thacker, 1973; Joyce et al., 2011). Similarly, it was suggested that the larger cells of \textit{K. pneumoniae} NCTC 418 were more sensitive to ultrasound treatment because of their large cross-sectional area (Nesaratnam et al., 1982). Rod-shaped bacteria are also reported to be more sensitive than coccus-shaped cells (Alliger, 1975) due to their cell surface to volume ratio (Chemat et al., 2011). However, as for gram-status, it was also reported that there was no direct relationship between the size or shape of cells and the effects of ultrasonication (Cameron, 2007).

2.4.3.3 Bacteria with spores

Microbial spores are resistant to detrimental conditions including extreme temperatures and high or low pH (Moir and Smith, 1990; Chemat et al., 2011). For example it would require 4 h at 100°C to destroy \textit{Bacillus thermophilus} spores and \textit{B. subtilis} spores have been considered an indicator for the sterilisation process because of their high resistance to thermal treatment (Chemat et al., 2011). Similarly, spore formers were also found to be more resistant to ultrasound than vegetative bacteria (Earnshaw et al., 1995; Drakopoulou et al., 2009). However, ultrasonicated \textit{Bacillus} spores were found to be more sensitive to heat treatment than non-ultrasonicated ones (Ordoñez and Burgos, 1976), since the cells had been pre-damaged by ultrasound treatment. In another study, scanning electron microscope (SEM) images confirmed the disruption and lysis of the \textit{Bacillus globigii} spores and cracked spores and “pustules” near the surface of spores when subjected to ultrasound treatment (Chandler et al., 2001).

2.4.3.4 Growth phases

The effect of ultrasound on microorganisms in their different growth phase has not been extensively considered. It was however reported that dividing yeast cells were more vulnerable to ultrasonication than when in their stationary phase, because the
dividing yeast cells or diploid cells were much larger than haploid cells (Thacker, 1973). Manosonication (20 kHz, pH 7.0 buffer, 117 μm, 200 kPa) of *C. sakazakii* showed that this bacterium was 1.6 times more resistant when in the stationary and death phases than when manosonicated in the log phase (Arroyo et al., 2011b).

2.4.3.5 Viruses

Viruses were also investigated as targets for ultrasound treatment. Three species of virus surrogates were examined including *Murine norovirus* (MNV-1), *Feline calicivirus* (FCV-F9), and MS2 bacteriophage, by using high-intensity ultrasound treatment at 20 kHz for 2 to 30 min (Su et al., 2010). However, the ultrasound treatment alone was not sufficient to inactivate these viruses in food such as orange juice. Thus, it was recommended that a combination of ultrasound with other processing techniques, such as heat and high pressure, is normally required to achieve viruses inactivation. The bacteriophages ΦX174 and MS2 were treated different high frequency ultrasound (582, 862, and 1142 kHz, 133 W) in combination with visible light 13 (Chrysikopoulos et al., 2013). It was found that these bacteriophages were sensitive to ultrasound treatment at the lower frequencies (582 and 862 kHz), and that ΦX174 was more resistant to ultrasound than MS2.

2.4.4 Ultrasound as a hurdle technology

Antoniadis et al. (2007) have reported that although ultrasound treatment alone can powerfully inactivate some bacteria, it requires high power to reach a 100% kill rate, which renders the technique to be expensive. As a result, combinations with other inactivation techniques can be used to lower the processing cost and improve its efficiency. Typical methods which can be combined with ultrasound are thermal, high pressure or both, resulting processes are termed manosonication (MS), thermosonication (TS), manothermosonication (MTS) as mentioned above, respectively. Other technologies include pulsed magnetic fields (PMF), pulsed electric

13 The specific conditions of the visible light were not mentioned in the Chrysikopoulos et al.’s paper.
fields (PEF), high intensity light pulses (HILP), ultraviolet (UV), supercritical carbon dioxide (SC-CO₂), and the addition of catalyst such as titanium dioxide (TiO₂) or disinfectants.

Some applications of MS and MTS have been covered previously in this literature review. In addition, MTS treatment at 300 kPa for 12 min inactivated 75% to 99.9% of *B. subtilis* spores at ultrasound amplitudes of 90 to 150 µm, and the inactivation of *B. subtilis* increased further when the processing temperature was increased to 70°C (Raso et al., 1998b).

Ultrasound (20 kHz) and pulsed electric fields or high intensity light pulses, emerging non-thermal technologies, were also used for the inactivation of *E. coli* and *L. innocua* (Muñoz et al., 2012b). The combination of these two processes of ultrasound and pulsed electric fields achieved a 4.0-log reduction for *E. coli*, compared to a 2.6-log reduction for each process alone. While the application of pulsed electric fields and ultrasound achieved only a 1.6-log reduction for *L. innocua*, this remained much higher than the reduction achieved by pulsed electric fields (0.3-log reduction) or ultrasound (1.1-log reduction) alone (Muñoz et al., 2012b). In combination with high intensity light pulses, the inactivation of *E. coli* (3.9-log reduction) was not much different from the levels of inactivation achieved by high intensity light pulses alone (3.5-log reduction). In contrast, combination of ultrasound and high intensity light pulses resulted in a 4.1-log reduction of *L. innocua* compared to a 2.7-log reduction by high intensity light pulses alone and a 1.1-log reduction by ultrasound alone. In addition, the combination of ultrasound and high intensity light pulses resulted in a marked increase in the inactivation of *E. coli*, from a 2.7 log reduction for thermosonication (24 kHz, 100 µm, 50°C, 5 min) to nearly a 6 log reduction when thermosonication and high intensity light pulses treatments were used (Muñoz et al., 2012a). Ultrasound was also combined with pulsed magnetic fields treatment to inactivate *E. coli* ATCC 11775, but this did not achieve any significant difference compared to ultrasound treatment alone (20 kHz, 70 W) (San Martin et al., 2001).
The combination of UV-C (254 nm)\textsuperscript{14} and ultrasound (20 kHz) was also reported to be very effective on the inactivation of common fish pathogens such as heterotrophic bacteria and *Paramecium* spp. in recirculating aquaculture systems\textsuperscript{15}, compared to ultrasound alone which was ineffective in inactivating these pathogens (Bazyar Lakeh et al., 2013).

The SC-CO\textsubscript{2} technique is another non-thermal processing method used for bacteria inactivation. SC-CO\textsubscript{2} (350 bar, 36°C) caused a 0.3-log reduction for *E. coli* DH1 in 5 min and it achieved a 1-log reduction using high-power ultrasound treatment (30 kHz, 40 ± 5 W); the combination of these two methods resulted in a 8.5-log reduction (Ortuñ\~no et al., 2012). Similarly, 2 min application of ultrasound and SC-CO\textsubscript{2} resulted in 7-log reduction for *S. cerevisiae* (Ortuñ\~no et al., 2013). The main reasons for such a synergistic effect could be due to the solubilisation of CO\textsubscript{2} and the transfer of cell-medium mass as a result of ultrasound treatment (Ortuñ\~no et al., 2012).

TiO\textsubscript{2} is sometimes used when ultrasonication is used for disinfection purposes. (Dadjour et al., 2006; Drakopoulou et al., 2009; Rahman et al., 2010). The presence of TiO\textsubscript{2} is believed to promote the formation of hydroxyl radicals by heterogeneous nucleation, and that it can also form TiO\textsubscript{3} that can aggravate oxidative damage to bacteria. In addition, excitation of TiO\textsubscript{2} by the thermal and sonoluminescence effects can lead to light-induced photocatalytic inactivation (Dadjour et al., 2006; Drakopoulou et al., 2009; Rahman et al., 2010). The addition of TiO\textsubscript{2} during ultrasonication was reported to cause damage and accelerated peroxidation of the cell membrane of *E. coli* (Rahman et al., 2010). Another disinfectant trialled with ultrasound treatment is Kohrsolex AF\textsuperscript{16} (Jatzwauk et al., 2001). The combination of ultrasound and Kohrsolex AF resulted in the inactivation of *S. aureus, P. aeruginosa*

\textsuperscript{14} Ultraviolet C (UV-C) means ultraviolet radiation with wavelengths between 200 and 290 nm.

\textsuperscript{15} The water used in the experiments was obtained from recirculating aquaculture systems.

\textsuperscript{16} Kohrsolex AF was an aldehyde-free disinfectant that contained 5.1% dodecylbispropylene-diamine and 16.5% laurylpropylene-diamine.
and *Candida albicans*, while ultrasound alone (35 kHz, 0.66 W/cm² for 1 h) did not cause significant reduction in the case of these bacteria.

Biofilm was also treated by ultrasound, but ultrasound alone was not sufficient to inactive the biofilm *E. coli* at either 70 kHz or 500 kHz, but it enhanced the killing by the antibiotic gentamicin (Peterson and Pitt, 2000).

### 2.5 Effects of ultrasound treatment on food

#### 2.5.1 Ultrasound processing of food systems

As a novel processing technology, ultrasound treatment has the potential for a wide range of food and other industrial applications, including mixing and homogenisation, separation, crystallisation, drying, filtration, viscosity alteration, defoaming, and enzyme and microbial inactivation (Table 2.1). Some of these applications, such as emulsification, defoaming and extraction have already been industrially implemented (Patist and Bates, 2008), although not in the food industry. Most food related applications, including filtration (Chemat et al., 2011) and bacteria inactivation (Sagong et al., 2011) remain at the laboratory research stage.
Table 2.1 Examples of the applications of ultrasound in food systems.

<table>
<thead>
<tr>
<th>Application</th>
<th>Mechanism</th>
<th>Benefits</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>Increase the movement of solvents; cavitation causes the swelling of cells and breaks cell walls.</td>
<td>Reduction of preparation time; reduce reagent consumption.</td>
<td>(Vinatoru, 2001; Patist and Bates, 2008; Khan et al., 2010; Tadeo et al., 2010)</td>
</tr>
<tr>
<td>Mixing and homogenisation</td>
<td>Ultrasonic vibrations.</td>
<td>Produce fine particles and a smooth texture, increase efficiency.</td>
<td>(Mason et al., 2005; Chemat et al., 2011)</td>
</tr>
<tr>
<td>Filtration</td>
<td>Ultrasonic vibrations.</td>
<td>Reduce steady state membrane flux; improve filtration.</td>
<td>(Muthukumaran et al., 2007; Chemat et al., 2011)</td>
</tr>
<tr>
<td>Defoaming</td>
<td>The propagation of high power ultrasonic waves.</td>
<td>Avoid chemical contamination.</td>
<td>(Chemat et al., 2011)</td>
</tr>
<tr>
<td>Crystallisation</td>
<td>Influence nucleation, sonocrystallisation.</td>
<td>More efficient; produce smaller crystal sizes; reduce agglomeration.</td>
<td>(Li et al., 2006; Luquedecastro and Priegocapote, 2007; Narducci et al., 2011)</td>
</tr>
<tr>
<td>Drying</td>
<td>Ultrasonic vibration, uniform heat transfer.</td>
<td>Less time; reduce the loss of quality and characteristics of the food products.</td>
<td>(Mason et al., 2005; Chemat et al., 2011)</td>
</tr>
<tr>
<td>Viscosity alteration</td>
<td>Sonochemical modification.</td>
<td>No chemicals or additives needed, reduce the changing of the properties of product.</td>
<td>(Iida et al., 2008; Patist and Bates, 2008)</td>
</tr>
<tr>
<td>Enzyme and microbial inactivation</td>
<td>Cavitation induces mechanical effects and sonochemical reaction (formation of free radicals).</td>
<td>Reduce the loss of important nutritional components and minimally affects to physical characteristics of food including flavour, texture and colour.</td>
<td>(Mason, 1990; Earnshaw et al., 1995; Sala et al., 1995; Butz and Tauscher, 2002; Russell, 2002; Mason et al., 2005; Ashokkumar, 2011).</td>
</tr>
</tbody>
</table>

2.5.2 Inactivation of microorganisms in food systems

In this section inactivation of microorganisms by ultrasound in food related systems is reviewed. Inactivation of microorganisms in milks will be discussed in the next section, since this part is central to this thesis.
Microbial inactivation by ultrasound treatment is predominantly used in surface cleaning and disinfection as well as wastewater decontamination, rather than for the inactivation or inhibition of microbes in foods (Bilek and Turantas, 2013). Most of the research dealing with the inactivation of microorganisms in food systems was carried out in model liquid food matrices such as water, juice, milk, fruit, and solid or semi-solid foods such as vegetables, meat and eggs (Table 2.2). Bacteria inactivation in liquid foods has been covered in previous sections. In this section, applications in semi-solid or solid foods are reviewed. Applications of ultrasound for the treatment of solid foods include apples (Huang et al., 2006b), strawberries (Seymour et al., 2002; Cao et al., 2010), plums (Chen and Zhu, 2011), lettuce (Huang et al., 2006b; Sagong et al., 2011; Elizaquivel et al., 2012; Birmpa et al., 2013), carrots (Seymour et al., 2002; Alegria et al., 2009), cucumbers (Seymour et al., 2002), cherry tomatoes (Brilhante São José and Dantas Vanetti, 2012), red bell peppers (Alexandre et al., 2013), spinach leaves (Zhou et al., 2009), white cabbage (Seymour et al., 2002), spring onions (Seymour et al., 2002), and parsley (Seymour et al., 2002). Since cavitation, which is fundamental to bacteria inactivation, occurs in liquid media (Suslick, 1988), solid foods need to be immersed in aqueous media during ultrasonication.

As an example, treatment of strawberries was performed by immersing the fruit into a water bath in an ultrasonic chamber at 20°C, and ultrasonicated at different frequencies (25, 28, 40 or 59 kHz) for 10 min (Cao et al., 2010). As a result approximately a 0.3 to 0.9-log reduction for bacteria and a 0.2 to 1.1-log reduction for mould and yeast was achieved. The ultrasound treatment at 40 kHz resulted in a significant reduction of decay after 8 days of storage at 5°C compared to non-treated strawberries.

Because the rate of inactivation in solid or semi-solid foods is very low, sanitisers are used in the aqueous medium to improve bacterial inactivation. For example, cherry

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17 The specific names of the microorganisms were not mentioned in Cao et al.’s paper.
tomatoes were ultrasonicated in the presence of commercial sanitisers (200 mg/L sodium dichloroisocyanurate or 40 mg/L peracetic acid) for 10 min, and an average of 3.9-log reduction of *Salmonella* Typhimurium ATCC 14028 was achieved, compared to ultrasonication or immersion in the sanitisers alone (0.4-2.7-log reduction) (Brilhante São José and Dantas Vanetti, 2012). When ultrasound treatment of lettuce was performed in the presence of organic acids, an additional 0.8 to 1.0-log reduction of *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. monocytogenes* was achieved (Sagong et al., 2011).

In addition to the use of sanitisers, hurdle technologies are also employed. For instance, TS, the combination of thermal and ultrasound treatment (54°C, 24 kHz, 400 W, 60 μm), was used to decontaminate eggshells, by sonicating a single egg immersed in 200 ml of water (Cabeza et al., 2011). This process was found to be effective against *Salmonella enterica*, *Salmonella* Enteritidis and *Salmonella* Typhimurium, (approximately a 5-log reduction was achieved on *Salmonella* Enteritidis), without effecting the physical characteristics of the eggshells.

In the case of meat samples, the effect of ultrasonication is related to the part of the cut that has been sonicated. The MS of part of fresh pork jowl inoculated with bacteria, showed that 0.6 to 3.6-log reductions were achieved for *E. coli*, *Salmonella* Typhimurium, and *Y. enterocolitica* on the skin surface (0.5–2 s). In contrast, 0.4 to 2.5-log reductions were obtained for the meat surface of the pork jowl (Morild et al., 2011).
## Table 2.2 Ultrasound inactivation of microbes in foods.

<table>
<thead>
<tr>
<th>Product</th>
<th>Microbes</th>
<th>Initial number</th>
<th>Media volume</th>
<th>Treatments</th>
<th>Ultrasound conditions</th>
<th>Microbial log reduction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model apple juice</td>
<td><em>Escherichia coli</em> ATCC 25922</td>
<td>1×10⁶ CFU/ml</td>
<td>50 ml</td>
<td>US</td>
<td>20 kHz, 0.4-37.5 μm; &lt;30°C; 0-15 min</td>
<td>0.4μm: 3 7.5μm: total inactivation in 6 min 37.5μm: total inactivation in 3 min</td>
<td>(Patil et al., 2009)</td>
</tr>
<tr>
<td>Apple juice</td>
<td><em>Alicyclobacillus acidophilus</em> DSM14558T, <em>Alicyclobacillus acidoterrestris</em> DSM 3922T</td>
<td>1.9×10⁵-5.8×10⁸ CFU/ml</td>
<td>500 ml</td>
<td>US</td>
<td>25 kHz; 200-600 W; &lt;50°C; 1-30 min</td>
<td>Up to 4.56</td>
<td>(Wang et al., 2010)</td>
</tr>
<tr>
<td>Apple juice</td>
<td><em>Saccharomyces cerevisiae</em> ATCC 4113</td>
<td>&gt;10⁷ CFU/ml</td>
<td>500 ml</td>
<td>TS</td>
<td>24 kHz; 120 μm; 60°C; 30 min</td>
<td>Total inactivation</td>
<td>(Marx et al., 2011)</td>
</tr>
<tr>
<td>Apple juice</td>
<td><em>Escherichia coli</em> K12 DSM 1607</td>
<td>N/A</td>
<td>40 ml</td>
<td>TS+LP</td>
<td>24 kHz; 100 μm (85 W/cm²); 40, 50°C; 2.9, 5 min</td>
<td>TS: up to 2.7 PL : up to 4.9 TS+PL: up to 6</td>
<td>(Muñoz et al., 2012a)</td>
</tr>
<tr>
<td>Apple juice</td>
<td><em>Escherichia coli</em> O157:H7, <em>Listeria monocytogenes</em>, <em>Salmonella</em> spp., Yeast mixture</td>
<td>Stationary phase 1000 ml</td>
<td>US (Dynashock) /US+UV-C</td>
<td>28, 45, 100 kHz; Up to 600 W (50–100%); 18.07-44.03°C; 0-30 min</td>
<td>US: up to about 1.8 US+UV-C: 2.59 for <em>E. coli</em> O157:H7 in 15 min</td>
<td>(Gabriel, 2012)</td>
<td></td>
</tr>
<tr>
<td>Model orange juice</td>
<td><em>Escherichia coli</em> (ATCC 25922, NCTC 12900)</td>
<td>1×10⁶ CFU/ml</td>
<td>50 ml</td>
<td>US</td>
<td>20 kHz; 0.4-37.5 μm; &lt;30°C; 15 min</td>
<td>ATCC 25922: 7.5 and 37.5 μm: total inactivation, NCTC 12900: 7.5 μm: 2.5; 37.5 μm: 2.7</td>
<td>(Patil et al., 2009)</td>
</tr>
<tr>
<td>Orange juice</td>
<td>Total mesophilic aerobes</td>
<td>N/A</td>
<td>1.4 L</td>
<td>US+Thermal</td>
<td>23, 500 kHz; 120-600 W (60%); 17.9-88%; 15 min</td>
<td>0.1-1.7</td>
<td>(Valero et al., 2007)</td>
</tr>
<tr>
<td>Orange juice</td>
<td><em>Salmonella</em> spp.</td>
<td>6–7 log CFU/ ml</td>
<td>250 ml</td>
<td>US+ Osmotic pressure</td>
<td>20 kHz; 50±0.2 W, 48 μm (40%), 0.83 W/ml; 25±2°C; 6.8-20.4 min</td>
<td>0.3-4.1</td>
<td>(Wong et al., 2012)</td>
</tr>
<tr>
<td>Tomato juice</td>
<td><em>Pichia fermentans</em> DSM 70090</td>
<td>Stationary phase</td>
<td>80 ml</td>
<td>US</td>
<td>20 kHz; 0.33-0.81 W/ml, 24.4-61 μm; 25±1.0°C; 10 min</td>
<td>About 1 log to total inactivation</td>
<td>(Adekunte et al., 2010a)</td>
</tr>
<tr>
<td>Milk</td>
<td><em>Pseudomonas fluorescens</em> NIZO B337, <em>Streptococcus thermophilus</em> B8</td>
<td>10⁵ CFU/ml</td>
<td>up to 100 ml</td>
<td>US</td>
<td>20 kHz; Up to 120 μm; 33-76°C</td>
<td><em>P. fluorescens</em>: 0.2-4.2 <em>S. thermophilus</em>: 0-2.7</td>
<td>(Villamiel and de Jong, 2000a)</td>
</tr>
<tr>
<td>Ultra high temperature (UHT) milk</td>
<td><em>Escherichia coli</em>, <em>Lactobacillus acidophilus</em>, <em>Saccharomyces cerevisiae</em></td>
<td>1×10⁶ &amp; 1×10⁷ CFU/ml</td>
<td>40 ml</td>
<td>US</td>
<td>20 kHz; 750 W, 124 μm (100%); 24-26°C; 10 min</td>
<td><em>E. coli</em>: 4.4 <em>L. acidophilus</em>: 0.8 <em>S. cerevisiae</em>: 2.1</td>
<td>(Cameron et al., 2008)</td>
</tr>
<tr>
<td>Raw whole milk</td>
<td><em>Listeria innocua</em> ATCC 51742</td>
<td>Around 10⁷ CFU/ml</td>
<td>500 ml</td>
<td>TS/Thermal</td>
<td>24 kHz; Up to 400 W, 40-120 μm (0.86-2.85 W/cm²); 63±0.5°C; 2-30 min</td>
<td>TS: 5 (10 min)  Thermal: 5.3 (30 min)</td>
<td>(Bermúdez-Aguirre et al., 2009)</td>
</tr>
<tr>
<td>Product</td>
<td>Microbes</td>
<td>Initial number</td>
<td>Media volume</td>
<td>Treatments</td>
<td>Ultrasound conditions</td>
<td>Microbial log reduction</td>
<td>References</td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>----------------</td>
<td>--------------</td>
<td>------------</td>
<td>-----------------------</td>
<td>------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Reconstituted powdered infant formula</td>
<td><em>Cronobacter sakazakii</em> (NCTC 08155 and ATCC 11467)</td>
<td>About $10^9$ CFU/ml</td>
<td>50 ml</td>
<td>US</td>
<td>20 kHz; 24.4-61 μm; 25-50°C; 10 min</td>
<td>&gt;3</td>
<td>(Adekunte et al., 2010b)</td>
</tr>
<tr>
<td>Raw milk</td>
<td>Total aerobic mesophilic bacteria (TAMB), Coliform bacteria (CB)</td>
<td>TAMB: 8.6 log CFU/ml; CB: 5.31 log CFU/ml</td>
<td>200 ml</td>
<td>US+ UV-C /Thermal</td>
<td>24 kHz; 120 μm (240 W); &lt;30/65°C; 15/30 min</td>
<td>US+ UV-C (15 min); TAMB: 4.79; CB: 5.31. US (15 min). TAMB: 1.31; CB: 4.01. Thermal (30 min): TAMB: 3.28; CB: 5.31.</td>
<td>(Şengül et al., 2011)</td>
</tr>
<tr>
<td>Liquid whole egg</td>
<td><em>Salmonella enteritidis</em></td>
<td>Stationary phase</td>
<td>12.5-25 ml</td>
<td>US /US+Thermal /US+PEF /US+HHP</td>
<td>20 kHz; 34.6-40 W; 5-55°C; 30 s-5 min</td>
<td>US (5 min): 1 US+ Thermal (5 min): 1-2.3 US+PEF: 2.25-2.3 US+HHP (30-150 s): up to 3</td>
<td>(Huang et al., 2006a)</td>
</tr>
<tr>
<td>Liquid whole egg</td>
<td><em>Escherichia coli</em> K12 DH 5a, <em>Listeria seeligeri</em> NCTC 11289</td>
<td><em>E. coli</em> ~ $10^8$ CFU/ml; <em>L. seeligeri</em> ~ $10^7$ CFU/ml</td>
<td>10 ml</td>
<td>US / US + HHP</td>
<td>20 kHz; 24.6-42.0 W; Iced water bath; Up to 300 s</td>
<td><em>E. coli</em>: 1-2 <em>L. seeligeri</em>: no inactivation</td>
<td>(Lee et al., 2003)</td>
</tr>
<tr>
<td>Tomato paste</td>
<td><em>Lactobacillus sporogenes</em></td>
<td>1×$10^7$ CFU/ml</td>
<td>N/A</td>
<td>US + $^{60}$Co-γ irradiation</td>
<td>40 kHz; 60-120 W; 20°C; 0-25 min</td>
<td>Up to total inactivation</td>
<td>(Ye et al., 2009)</td>
</tr>
<tr>
<td>Cherry tomatoes</td>
<td><em>Salmonella enteritidis</em> serovar Typhimurium ATCC 14028</td>
<td>6-7 log CFU/ml</td>
<td>N/A</td>
<td>US+ Commercial sanitisers</td>
<td>40 kHz;10 min</td>
<td>3.9</td>
<td>(Brilhante São José and Dantas Vanetti, 2012)</td>
</tr>
<tr>
<td>Lettuce</td>
<td><em>Escherichia coli</em> O157:H7, <em>Salmonella Typhimurium, L. monocytogenes</em></td>
<td>$10^6$ CFU/ml</td>
<td>N/A</td>
<td>US + Organic acids</td>
<td>40 kHz; 30 W/L; 5 min</td>
<td>2.75, 3.18, and 2.87</td>
<td>(Sagong et al., 2011)</td>
</tr>
<tr>
<td>Strawberry</td>
<td>Bacteria, Mould and yeast.</td>
<td>N/A</td>
<td>N/A</td>
<td>US</td>
<td>25, 28, 40 or 59 kHz; 20°C; 10 min</td>
<td>According to frequency: <em>Bacteria</em>: 0.88, 0.79, 0.66, 0.26 <em>Mould and yeast</em>: 1.07, 0.97, 0.54, 0.22</td>
<td>(Cao et al., 2010)</td>
</tr>
<tr>
<td>Skin and meat surfaces of pork jowl</td>
<td><em>Escherichia coli</em>, <em>Salmonella Typhimurium, V. enterocolitica</em></td>
<td>$10^5$ or $10^6$ CFU/cm²</td>
<td>N/A</td>
<td>US + high-pressure steam</td>
<td>30 to 40 kHz; 130°C; 0.5-2 s</td>
<td><em>E. coli</em> on skin 0.9-3.3; meat 1.0-2.5; <em>Salmonella</em> on skin 0.6-3.2; meat 0.4-2.1; <em>V. enterocolitica</em> on skin 0.8-3.6; meat 0.6-2.1</td>
<td>(Morild et al., 2011)</td>
</tr>
<tr>
<td>Egg and eggshell</td>
<td><em>Salmonella enteritidis</em></td>
<td>7.78 log CFU</td>
<td>200 ml water</td>
<td>TS</td>
<td>24 kHz; 400 W, 60 μm; 54°C; 5 min</td>
<td>4.8</td>
<td>(Cabeza et al., 2011)</td>
</tr>
</tbody>
</table>

Note: The list of abbreviations in the table are: CFU, colony forming units of microorganisms; US, ultrasound treatment; TS, thermosonication; LP, light pulses; UV-C, Ultraviolet C; PEF, pulsed electric fields; HHP, Hydraulic high pressure; N/A, not available.
2.6 Ultrasonic inactivation in milk and effects on milk properties

2.6.1 Milk

Milk is considered one of the most complete foods, as it is highly nutritious, readily digested and rich in essential components such as protein, minerals and vitamins. Having a high-water content, milk is highly perishable because it can be easily contaminated by microorganisms. Thermal treatment is always involved in milk processing to reduce microbial spoilage, however, this causes protein denaturation and modifications (Corzo et al., 1994; Anema, 2008a; Lan et al., 2010). Thermal treatment also results in protein nutrient quality to decrease (AlKanhal et al., 2001) and produce undesirable flavours and colours (Barbosa-Cánovas et al., 2010). As a result, non-thermal treatments such as high pressure and ultrasound are considered to be promising techniques for milk pasteurisation without causing the loss of nutritional and sensorial properties of milk and milk products under controlled sonication conditions.

While the published literature about milk is exhaustive, only aspects of milk which are most relevant to the thesis are discussed here. For example, since the work involving milk in this thesis was made from reconstituted low-heat skim milk powder, the fat component of milk will not be discussed.

2.6.1.1 Milk composition

Milk is a biological fluid with a complex composition (Reimerdes and Mehrens, 1994; Varnam and Sutherland, 1994). Milk is regarded as a colloidal dispersion, and the dispersed medium is casein micelles and fat globules, while the dispersion phase is an aqueous solution which includes whey proteins and lactose (Bienvenue et al., 2003). For bovine milk, the constituents of milk may vary among the cow’s origin, but normally they have average levels. Bovine milk includes about 87.1% water, 3.3% proteins, 4.0% fat, 4.6% lactose and some minerals, vitamins and enzymes (Walstra et
Vitamins and enzymes can act as antioxidants that can scavenge radicals or hydrogen peroxide (Lindmark-Månsson and Åkesson, 2000). Milk proteins were also reported to have antioxidative activities (Pihlanto, 2006).

2.6.1.2 Proteins

The proteins in milk play important roles in human nutrition and their physical and chemical characteristics significantly influence the behaviour and properties of dairy products (Whitney, 1995). Bovine milk consists of 3.0–3.5% (w/v) proteins, mainly ~80% caseins and ~20% whey proteins (Wang et al., 2009). The caseins consist of four types: αs1-casein (αs1-CN), αs2-casein (αs2-CN), β-casein (β-CN) and κ-casein (κ-CN) with a ratio of 4:1:4:1, and the whey proteins contain α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lg) with the proportion of 1:3 (Visser et al., 1991; Marchin et al., 2007; Bonfatti et al., 2008; Wang et al., 2009; McMahon and Oommen, 2013). Bovine serum albumin (BSA), immunoglobulins (Igs) and lactoferrin are also part of the whey proteins although present in very small amounts (Singh and Creamer, 1991; Farrell et al., 2004). The solubility behaviour is different for caseins and whey protein in acidic condition, and caseins precipitate at pH 4.6 (Walstra et al., 2006).

A. Caseins

Caseins are phosphoproteins, and each of these caseins has several variants (Walstra et al., 2006). Each one of them is made of a single polypeptide chain (Horne, 2002). Caseins are hydrophobic but have a high charge, which keep them stable in milk (Walstra et al., 2006). Caseins are heterogeneous because of the effects of post-translational modifications for individual casein (Ginger and Grigor, 1999). Two major post-translational modifications including glycosylation and phosphorylation, which are critical for the assemblage of individual caseins and their physic-chemical properties (Horne, 2002). They do not undergo denaturation as they have little secondary and tertiary structure (Walstra et al., 2006).
The unique characteristic of caseins is their relative sensitivity to calcium based on the phosphoserine clusters and post-translational modifications (Horne, 2002). Two groups of caseins are defined according to their sensitivity to calcium, the calcium-sensitive caseins including $\alpha_{s1}$-CN, $\alpha_{s2}$-CN and $\beta$-CN and the calcium-insensitive caseins is $\kappa$-CN (Ginger and Grigor, 1999; Horne, 2002). The calcium-sensitive caseins consist of phosphoserine residues in clusters in sequences while $\kappa$-CN only contains one phosphoserine residue and has low capacity for calcium phosphate binding (Gaucheron, 2005; Huppertz, 2013).

$\alpha_{s1}$-CN is the dominant protein fraction in milk (up to 40% of total caseins) (Farrell et al., 2004) and it is highly phosphorylated (Huppertz, 2013). $\alpha_{s1}$-CN contains one major and one small phosphorylated form consisting of 8 to 9 bound phosphate per residue respectively (Ginger and Grigor, 1999; Farrell et al., 2004). It was also reported to possess radical scavenging and antioxidant properties (Kitts, 2005). $\alpha_{s2}$-CN is also highly phosphorylated peptide (Ginger and Grigor, 1999). Unlike the other caseins, the main forms of $\alpha_{s2}$-CN contain intramolecular disulfide bonds (Farrell et al., 2004). $\alpha_{s2}$-CN is the most hydrophilic form of the caseins, as it contains anionic substances including phosphoseryl and glutamyl residues (Farrell et al., 2004). The primary structure of $\beta$-CN consists of 209 amino acid residues (Huppertz, 2013). $\beta$-CN is highly hydrophobic compared to the other caseins (Farrell et al., 2004). $\kappa$-CN is distinct amongst the caseins, as it is the smallest protein and shows calcium-insensitive properties (Huppertz, 2013).

B. Casein micelles

Almost all the caseins in milk are aggregated into large complex colloidal particles, which are known as micelles, consisting of the four caseins mentioned above (Marchin et al., 2007; McMahon and Oommen, 2013). On a total solid basis, approximately 94% of the micelle is made of proteins, while the remaining correspond to the colloidal calcium phosphate (CCP) that mainly contains calcium, phosphate, magnesium and other small amounts of low molecular mass species.
(O’Mahony and Fox, 2013). Electron microscopy shows that casein micelles are spherical in shape with a size range of 50–500 nm and their average size is 120 nm in diameter (O’Mahony and Fox, 2013). The molecular weight of casein micelles is in a range of $10^8$ to $10^9$ Da (O’Mahony and Fox, 2013).

The structure of casein micelles has not been unequivocally elucidated, and several models were proposed based on their biochemical and physical properties, including the sub-micelle model (Slattery and Evard, 1973; Slattery, 1977; Schmidt, 1980), the nanocluster model (Holt, 1992; Holt et al., 1998; Kruif and Holt, 2003), and the dual-binding model (Horne, 1998; Horne, 2002; Horne, 2006). The dual-binding model is the most recent model and it has become well known (Figure 2.9). Hydrophobic interactions and electrostatic repulsion are proposed as two distinct types of bonding for assembly and growth of micelles, and they provide a balanced attraction and repulsion forces within the micelles for their association (Horne, 1998). Hydrophobic interactions enable different molecules of caseins assembled together as a positive force. The degree of polymerization of caseins is defined by electrostatic repulsion and further growth is inhibited (Horne, 2008). During the process of self-association, $\alpha_{s1}$-CN, $\alpha_{s2}$-CN and $\beta$-CN bond to calcium phosphate residues and polymerise through their hydrophobic regions. In contrast, $\kappa$-CN only generates linkages through its hydrophobic N-terminal end, and thus they terminates the growth of the micelles because they lack phosphoserine clusters for linkage and do not have another hydrophobic region for extending the chains (Horne, 1998). Therefore, $\kappa$-CN is present on the surface of casein micelles according to this model.
Figure 2.9 Dual bonding model proposed for the casein micelle structure (Horne, 1998).

The structure of casein micelles is dynamic that changes with temperature and pressure or the micellar environment such as pH, ionic charge or presence of chelatant (Horne, 2008). For example, the structure will completely dissociate and each single casein will be released from the micelles when a strong calcium sequestrant such as ethylenediaminetetraacetic acid (EDTA) is added (Griffin et al., 1988).

C. Whey proteins

Whey proteins, also known as serum proteins, are typically globular proteins and do not form micelles (Walstra et al., 2006). α-Lac is a highly structured and compact globular protein consisting of 123 amino acids (Edwards et al., 2008). The stability of α-Lac structure depends highly on its specific nonexposed binding site (Walstra et al., 2006). α-Lac is stable under thermal treatment due to binding of calcium ions (Wehbi et al., 2005). β-Lg is the principal whey protein in bovine milk and contains 162 amino acids (Edwards et al., 2008). Ten genetic variants of β-Lg are known and the most common variants are β-Lg A and B (Farrell et al., 2004). The monomolecular β-Lg consists of two disulphide bridges and a free thiol group which was reported to be linked to the surface of casein micelles during thermal treatment (Singh and Waungana, 2001; Sawyer, 2013).
Whey protein denaturation can cause some undesirable effects including deposit formation, milk instability and gelling, although it can enhance modifications of functional behaviour (Kessler and Beyer, 1991; Law and Leaver, 1999; Anema, 2008a). Casein micelle systems are difficult to be damaged by high temperature, pressure or high shear, while whey proteins are sensitive to thermal treatment (Anema, 2008a). When skim milk is heated at 120°C for 2 min, only 34% of α-Lac were denatured, while 85% of β-Lg were denatured and serum albumin and immunoglobulins were denatured completely (Singh and Creamer, 1991). For the low-heat preheat treatment of skim milk power (72°C for 15 s) only about 1–2% of α-Lac and 6–8% of β-Lg is denatured (Singh and Creamer, 1991).

2.6.2 Microbial inactivation in milk

Although ultrasound inactivation of bacteria and other microorganisms has been studied widely, there have been limited studies conducted in milk which uses it as the bacterial medium. Typical examples of ultrasonic inactivation in milk are reported in Table 2.2. Most previous researches have focused on TS of microbial inactivation in milk instead of using ultrasound treatment alone. This is probably because sonication alone could not inactivate bacteria to desired levels. The occurrence of cavitation is lower in high viscous media such as milk than low viscous media such as water, due to acoustic impedance by high viscous liquids (Salleh-Mack and Roberts, 2007). Therefore, the rate of inactivation decreases in milk compared with water. For instance, studies on milk with *L. monocytogenes* and total aerobic bacteria have shown that inactivation of bacteria was possible with TS (20 kHz, 150 W, 118 W/cm², 18 min) (D'Amico et al., 2006). A 4.82-log reduction of total aerobic bacteria was achieved with mild heat (57°C) in raw milk compared to a 3.13-log reduction by ultrasound treatment alone. TS caused a 5.34-log reduction for *L. monocytogenes* in UHT milk while only a 3.69-log reduction was achieved by sonication alone. It was also reported that the inactivation of *B. subtilis* in milk was more efficient by TS than either heat or ultrasonication alone (20 kHz, 150 W, 70–95°C) (Garcia et al., 1989).
The inactivation of *Listeria innocua* and mesophilic bacteria in raw whole milk by ultrasound treatment was studied using an ultrasonic processor (24 kHz, 120 μm, 400 W) (Bermúdez-Aguirre et al., 2009). When sonicated for 10 min, a 5-log reduction was achieved under mild temperature (63°C); in contrast, a 5.3-log reduction was achieved using thermal pasteurisation alone.

The amount of solids present in the milk system also markedly affects bacteria inactivation by ultrasound. *Listeria innocua* ATCC 51742, in milks with different butter fat contents, was ultrasonicated (24 kHz, 120 μm amplitude, 63°C) for up to 30 min (Bermúdez-Aguirre and Barbosa-Cánovas, 2008). The rate of inactivation increased with the decrease of fat content, and 2.5, 3.2, 4.5 and 4.9 log-reductions were obtained for milk with 3.47%, 2%, 1% butter fat, and fat free milk, respectively (Bermúdez-Aguirre and Barbosa-Cánovas, 2008). This is probably due to the fact that viscosity of the milks is expected to increase with the fat content. It was also reported, in the same study, that fat globules can be broken into small particles by ultrasonication and that bacteria may adhere to these particles or hide inside of them for protection (Bermúdez-Aguirre and Barbosa-Cánovas, 2008).

### 2.6.3 Effects of ultrasound on the physical and chemical properties of milk

Several studies have considered the physical effects generated by ultrasound on milk. Most studies reported that the viscosity of skim milk was not affected at low ultrasonication powers (20 kHz, 20 and 41 W, 15–60 min) (Shanmugam et al., 2012), while the viscosity of concentrated skim milk decreased by about 10% at higher powers (20 kHz, 40–80 W) (Zisu et al., 2013). The viscosity of whey proteins and whey protein retentate also decreased with low-frequency ultrasound treatment (Zisu et al., 2010; Chandrapala et al., 2012). It was suggested that the reduction of the viscosity of skim milk was probably due to the breakup of fat globules and the casein-whey protein aggregates (Chandrapala et al., 2012). In extreme conditions with higher power and longer ultrasound time, the viscosity of milk could increase (Ashokkumar et al., 2009; Zisu et al., 2010). The viscosity of the solutions of whey
protein isolate (WPI) and whey protein were reported to increase significantly by ultrasonication at 20 kHz and 43–48 W/cm² (Krešić et al., 2008).

The overall particle size of the skim milk was also reported to change with ultrasound treatment. Ultrasonication resulted in a decrease of the size of skim milk (Nguyen and Anema, 2010; Chandrapala et al., 2012). The size reduction of the skim milk was attributed to the breakup of fat globules (Chandrapala et al., 2012). In the case of whey protein-based systems, the particle size of 33 % whey protein retentate (w/w) decreased from 0.87 µm to 0.66 µm due to ultrasonication (20 kHz at 60 % amplitude) (Zisu et al., 2010). Whey protein aggregates were also reported to decrease in size, when reconstituted 5% (w/w) whey proteins were sonicated at 20 kHz and 31 W (Zisu et al., 2011). However, bovine serum albumin (BSA) was reported to increase 3.4 times in size under ultrasonication (Gulseren et al., 2007).

The structure of casein micelles and whether they are disrupted by ultrasound treatment are still controversial. It was found the size and structure of casein micelles were not affected or only had a slight decrease after ultrasonication (Chandrapala et al., 2012; Shanmugam et al., 2012). A small decrease (1 to 5 nm) in the size of casein micelles was found after sonication of skim milk (20 kHz, 20–41 W, 60 min) (Shanmugam et al., 2012). However, it was previously reported that casein micelles were probably disrupted and free caseins were released by ultrasound treatment\(^{18}\), which resulted in the increase in the antioxidant activities of skim milk (Taylor and Richardson, 1980b).

2.7 Concluding remarks

In this literature review, the topics related to ultrasound and bacteria are covered. Microorganisms are well studied and a concise literature exists. Similarly, fundamentals related to ultrasound, particularly ultrasound cavitation, are also well

\(^{18}\) The specific frequency and power were not mentioned in Taylor and Richardson’s paper. The sample was treated by a Branson sonifier (Branson Instruments, Inc., Stamford, USA) at the highest acoustic power.
understood. However, there are clearly several gaps in the research works related to the inactivation of bacteria by ultrasound. Firstly, the exact mechanisms are still not elucidated. While cavitation effect is generally offered as an explanation, the exact physics behind the break-up of a microorganism by cavitation is not well understood. Secondly, there are a large number of microorganisms, the majority relevant to food safety, treated by ultrasound. Unfortunately, the ultrasound treatments are not always performed under the same conditions (e.g. bacteria number, medium pH, ultrasound frequency and power). In fact in some publications the ultrasound conditions are not even given. Further, while ultrasound treatment is known to increase the temperature of the medium, most studies do not record the maximum temperature reached during sonication. This is an important parameter, since temperature also contributes to the inactivation of bacteria. Thus comparison between published literatures is very difficult, and in turns this makes the elucidation of the mechanisms behind ultrasound inactivation of bacteria even more difficult.
CHAPTER 3

Inactivation of microorganisms by low-frequency high-power ultrasound: I. Effect of growth phase and capsule properties of the bacteria

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The content of this chapter has been published

3.1 Abstract

The aim of this study was to determine the effects of high-intensity low-frequency (20 kHz) ultrasound treatment on the viability of bacteria suspension. More specifically, we have investigated the relationship between the deactivation efficiency and the physical (size, hydrophobicity) and biological (gram-status, growth phase) properties of the microbes. *Enterobacter aerogenes, Bacillus subtilis, Staphylococcus epidermidis, Staphylococcus epidermidis* SK and *Staphylococcus pseudintermedius* were chosen for this study owing to their varying physical and biological properties. The bacteria were cultured in Nutrient broth and were sonicated in broth or physiological salt solution. The survival ratio of the bacteria suspension was measured as a function of the ultrasound power (up to 13 W) for a constant sonication time of 20 min. Transmission electron microscopy was used to evaluate the ultrasound-induced damages to the microbes. Ultrasound treatment resulted in lethal damage to *E. aerogenes* and *B. subtilis* (up to a 4.5-log reduction), whereas *Staphylococcus* spp. were not affected noticeably. Further, *E. aerogenes* suspensions were more sensitive to ultrasonication in the exponential growth phase than when they were in the stationary phase. It was found that the effectiveness of ultrasound for inactivating bacteria did not depend on the size, gram-status or hydrophobicity of the bacteria. The results of this study demonstrate that the main reason for bacterial resistance to ultrasonic deactivation is due to the properties of the bacterial capsule (extracellular polysaccharides). Microbes with a thicker “soft” capsule are highly resistant to the ultrasonic deactivation process.

**Keywords:** High-power ultrasound; Bacteria inactivation; *Enterobacter aerogenes; Bacillus subtilis; Staphylococcus epidermidis; Bacterial capsule.*
3.2 Introduction

Thermal processing, such as pasteurisation and ultra-high temperature treatment (UHT), have long been involved in bacterial inactivation in the manufacture of food products (Piyasena et al., 2003). However, they are also known to cause loss of vitamins, nutrients and flavours (Kadkhodaee and Povey, 2008). As a result, non-thermal processing technologies, including ultrasound, ultra-high pressure, dense phase CO₂, ozonation, high voltage-pulsed electric fields and magnetic fields treatments, have attracted a lot of interest as they offer an alternative to thermal processing methods. Specifically, ultrasound treatment is known to have a reduced impact on nutritional content and overall food quality under controlled sonication conditions (Chemat et al., 2011), and has been considered as an alternative method for microbial pasteurisation and sterilisation. Ultrasound is a kind of vibrational energy that normally has a frequency of 20 kHz or more (Mason, 1990). Low-frequency (20–100 kHz) ultrasound is also termed high-power ultrasound, while low-power ultrasound generally corresponds to a frequency range in excess of 100 kHz (Mason, 1990; Butz and Tauscher, 2002; Piyasena et al., 2003; Patist and Bates, 2008).

Bacteria inactivation using ultrasound treatment was first reported in the 1920s (Harvey and Loomis, 1929) and the investigation on the mechanism of microbial inactivation began in the 1960s (Earnshaw et al., 1995). There are numerous theories available on the mechanism of biocidal effects of ultrasound. Researchers believe that it is due to acoustic cavitation, which causes mechanical effects and sonochemical reactions such as the generation of highly reactive radicals and molecular products such as H₂O₂ (Mason, 1990; Earnshaw et al., 1995; Sala et al., 1995; Butz and Tauscher, 2002; Russell, 2002; Mason et al., 2005; Kadkhodaee and Povey, 2008; Wu and Nyborg, 2008; Drakopoulou et al., 2009; Adekunte et al., 2010c; Ashokkumar, 2011). Acoustic cavitation refers to the growth and collapse of microbubbles in liquid media. In a liquid environment, cavitation bubbles are produced throughout the liquid due to the pressure fluctuations generated by the ultrasound wave. The collapse of
these cavitation bubbles will generate mechanical effects (shock waves, shear forces and micro-jettings), which can damage microorganisms (Wu and Nyborg, 2008; Collis et al., 2010). For low-frequency high-power ultrasound, it is generally speculated that the main effect of bacterial inactivation is the result of acoustic cavitation (Joyce et al., 2003a; Mason et al., 2003; Mason et al., 2005; O’Donnell et al., 2010; Soria and Villamiel, 2010).

Although the inactivation of bacteria by high-power ultrasound is well known and extensively studied, the relationship between the effectiveness of ultrasound to inactivate bacteria and their physico-chemical properties is not yet well understood. For instance, some reports showed that gram-negative bacteria were more sensitive to ultrasonic inactivation than gram-positive bacteria (Ahmed and Russell, 1975; Alliger, 1975; Cameron et al., 2008; Drakopoulou et al., 2009; Monsen et al., 2009), while other researchers reported no significant relationship between the gram-status of bacteria and ultrasonic inactivation (Scherba et al., 1991; Cameron, 2007). It was reported that larger cells were more susceptible to ultrasound than smaller ones (Thacker, 1973; Alliger, 1975; Nesaratnam et al., 1982) and rod-shaped bacteria were more sensitive than coccus-shaped cells (Alliger, 1975), while it was also reported that there was no direct relationship between the size and shape on the effect of ultrasonication (Cameron, 2007). In summary, based on the information available in the literature, it is obvious that there are mixed opinions and different speculations on the mechanism of ultrasonic inactivation of pathogens with regard to their properties.

Hence, the aim of this study was to investigate how ultrasound inactivation of bacteria might be affected by some physical and biological properties of five different microorganisms, namely Enterobacter aerogenes, Bacillus subtilis, Staphylococcus epidermidis, S. epidermidis SK and Staphylococcus pseudintermedius. These bacteria were chosen owing to their different sizes and gram-statuses. E. aerogenes is a gram-negative rod-shaped bacterium and it belongs to the family of Enterobacteriaceae, which has a size range of 0.3–1.0 μm × 1.0–6.0 μm (Imhoff,
2005). *B. subtilis* is a gram-positive rod-shaped bacterium, and the normal size range is about 0.7–0.8 µm × 2.0–3.0 µm (Schleifer, 2009). *S. epidermidis* is a gram-positive coccus and the size range is 0.8–1.0 µm in diameter (Schleifer, 2009). These bacteria were sonicated using high-power low-frequency (20 kHz) at different phases of their growth. Note that the ultrasound inactivation of *B. subtilis* and *S. epidermidis* were previously reported in the literature (Singer et al., 1999; Joyce et al., 2003b). However, in this study the ultrasound inactivation at different stages of their growth phase was considered, in addition two other species/strains of *Staphylococcus* had been used. To the best of our knowledge, ultrasound treatment of *E. aerogenes* is for the first time reported in this study. In addition, a direct comparison of the ultrasonic deactivation of these bacteria in relation to their physico-chemical properties has never been studied.

3.3 Material and methods

3.3.1 Preparation of bacterial suspensions and determination of their growth phases

*E. aerogenes, B. subtilis, S. epidermidis* and two additional species/strains of *Staphylococcus, S. epidermidis SK* (from human skin) and *S. pseudintermedius*\(^ {19} \), were obtained from the Microbiology Laboratory of the School of Biological Sciences at the University of Auckland, New Zealand. These bacteria were kept as stocks at –80°C. For each bacterium, 1 ml of stock was spread on a Nutrient agar (Difco\(^ {TM} \)) plate and incubated overnight at 37°C. Following this process, a loop of the bacterial colony was transferred into 50 ml of Nutrient broth (Difco\(^ {TM} \)) and then incubated at 37°C overnight in a shaking room (200 rev min\(^ {−1} \)) to produce a working stock bacterial culture. 4 ml of working culture was then added into 100 ml of fresh Nutrient broth and incubated with shaking (200 rev min\(^ {−1} \)) at 37°C to make final bacterial suspensions. The incubation time for this last step was fixed in such a way that the bacteria culture was at the chosen growth phase. These bacteria suspensions

\(^ {19} \) *Staphylococcus epidermidis, Staphylococcus epidermidis SK* and *Staphylococcus pseudintermedius* were identified by using molecular methods that are shown in Appendix A1.5.
were used for the ultrasonication experiments. The growth curves of the microorganisms were monitored by measuring the optical density change of the microorganism suspensions with time using a Spectrophotometer (HeλIOS β UV Visible, Thermo Electron Corporation, UK) at a wavelength of 600 nm.

When required, the bacteria suspensions were washed prior to ultrasonication. Two washing media were used, fresh Nutrient broth or physiological salt solution (0.9% NaCl, PSS). Fresh Nutrient broth was used to get rid of waste produced during the bacteria growth while ensuring enough nutrients for the survival of the bacteria. PSS was used to ensure that there was no excess nutrient and that the bacteria were suspended in a medium similar to water. The washing procedure was carried out as follows: bacterial suspensions at the stationary phase were transferred into 50 ml tubes and centrifuged using Biofuge Stratos (Heraeus, Thermo Electron Corporation, Germany) at 10,000 g for 10 min at 4°C to obtain a bacterial cell pellet. The supernatant was discarded and replaced by an exact amount of either fresh Nutrient broth or PSS. The centrifuge tube was thoroughly vortexed to resuspend the bacteria. This centrifugation followed by the resuspension step was repeated three times.

During this work, to ensure that the bacteria cultures were not contaminated by other microorganisms, their purities were confirmed by using Gram staining (Magee et al., 1975) and a catalase test (Serra et al., 2008).

3.3.2 Ultrasound treatment

15 ml of bacterial suspensions were placed in 20 ml columniform glass vials, and ultrasonicated by an ultrasound homogeniser (Sonic Ruptor 250, Omni International, USA) at 20 kHz. The processing tip (diameter 12.7 mm) of the ultrasonic horn was always positioned 1 cm below the surface of the bacterial suspensions. The bacterial suspensions were surrounded by an ice bath, resulting in the temperature in the vessel not exceeding 30°C under the sonication condition used in this study. The ultrasound
power delivered, $P$, was determined using the calorimetric method (Koda et al., 2003):

$$ P = mC_p(\Delta T/\Delta t) $$

(3.1)

where $C_p (=4.18 \text{ J/(g K)})$ is the specific heat capacity of water, $m (=15 \text{ g})$ is the mass of sonicated water, $\Delta T$ is the increase in the temperature, and $\Delta t$ is the applied sonication time.

All bacteria suspensions were ultrasonicated at different powers (0.7, 3.6, 5.0, and 12.7 W) for a constant time of 20 min. All sonication treatments were performed at least in duplicate.

3.3.3 Bacterial enumeration

A slightly modified Miles-Misra method (Miles et al., 1938) was used to count the viable bacterial cells. Serial 1:10 dilutions from $10^0$ to $10^{-8}$ times were made in 96 Well Tissue Culture plates (Cellstar, Greiner bio-one, Germany) by mixing sterile Nutrient broth or PSS with bacterial samples. Dried sterile Nutrient agar (Difco™) plates were sectioned into 6 parts in advance by a permanent marker. Each diluted sample (50 µl $\times$ 3) was dropped onto 3 sectors and then the plates were incubated at 37°C overnight. The number of colonies for the sectors where the colonies were countable (15–150) was enumerated under a light microscope, and the CFU per millilitre for the original bacterial suspensions was calculated using:

$$ N = \bar{n} \times d_i \times 20 $$

(3.2)

where $N$ is the number of total colonies (CFU/ml), $\bar{n}$ is the average number of colonies for a dilution and $d_i$ is the dilution factor.

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20 The details of the method of enumeration are shown in Appendix A1.6.
3.3.4 Size measurement

The Malvern Mastersizer 2000 (Malvern Instruments, UK) was used for the particle size measurement of bacterial suspensions. Milli-Q water with a refractive index of 1.33 was used as the dispersant medium, and the value of the refractive index of bacteria was 1.38 (Bae and Hirleman, 2007; Foladori et al., 2008). Note that the Mastersizer 2000 software used to obtain the particle size values assumes that the bacteria suspension is made of spherical particles.

3.3.5 TEM analysis

Bacterial samples were examined under transmission electron microscope (TEM) with negative staining. Carbon-coated copper TEM grids were glow discharged at 500 V for 15 s to maintain grids’ surface hydrophilicity. One drop (~40 µl) of bacterial suspension, 2 drops of Milli-Q water and one drop of 2% aqueous uranyl acetate were dripped on a piece of Parafilm in succession. These 4 drops were positioned separately on the Parafilm. The discharged grid was then immersed into a bacterial mixture drop for 30 s, then washed quickly by immersing into the Milli-Q drops and dried using filter paper. The dried grid was then immersed into the uranyl acetate drop for 30 s to be stained. The stained grids were air dried for 1 min and then kept in petri dishes lined with filter paper until TEM observations. The grids were placed into a specimen holder (CM12 TEM, Philips, Netherlands) and then examined at 120 kV (Patterson et al., 2009).

3.3.6 Hydrophobicity measurement

The hydrophobicity measurement used in this study was previously reported by several researchers (Busscher et al., 1984; Loosdrecht et al., 1987; Daffonchio et al., 1995). Bacterial suspensions incubated overnight (20–40 ml) were first washed in PSS using centrifugation/resuspension steps as described above. An extra centrifugation/resuspension step was performed to concentrate the bacteria suspension.
In this step only 2 ml of water was added to the bacteria pellet before redispersion by the use of a vortex. The washed concentrated bacterial suspension was then deposited on a micropore filter paper (0.45 μm pore size). In order to standardise the moisture content, the filters carrying bacterial cells were placed on the surface of agar (1% weight/volume containing 10% (volume/volume) glycerol) for 1–2 h. Then the filters were stuck on a glass slide with double-sided sticky tape and air dried for 1–3 h. Water droplets were dropped on the dried filters, and water contact angles were measured using a contact angle meter (KSV Instruments, Finland). For each bacterium, at least 40 measurements were carried out.

### 3.4 Results

In previous studies on ultrasonic deactivation of pathogens, the significance of the physical and biological properties of the microbes has generally been ignored. Since the main aim of this study is to find out the importance of the properties of the microbes on the efficiency of ultrasonic deactivation, these properties (particle size, gram-status and hydrophobicity) were measured. In addition, because the properties of some bacteria are known to vary with their growth phases, bacteria cultures at different phases were also considered. In order to ensure that the suspending medium (nutrients) did not affect the ultrasound efficiency due to a change in the solution viscosity, bacteria suspensions were also washed prior to ultrasound treatment.

#### 3.4.1 Physical and biological properties of the different bacteria suspensions

The growth of microorganisms reproducing normally has four phases: a lag phase, an exponential phase (log phase), a stationary phase, and a death phase (Monod, 1949; Novick, 1955). In the lag phase, there is no marked increase in the cell number, while in the exponential phase, cells are most active and begin to divide at a maximal rate. In the stationary phase, cell growth ceases and the population number remains constant. In the death phase, the cell number diminishes logarithmically (Monod, 1949; Novick, 1955; Prosser and Tough, 1991; Kolter et al., 1993; Russell and Cook,
Although bacteria reproduction is most active in the exponential phase, they are also more susceptible to detrimental conditions such as radiation and antimicrobial drugs (Twentyman and Bleehen, 1973; Abedi-moghaddam et al., 2004). It was observed using both light and electron microscopes that \textit{E. aerogenes} changed from a rod-like shape in the exponential phase to a near coccus (spherical) shape in the stationary phase (Figure 1.1A and inset Figure 1.1a). These differences in the biological and physical properties of the bacteria at different phases might affect the efficiency of ultrasound to inactivate these bacteria.

The growth curves of \textit{E. aerogenes}, \textit{B. subtilis} and \textit{S. epidermidis} are shown in Figure 3.1. It could be clearly seen that \textit{E. aerogenes}, \textit{B. subtilis} and \textit{S. epidermidis} suspensions reached the stationary phase after 5 h, 14 h and 8 h incubation, respectively. Thus, in order to investigate the effect of the growth phase, bacterial suspensions for \textit{E. aerogenes}, \textit{B. subtilis} and \textit{S. epidermidis} in the exponential phase were obtained after 2 h, 5 h and 3 h of incubation, respectively. These bacteria were sonicated without further delay. For the stationary phase, all bacterial suspensions were incubated for 20 h and then sonicated.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.1.png}
\caption{Growth curves of \textit{Enterobacter aerogenes} (■), \textit{Bacillus subtilis} (○) and \textit{Staphylococcus epidermidis} (▲). Error bars correspond to standard deviations.}
\end{figure}
Particle size distributions of the different bacteria are shown in Figure 3.2. The particle size distributions of *S. epidermidis* and *B. subtilis* are monomodal, while that of *E. aerogenes* suspension is bimodal, with a small volume distribution at low sizes (100–400 nm) and a main distribution with a maximum peak at ~1 µm. It is likely that the small distribution is due to the bacteria waste, made mainly of polysaccharides, while the main distribution corresponds to the bacteria cells. The Sauter mean diameters ($D_{3,2}$) are 1.27 ± 0.01, 1.29 ± 0.07 and 1.92 ± 0.02 µm for *E. aerogenes*, *B. subtilis*, and *S. epidermidis*, respectively (Table 3.2). A possible explanation of the large size of *S. epidermidis* is the tendency of these bacteria to cluster into small aggregates as observed by light microscopy (Figure 1.1C). These sizes are different from those reported in the literature (Imhoff, 2005; Schleifer, 2009). This is not surprising since the light scattering technique used here assumes that the bacteria are spherical, and the value found in the literature are usually obtained by microscopy observations. It can also be shown the aggregation effects of bacterial fragments by ultrasonication from light micrographs (not shown).

![Figure 3.2](image)

**Figure 3.2** Particle size distribution for *Enterobacter aerogenes* (■), *Bacillus subtilis* (●) and *Staphylococcus epidermidis* (▲).
Table 3.1 Some physical and biological characteristics of the bacteria used in this study.

<table>
<thead>
<tr>
<th></th>
<th><em>Enterobacter aerogenes</em></th>
<th><em>Bacillus subtilis</em></th>
<th><em>Staphylococcus epidermidis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Shape</strong></td>
<td>Rod-like (tend to be cocci in the stationary phase)</td>
<td>Rod</td>
<td>Cocci</td>
</tr>
<tr>
<td><strong>Size, D_{3,2} (µm)</strong></td>
<td>1.27±0.01</td>
<td>1.29±0.07</td>
<td>1.92±0.02</td>
</tr>
<tr>
<td><strong>Hydrophobicity</strong></td>
<td>Hydrophilic (24.0°±6.4)</td>
<td>Moderately hydrophobic (50.1°±7.2)</td>
<td>Hydrophilic (24.7°±5.5)</td>
</tr>
<tr>
<td><strong>Cell wall type</strong></td>
<td>Gram-negative</td>
<td>Gram-positive</td>
<td>Gram-positive</td>
</tr>
<tr>
<td><strong>Arrangement</strong></td>
<td>Clusters</td>
<td>Pairs or single</td>
<td>Clusters</td>
</tr>
</tbody>
</table>

3.4.2 Effect of ultrasound treatment on bacteria survival ratio

One of the aims of this study was to investigate if the inactivation of bacteria by ultrasound treatment depends on the bacteria growth phases (Figure 3.1). The properties of bacteria are known to change during their growth phases. As indicated previously, *E. aerogenes* is found to change from a rod-like shape in the exponential phase to a near coccus shape in the stationary phase. Based on the growth curves (Figure 3.1), suspensions of bacteria collected at different times during their growth were prepared for ultrasound treatment. *E. aerogenes*, *B. subtilis* and *S. epidermidis* suspensions were ultrasonicated in both exponential and stationary phases. Further, to investigate the effect of the culture medium, bacteria suspensions in the stationary phase were also washed with fresh Nutrient broth using successive centrifugation steps.

Figure 3.3 reports the logarithmic of the survival ratio, Log \((N/N_0)\), as a function of sonication power for the three different bacteria in their exponential growth phase or in their stationary phase before and after washing with fresh Nutrient broth. The results showed that ultrasonication of *E. aerogenes*, in the exponential or stationary phase and before or after washing, resulted in a marked decrease in live bacteria.
(Figure 3.3A). Up to a 4-log reduction was obtained when *E. aerogenes* suspensions in the stationary phase were ultrasonicated at 12.7 W. Within experimental errors, there were no considerable differences in the effect of ultrasonication between the washed and unwashed *E. aerogenes* suspensions. Sonication of *E. aerogenes* suspensions in the exponential phase resulted in even a higher (~4.4-log) reduction in the number of live bacteria. It was previously reported that the bacteria were more susceptible to detrimental conditions, such as radiation and some antimicrobial drugs during the exponential phase (Twentyman and Bleehen, 1973; Abedi-moghaddam et al., 2004). Figure 3.3A also shows that there was a slight increase in the viable numbers of *E. aerogenes* at 3.6 W. This increase could be due to the fact that at low power, ultrasound treatment is able to break or disperse the bacteria clusters but is not strong enough to cause damage to *E. aerogenes* cells. Previous studies found that both higher frequency and lower power ultrasound had an effect on disaggregating bacterial clusters (Joyce et al., 2003b; Joyce et al., 2011). The dispersion of the *E. aerogenes* clusters increased the availability of nutrients to the bacteria thus increasing their number. In addition, it was reported that low intensity ultrasound improved microbial metabolism and stimulated physiological activities (Schläfer et al., 2000; Lin and Wu, 2002; Chisti, 2003; Liu et al., 2007). However, the increase in the bacteria number for the washed bacteria suspension at 3.6 W was not observed. It is not yet clear why the washed bacteria suspension behaved differently from the unwashed suspension, since both showed aggregation before ultrasound treatment. It can be speculated that a longer time might be needed for the washed bacteria to adapt to its new environment.
Figure 3.3 Log of survival ratio (Log ($\frac{N}{N_0}$)) for sonicated (A) *Enterobacter aerogenes*, (B) *Bacillus subtilis* and (C) *Staphylococcus epidermidis*. Symbols are: Bacteria at stationary phase (■); Washed bacteria at stationary phase (●); Bacteria at exponential phase (▲). The bacteria suspensions were sonicated at different powers for 20 min (20 kHz). Error bars correspond to standard deviations.
In the case of *B. subtilis*, ultrasound treatment also resulted in a similar bacteria inactivation to *E. aerogenes* suspensions. Up to a ~4.5-log reduction was obtained when a suspension of *B. subtilis* was sonicated at 12.7 W for 20 min (Figure 3.3B). However, ultrasound inactivation of these bacteria was not affected by the growth phase or by the washing step. This is not surprising since there was no indication that the properties of these bacteria, contrary to *E. aerogenes*, changed as a function of the growth phase. However, *S. epidermidis* behaved differently to the other two bacteria in its response to ultrasound (Figure 3.3C). For unwashed *S. epidermidis*, at the exponential phase and stationary phase, the survival ratio slightly increased at lower ultrasound powers and there was only a very small reduction in bacteria number (0.2-log reduction) when the highest ultrasound power was applied (12.7 W). Washed *S. epidermidis* increased slightly at all the ultrasonication powers. This slight increase also might be explained in terms of the increase in the accessible nutrients since ultrasound induces the disaggregation of the bacteria clusters (Joyce et al., 2003b; Joyce et al., 2011), as well as improves microbial metabolism and stimulates physiological activities (Schläfer et al., 2000; Lin and Wu, 2002; Chisti, 2003; Liu et al., 2007). Microscopic observation of *S. epidermidis* also showed that these bacteria form clusters (Figure 1.1C).

### 3.4.3 Transmission electron microscopy (TEM) observations

TEM is a very powerful technique extensively used to visualise the microstructure of single bacterial cells. TEM images of *E. aerogenes*, *B. subtilis* and *S. epidermidis* before and after ultrasonic treatments are shown in Figure 3.4. The TEM images of the non-sonicated bacteria suspensions confirm that *B. subtilis* is rod-like while *S. epidermidis* is spherical and *E. aerogenes* tends to be coccus at the stationary phase. It can also be clearly seen that ultrasonic treatments had a considerable effect on the cell structure of *E. aerogenes* and *B. subtilis*. The cell surfaces before ultrasonication were intact and smooth (Figure 3.4A1 and B1). Ultrasound treatment resulted in a suspension of misshapen structures and fragments for these two bacteria. For *E.*
*aerogenes* and *B. subtilis*, ultrasound treatment induced cell damage, where the structure integrity of bacterial cells was destroyed, the cytoplasmic membrane dislocated, and the intracellular content leaked (Figures 3.4A2, A3, B2 and B3).

![Transmission electron micrographs of (A) Enterobacter aerogenes, (B) Bacillus subtilis and (C) Staphylococcus epidermidis before (1) and after (2 and 3) 20 min ultrasound treatments (20 kHz). Ultrasound powers were: A2: 5.0 W; A3: 12.7 W; B2, B3, C2 and C3: 5.0 W.](image)

**Figure 3.4** Transmission electron micrographs of (A) *Enterobacter aerogenes*, (B) *Bacillus subtilis* and (C) *Staphylococcus epidermidis* before (1) and after (2 and 3) 20 min ultrasound treatments (20 kHz). Ultrasound powers were: A2: 5.0 W; A3: 12.7 W; B2, B3, C2 and C3: 5.0 W.

TEM observations were previously carried out to investigate the effect of ultrasound on microorganisms such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Lactobacillus acidophilus* (Cameron et al., 2008). TEM analysis of *E. coli* and *Streptococcus mutans* and their cytoplasmatic contents showed different kinds of
damage, caused by ultrasound treatment (Koda et al., 2009). In the case of *S. epidermidis*, however, TEM did not show any change in the structure of this bacterium (Figures 3.4C1, C2 and C3). This confirms the survival ratio measurements (Figure 3.3C), which did not show any marked decrease in the number of bacteria with ultrasound treatment.

### 3.5 Discussion

This study showed that ultrasound treatment resulted in marked reduction number (up to a 4.5-log reduction) of *E. aerogenes* and *B. subtilis*. Further, in the case of *E. aerogenes*, ultrasound reduction was more effective when the bacteria were in the exponential phase. This can be explained in terms of the difference in shape between the exponential phase (rod-like) and stationary phase (cocci). However, the survival ratio of *S. epidermidis* was not greatly affected by ultrasound treatment. The published literature previously reported that ultrasound treatment was ineffective for the inactivation of some bacteria species (Gogate, 2007; Monsen et al., 2009). However different explanations, based on the bacteria characteristics, have been put forward. For example it was suggested that gram-positive bacteria were more resistant to ultrasonication than the gram-negative ones (Ahmed and Russell, 1975; Alliger, 1975; Cameron, 2007; Cameron et al., 2008; Drakopoulou et al., 2009; Monsen et al., 2009). The size and shape also were reported to influence the effect of sonication on bacteria (Thacker, 1973; Alliger, 1975; Nesaratnam et al., 1982; Cameron, 2007). In some cases the sensitivity to ultrasonic cavitation was simply attributed to the species of bacteria, without further explanation (Scherba et al., 1991).

A close examination of Table 3.1 shows that the effectiveness of ultrasound for inactivating bacteria does not depend on the size, gram-status or hydrophobicity of the bacteria. For instance, both *B. subtilis* and *S. epidermidis* are both gram-positive, and *E. aerogenes* and *S. epidermidis* are hydrophilic. However, while both *E. aerogenes* and *B. subtilis* can be inactivated by ultrasound under the sonication condition used in this study, *S. epidermidis* is more resistant to ultrasound. One physical characteristic
that might be responsible for this observation is the thickness of the capsule. This has been previously suggested to explain the effect of the gram-status on ultrasound efficiency to inactivate bacteria, with gram-positive likely to have a thicker cell wall than gram-negative bacteria (Drakopoulou et al., 2009). However, our current investigation has provided first-time evidence that, independent of the gram-status, it is the size of the capsule that is important. To further investigate the importance of the thickness of the bacteria capsule, two bacteria from the same genus (*Staphylococcus*) as *S. epidermidis* were cultured and sonicated. These bacteria are *S. epidermidis* SK present on human skin and *S. pseudintermedius*, and their TEM micrographs are reported in Figure 3.5. These two bacteria are also cocci and possess thick capsules. The results of the ultrasound treatment on these two bacteria are shown in Figure 3.6 and compared with the results obtained for *S. epidermidis* (reported in Figure 3.4C). It can be clearly seen that ultrasonication of these two bacteria also did not result in a marked decrease in bacteria number. The most reduction in bacteria number was observed for *S. pseudintermedius* where the bacteria number decreased by less than 30% at the highest power used.

![Figure 3.5](image)

**Figure 3.5** Transmission electron micrographs of different species/strains of *Staphylococcus*: (A) *Staphylococcus epidermidis*, (B) *Staphylococcus epidermidis* SK, and (C) *Staphylococcus pseudintermedius*. Scale bars correspond to 0.5 µm.

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21 The capsules (slime) of *Staphylococcus* spp. and *Enterobacter aerogenes* were observed by light microscope after using the methods of Anthony’s staining/India ink staining that are shown in Appendix A1.4. Slime was found from *E. aerogenes*. 

75
Figure 3.6 Log of survival ratio (Log \(N/N_0\)) for different species/strains of *Staphylococcus*: *Staphylococcus epidermidis* (□); *Staphylococcus epidermidis* SK (■); and *Staphylococcus pseudintermedius* (○). The bacteria suspensions were sonicated at different powers for 20 min (20 kHz). Error bars correspond to standard deviations.

A possible explanation of the resistance of the bacteria to break up by ultrasound is the stiffness related to the Young’s modulus, which can be measured by atomic force microscopy (AFM) and tensile strength measurements (Deng et al., 2011; Tuson et al., 2012). Although values of the Young’s modulus for *E. aerogenes*, *B. subtilis* and *S. epidermidis* used in this study are not known, values for similar microorganisms have been reported in the literature. The Young’s modulus of *B. subtilis* FJ7 is 10 to 30 MPa in different humidities and salt concentrations (Thwaites and Mendelson, 1989; Thwaites and Surana, 1991) and that of *B. subtilis* BB11 cells is 100 to 200 MPa (Tuson et al., 2012). A value of 95.4 MPa for the Young’s modulus of *Staphylococcus aureus* NCTC 8532, a gram-positive bacterium, was found to be lower compared to the measured value of 221.4 MPa for *E. coli*, a gram-negative bacterium (Eaton et al., 2008). From these values of the Young’s modulus it is difficult to explain the resistance of bacteria with capsules to ultrasound inactivation in terms of the stiffness.
of the bacteria. In fact, *Staphylococcus*, which has one of the lowest values of the Young’s modulus, is more resistant to ultrasound than *E. coli*, known to be easily inactivated by ultrasound treatment, with a higher value of the Young’s.

The bacterial capsule is a layer external to the plasma membrane and contributes to maintain cellular integrity. It consists of homogeneous polysaccharides and proteins (Schleifer and Kandler, 1972; Tuson et al., 2012). This layer can be removed without affecting the viability of bacterial cells (Wilkinson, 1958). The bacteria capsule is also highly hydrated and can contain up to 99.9% water (Sutherland, 1979). Thus this layer can be considered as “soft” material similarly to aqueous polysaccharide solutions. To explain the resistance of *S. epidermidis*, *S. epidermidis* SK and *S. pseudintermedius* to ultrasound deactivation, we propose two mechanisms. Firstly, these bacteria contain a relatively large biomaterial layer (~100 nm from TEM observations) from the plasma membrane (cell death occurs when the plasma membrane is damaged). The closest position of the collapsing cavitation to the plasma membrane is limited to the size of the capsule layer. As a consequence, the shear force due to the collapse of the cavitation bubble acting on the bacteria is reduced, since this shear force is inversely proportional to the distance between the collapsing cavitation bubble and the plasma membrane (Maisonhaute et al., 2002b). Secondly, the bacteria capsule is very soft and this will result in dampening the mechanical effects due to cavitation. It has been recently reported on the effect of cavitation on starch granules, where removal of the proteins from the starch granules through SDS and ethanol washing resulted in a decrease in starch granule damage (Zuo et al., 2012). The removal of the protein and lipids from the starch granule surface was expected to result in a flexible granule surface mainly made of hydrated starch polysaccharides. Hence, it is suggested that the resistance to ultrasonic deactivation of *S. epidermidis*, *S. epidermidis* SK and *S. pseudintermedius* is due to the thickness as well as “softness” of the capsule.
3.6 Conclusions

The deactivation of bacteria suspensions using high-power ultrasound was investigated to relate the efficiency of ultrasound treatment to the physical and biological properties of three bacteria, namely, *E. aerogenes*, *B. subtilis*, and *S. epidermidis*. These three bacteria were chosen as they have different physical and biological properties. The state of the growth (i.e. bacteria suspensions in the exponential phase or in the stationary phase) was also considered. The study showed that high-power ultrasound was very efficient in reducing the number of live cells in the case of *E. aerogenes*, *B. subtilis*. The rate of inactivation was also found to be dependent on the growth phase of *E. aerogenes*. This was attributed to its change in morphology from rod-like in the exponential phase to a coccus shape in the stationary phase.

In contrast, *S. epidermidis* was found to be very resistant to inactivation by high-power ultrasound. This was attributed to the presence of a biopolymer capsule surrounding the bacteria. TEM observation provided a direct indication that these bacteria possess a thick capsule layer (in the order of 100 nm) and that the cells were not damaged by ultrasound treatment. To demonstrate the protective effect of the bacteria capsule, two other bacteria, *S. epidermidis* SK and *S. pseudintermedius*, from the same genus/species were also subjected to ultrasound treatment. The experimental results showed that these two bacteria were also resistant to the ultrasound treatment. We believe that this is due to the protective effect of the bacteria capsule against mechanical damage resulting from ultrasound cavitation. The presence of a thick capsule (biopolymer layer) prevents cavitation bubbles from collapsing near the plasma membrane thus preventing the break-up of the bacterial cell. On the other hand, being highly hydrated the capsule would be very soft and hence might help absorb the mechanical forces exerted on the bacterial cell. Overall, in light of the findings of this study, we propose to consider the thickness and softness of the
bacteria capsules as one of the most important parameters when using high-power ultrasound for the deactivation of microbes.

3.7 Acknowledgements

S. Gao is supported by a New Zealand China Food Safety Scholarship, funded by the New Zealand Ministry of Foreign Affair and Trade. YH and MA thank the University of Auckland for the award of the Distinguished Visitor Award (Project number 3702372).
CHAPTER 4

Inactivation of microorganisms by low-frequency high-power ultrasound: II. A simple model for the inactivation mechanism

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The content of this chapter has been published
4.1 Abstract

A simple theoretical model based on shear forces generated by the collapse of ultrasound cavities near the surface of a microorganism is proposed. This model requires two parameters that take into account the number of acoustic cavitation bubbles, and the resistance of the cell wall of the microorganism to the shear forces generated by bubble collapse. To validate the model, high-power low frequency (20 kHz) ultrasound was used to inactivate two microorganisms with very different sizes, viz., a bacterium, *Enterobacter aerogenes* and a yeast, *Aureobasidium pullulans*. The microorganisms were sonicated at 4 W to 12 W for 5 min to 60 min. The inactivation ratio was experimentally measured as a function of sonication time for different ultrasound power and for different initial cell numbers. For both *E. aerogenes* and *A. pullulans* the Log of the inactivation ratio decreased linearly with sonication time, and the rate of inactivation increased (*D*-value decreased) with the increase in sonication power. The rate of inactivation was also found, for both microorganisms, to increase with a decrease in the initial cell number. The fits, obtained using the proposed model, are in very good agreement with the experimental data.

**Keywords:** High-power ultrasound; Bacteria inactivation; Yeast; *Enterobacter aerogenes*; *Aureobasidium pullulans*; Theoretical model.
4.2 Introduction

Food safety is an important issue worldwide and hundreds of millions of people have suffered from foodborne diseases. Microbiological hazards involving bacteria, viruses, and yeasts are the most common causes of food spoilage and food poisoning. Traditionally, thermal treatments, such as pasteurisation and ultra-high temperature treatment (UHT), are extensively used in bacterial inactivation (Piyasena et al., 2003). However, these traditional microbial inactivation methods can cause the loss of important nutritional components, such as vitamins, and affect physical characteristics of food such as flavour, texture and colour (Kadkhodaei and Povey, 2008). As a result, non-thermal bacterial inactivation, including high-power ultrasound, pulsed electric field and high hydrostatic pressure, have attracted significant interest. Ultrasound processing has been recently used in a variety of food processing applications including food safety, preservation and extraction (Chemat et al., 2011). Bacteria inactivation by ultrasound treatment was reported as early as the 1920s (Harvey and Loomis, 1929), and investigations on the mechanism of microbial inactivation by ultrasound began in the 1960s (Earnshaw et al., 1995).

The effect of ultrasound treatment, either alone or in combination with heat treatment, has been investigated on numerous microorganisms. It was shown that bacteria such as *Escherichia coli* (Scherba et al., 1991), *Listeria monocytogenes* (Pagán et al., 1999), and *Listeria innocua* (Joyce et al., 2011) can be inactivated by ultrasound. Ultrasound inactivation of yeasts, such as *Saccharomyces cerevisiae* was also reported by several research groups (Ciccolini et al., 1997; Aronsson et al., 2001; Guerrero et al., 2001; Lanchun et al., 2003; Tsukamoto, 2004). Although the exact physico-chemical mechanism by which ultrasound inactivates microorganisms remains to be fully elucidated, it is accepted that it is a consequence of ultrasound cavitation; that is the formation, growth and collapse of microbubbles in the sonicated liquid, resulting in mechanical effects and sonochemical reactions (Mason, 1990; Earnshaw et al., 1995; Sala et al., 1995; Butz and Tauscher, 2002; Russell, 2002; Mason et al., 2005;
Kadkhodaee and Povey, 2008; Wu and Nyborg, 2008; Drakopoulou et al., 2009; Adekunte et al., 2010c; Ashokkumar, 2011). Sonochemical reactions, particularly the generation of hydrogen peroxide can contribute to the inactivation of bacteria. Mechanical effects due to cavitation include shear forces and micro-jetting or microstreaming (Wu and Nyborg, 2008; Collis et al., 2010), which can result in the breakup of the microorganism cell. For example, when a cavitation bubble collapses near a solid surface, a velocity gradient perpendicular to the surface is generated, resulting in shear forces acting on the surface (Maisonhaute et al., 2002b). Micro-jets are the results of a cavitation bubble collapsing in a non-symmetrical way near the surface. The solid surface blocks the liquid movement resulting in the liquid flow accelerating from the opposite side of the solid surface and passing through the microbubble (Zuo et al., 2012). In a study on the ultrasonic inactivation of Cryptosporidium oocysts, it was speculated that the shear forces generated by acoustic cavitation are primarily responsible for the inactivation process (Ashokkumar et al., 2003).

As indicated above, from an experimental viewpoint a large number of studies on the inactivation of bacteria and other microorganisms by ultrasound have been published. Unfortunately, theoretical analyses of the experimental results are scarce. In fact, most of the theoretical modellings of bacteria inactivation by ultrasound involve fitting the experimental data using numerical models usually borrowed from studies of the inactivation of bacteria by heat treatment. For instance, the Weibull distribution was found to be a good fit for the thermo-sonication inactivation of L. innocua in raw whole milk (Bermúdez-Aguirre et al., 2009) and the inactivation of Pichia fermentans, a yeast, by ultrasound treatment (Adekunte et al., 2010a). The Weinbull distribution was also found to be a good fit for the ultrasound inactivation of Alicyclobacillus acidiphilus DSM14558T, while the inactivation of Alicyclobacillus acidoterrestris DSM 3922T was better fitted by a biphasic linear model (Wang et al., 2010). Although these numerical models allow comparison between different bacteria species and between different ultrasound treatment conditions, they are not directly
related to the mechanism of ultrasound cavitation. In this paper, a simple theoretical model based on the inactivation of bacteria due to shear forces is proposed. This model relies on two parameters, namely, the resistance of the microorganism to break under the shear forces generated by acoustic cavitation, and the number of cavitation bubbles generated during sonication. To validate the model, two microorganisms were treated by high-power ultrasound at a frequency of 20 kHz, at different ultrasound powers and different times (up to 1 h). The effect of the initial cell number in the microorganism cultures was also considered. The two microorganisms chosen in this study were Enterobacter aerogenes and Aureobasidium pullulans, and to the best of our knowledge, there are no studies in the published literature about the effect of ultrasound on these two microorganisms. E. aerogenes is a gram-negative rod-shaped bacterium that belongs to the family of Enterobacteriaceae and the size of these bacteria are 0.3–1.0 µm × 1.0–6.0 µm (Imhoff, 2005). E. aerogenes is found ubiquitously in soil, water, dairy products, as well as human and animal faeces (Grimont and Grimont, 2006). A. pullulans is a yeast-like fungus and is also known as black yeast (Cooke, 1959; Hoog, 1993). A. pullulans exits widely in various environments including, soil, water, wood, rocks with high humidity and plant materials (Urzi et al., 1999; Chi et al., 2009). Isolates of A. pullulans are vegetative hyphae hyaline, smooth, thin-walled, transversely septate and 2–13 µm wide (Zalar et al., 2008).

4.3 Materials and methods

4.3.1 Preparation of microorganism suspensions

E. aerogenes and A. pullulans were obtained from the Microbiology Laboratory of the School of Biological Sciences at the University of Auckland (New Zealand). These microorganisms were maintained as stocks at −80°C. For each species of microorganism, a millilitre of the bacteria stock was spread on a Nutrient agar (Difco™) plate. For E. aerogenes, after overnight culture in a 37°C incubator, a loop
of the microorganism was transferred to 50 ml of Nutrient broth (Difco™) to be further incubated at 37°C overnight with shaking (200 rev min⁻¹) from which a working stock bacterial culture was prepared. Four millilitres of the mother culture was added to 100 ml of Nutrient broth and incubated once more in a shaking room (200 rev min⁻¹) at 37°C. The bacteria were grown for 20 h, at which time they reached the stationary phase. The purities of the bacteria were confirmed by using Gram staining (Magee et al., 1975). For *A. pullulans*, it was incubated at 28°C, and it needed 72 h growth to reach the stationary phase. The times at which the growth of these two microorganisms reached the stationary phase were determined from their growth curves, measured by monitoring the change in the optical density of the microorganism suspension with time. The optical density was measured using a HeλIOS β UV-visible spectrophotometer (Thermo Electron Corporation, UK) at a wavelength of 600 nm. Optical density measurements were also used to prepare microorganism suspensions with initial numbers of ∼10⁸ CFU/ml or ∼10⁷ CFU/ml.

Prior to sonication the microorganism suspensions were washed twice to get rid of the culture broth, which might influence the ultrasound treatment. To do so, the microorganism suspensions collected in their stationary phase were transferred into 50 ml tubes, and then centrifuged using Biofuge Stratos (Heraeus, Thermo Electron Corporation, Germany) at 4°C. Centrifugation conditions of 10,000g for 10 min, and 3,000g for 10 min were used for *E. aerogenes* and *A. pullulans* respectively. After centrifugation the supernatant was discarded and the pellet was resuspended in a physiological salt solution (0.9% NaCl, PSS). The centrifugation and resuspension steps were repeated twice.

4.3.2 Ultrasound treatment

The bacterial or yeast suspension (15 ml) was placed in a 20 ml columniform glass vial and ultrasonicated using a Sonic Ruptor 250 Ultrasonic Homogeniser (Omni International, USA) at 20 kHz. The processing tip (Diameter 12.7 mm) of the
ultrasonic homogeniser was always placed at 1 cm below the suspension surface. The bacterial suspensions were placed in an ice bath to ensure that the temperature was kept below 30°C. The bacterial suspensions were sonicated for 5, 10, 20, 40 and 60 min at three different ultrasound powers (4, 8, 12 W), and yeast suspensions were treated at four ultrasound powers (4, 8, 10 and 12 W). All the sonication treatments were performed at least in duplicate. The ultrasound power ($P$) was measured using the calorimetric method (Koda et al., 2003):

$$P = mC_p(\Delta T/\Delta t)$$  \hspace{1cm} (4.1)

where $C_p$ (=4.18 J/(g·K)) is the specific heat capacity of water, $m$ is the mass (=15 g) of water, $\Delta T$ is the increase in the temperature of the sonicated culture measured using a digital thermometer, and $\Delta t$ is the applied sonication time.

4.3.3 Enumeration of bacteria and yeast

Serial 1:10 dilutions from $10^0$ to $10^{-6}$ times were made in sterile Nutrient broth with samples taken from the bacteria suspensions either before or after the ultrasound treatment. The Miles-Misra method (Miles et al., 1938) was adapted slightly and used for counting the viable cells. A 96 Well Tissue Culture plate (Cellstar, Greiner bio-one, Germany) was used to mix bacterial suspensions and Nutrient broth. Diluted samples (50 µl) were dropped onto plates of dried sterile Nutrient agar (Difco™), and the plates were incubated overnight for 15 h at 37°C or for 24–36 h at 28°C in the case of *E. aerogenes* and *A. pullulans*, respectively. The colonies were counted using a light microscope.

4.3.4 Physical characterisation of the microorganism suspensions

The size of the microorganisms was obtained using a Malvern MasterSizer 2000 (Malvern Instruments, UK). The data were analysed using a refractive index of 1.33
for water and 1.38 for the microorganisms (Bae and Hirleman, 2007; Foladori et al., 2008).

Viscosity measurements were performed using an Ubbelohde capillary viscometer (Schott, SI Analytics, Mainz, Germany). The capillary viscometer (1.50 ± 0.01 mm diameter, and constant $K = 0.3 \text{ mm}^2/\text{s}$) containing the microorganism suspension was immersed in a water bath. A temperature controller (SD07R-20-A12E, Ply Science, USA) was used to maintain the water bath temperature constant at 25°C. The flow-through time in the capillary viscometer was measured by the Visco-Clock (Schott, SI Analytics, Mainz, Germany). Each sample was measured six times.

Microscopic observations of the bacteria suspensions were carried out using transmission electron microscopy using negative staining. Carbon-coated copper TEM grids (3.05 mm) were used to contain the bacterial samples. The TEM grids were glow discharged at 500 V for 15 s in order to maintain the grids’ surface hydrophilicity. One drop of microorganism suspension (40 µl), two drops of Milli-Q water (18.2 MΩ cm) and one drop of 2% uranyl acetate (aqueous) were dripped on Parafilm in succession. The grid was immersed into the bacterial suspension for 30 s, and then washed with the water drops. The grid was then transferred into the 2% uranyl acetate drop for 30 s to be stained and air dried for 1 min. The stained grids were inserted in the specimen holder of CM12 TEM (Philips, Netherlands) for examination at 120 kV (Patterson et al., 2009). In the case of yeast, microscopy observations were carried out on a light microscope (Leica DMRE, Germany) without staining. Note that, TEM was also trialled on the yeast suspension; unfortunately, TEM was not successful due to the large size of the yeast cells.

### 4.4 Model

In this model, the inactivation of bacteria is assumed to be the result of the high shear stress generated from the collapse of the cavitation bubbles (Figure 4.1A). The resulting shear stress $\sigma$ at the surface is given by (Maisonhaute et al., 2002b):
\[
\sigma = \eta v / d \tag{4.2}
\]

where \( \eta \) is the viscosity of the continuous phase, \( v \) is the velocity generated by the collapsing cavitation bubble, and \( d \) is the distance between the position of the collapsing bubble and the surface of the microorganism. In the case of a cavitation bubble collapsing in water when driven at 20 kHz, \( v \) was estimated to be approximately 200 m/s [24, 43]. Equation (4.2) shows that the closer the cavitation bubble is to the microorganism surface, the higher the shear stress \( \sigma \) acting on it. However, the minimum value of \( \sigma \) required to inflict damage to the surface of the microorganism depends on the cell strength of the microorganism, which is related to its different properties, such as elasticity of the membrane, presence of a capsule, hydrophobicity, and its size. To take into account the strength of the microorganism, a critical distance \( d_c \) is introduced. If the cavitation bubble collapses in a position in which \( d \leq d_c \), breakup of the microorganism will occur. Assuming that the cavitation bubble collapse occurs outside of the microorganism, then the probability \( \phi \) for a cavitation bubble to collapse at \( d \leq d_c \) is given by:

\[
\phi = \frac{(r+d_c)^3-r^3}{(r+\delta)^3-r^3} \tag{4.3}
\]

where \( 2 \times \delta \) is the surface-to-surface distance between two spherical microorganism cells. If the cells have the same size and are randomly distributed (see Figure 4.1B), then \( \delta \) is given by (Hemar and Horne, 1998):

\[
\delta = r \times \left( \frac{0.64}{\phi} \right)^{1/3} - 1 \tag{4.4}
\]

where \( r \) is the radius of the microorganism, 0.64 the value of the random-close packing and the volume fraction \( \phi \) of the microorganisms is given by:

\[
\phi = N \times (4\pi r^3 / 3)/V_T \tag{4.5}
\]
where \( N \) is the total number of microorganisms, and \( V_T \) is the total volume of the bacteria suspension.

\[
N_k = \mu(P) \times \varphi
\]  

(4.6)

For simplicity, equation (4.6) assumes that one collapsing cavitation bubble results in the inactivation of one individual microorganism only.

To compare the results of this model to the experimental data, this model is implemented in Microsoft Excel, where \( r \), the radius of the microorganism and the volume the sonicated suspension \( V_T \) are entered. \( N_0 \), the initial number of cells in the sonicated suspension is chosen to be close to the experimental value (e.g. \( 1 \times 10^8 \))
CFU/ml). Unfortunately, the critical distance $d_c$ and the number of cavitation bubbles per unit time $\mu$ are unknown. Obviously the measurement of the shear strength of a single live bacterium is challenging, while determination of the exact number of cavitation bubbles during ultrasound treatment is still under investigation (Sundaram et al., 2003; Dular et al., 2005). In this work a constant value is given for $d_c = 2 \mu m$.

The simulation using Excel is performed as follows: on the same Excel column (first column), different values of bacteria numbers $N$ in descending values are entered (e.g. $1 \times 10^7$ CFU/ml, $1 \times 10^6$ CFU/ml, $1 \times 10^5$ CFU/ml, $1 \times 10^4$ CFU/ml, etc.). In the second column the bacteria volume fraction $\phi$ corresponding to the bacteria number $N$ is calculated using Equation (4.5). In the third column, the bacteria surface-to-surface distance $\delta$ is calculated using equation (4.4) by taking into account the value of $\phi$ obtained in the second column. In the fourth column, the probability $\varphi$ for one cavitation bubble to collapse at $d \leq d_c$ and thus to kill a bacterium is calculated using Equation (4.3) and the values of $\delta$ (obtained in the third column) and the value of $d_c$ (=2 $\mu$m) and the radius $r$ of the bacteria. Thus in the fifth column the number of bacteria inactivated per unit time, $N_k$, can be calculated by using Equation (4.6), by multiplying the chosen value of $\mu$ by the probability, $\varphi$, calculated in the fourth column. Since the number of inactivated bacteria calculated in the fifth column is per unit time, the total time $\Delta t$ needed to inactivate 90% of the bacteria (that is from $1 \times 10^8$ CFU/ml to $1 \times 10^7$ CFU/ml, and from $1 \times 10^7$ CFU/ml to $1 \times 10^6$ CFU/ml and so on) is calculated in the sixth column by dividing the number of bacteria to be inactivated (e.g. $(1 \times 10^8-1 \times 10^7)$ CFU/ml) by $N_k$. From the simulated values obtained using Excel, Log ($N/N_0$) (where $N_0=1 \times 10^8$ CFU/ml and $N = 1 \times 10^8$, $1 \times 10^7$, $1 \times 10^6$ or $1 \times 10^5$ CFU/ml etc.) is plotted against time. The time is given by the summation of $\Delta t$. For example if $\Delta t = 1$ min to inactivate a bacteria suspension from $1 \times 10^8$ CFU/ml to $1 \times 10^7$ CFU/ml and $\Delta t = 2$ min to subsequently inactivate the same bacteria suspension from $1 \times 10^7$ CFU/ml to $1 \times 10^6$ CFU/ml, then values of Log ($N/N_0$) ($=\text{Log}((1 \times 10^7)/(1 \times 10^8)) = -1$) can be plotted against 1 min and of Log ($N/N_0$) ($=\text{Log}((1 \times 10^6)/(1 \times 10^8)) = -2$) can be plotted against 3 min, and so on. The plot of
Log \((N/N_0)\) against time was obtained by experiments. The value of \(\mu\) is varied and the calculation is performed until the best agreement is obtained between the measurements and the numerical calculations. The best agreement is assessed by maximising the \(R^2\) value calculated using experimental and numerical values. Note, that in this paper \(d_c\) is arbitrarily chosen, and it can be appreciated that a different value of \(d_c\) will result in a different value of \(\mu\). For instance, increasing \(d_c\) will result in a decrease in \(\mu\).

### 4.5 Results and discussion

As stated in the Introduction, a type of bacterium and a yeast were chosen to validate the proposed theoretical model. These were chosen mainly because they represent two different species of different sizes. Particle size measurements showed that *E. aerogenes* had a peak diameter of 1.1 \(\mu\)m and *A. pullulans* had a peak diameter of 7.6 \(\mu\)m. Figures 4.2A1 and 4.2B1 show the transmission electron (TEM) and light micrographs of these two microorganisms before and after ultrasound treatment. Microscopy observation confirmed the particle size measurements, where it could be clearly seen that *E. aerogenes* dispersions are made by bacteria cells with sizes close to 0.84 \(\times\) 1.0 \(\mu\)m while the dispersions of *A. pullulans* are made of yeast cells of sizes close to 5.3 \(\times\) 7.1 \(\mu\)m. TEM observation also shows that *E. aerogenes* cells are nearly spherical in the stationary phase, while *A. pullulans* cells are slightly elongated. Upon treatment with ultrasound, some of the microorganism cells are affected. In the case of *E. aerogenes* it can be clearly seen that the bacterial cell wall is broken, and that internal compounds leached out, resulting in a marked reduction of the bacterium size (Figure 4.2A2). In the case of *A. pullulans*, it can be seen that some yeast cells are broken (see arrows), however others retained their integrity (Figure 4.2B2).
Figure 4.2 (A) Transmission electron and light micrographs of *Enterobacter aerogenes* and (B) light micrographs *Aureobasidium pullulans* before (1) and after sonication (2). *E. aerogenes* was sonicated at 12 W for 20 min (20 kHz), and *A. pullulans* was sonicated at 12 W for 20 min (20 kHz). Scale bars correspond to 1 μm for *E. aerogenes* and 20 μm for *A. pullulans*. Inset A3 is the light micrograph of *Enterobacter aerogenes* before sonication, showing the extent of aggregation.

Both the bacteria *E. aerogenes* and the yeast *A. pullulans* suspensions were sonicated under different ultrasound power or initial microorganism number conditions. The experimental results of the sonication treatments are reported in Figures 4.3 and 4.4 for *E. aerogenes* and *A. pullulans*, respectively. In the case of *E. aerogenes*, the logarithm of the survival ratio Log \((N/N_0)\) as a function of sonication time for different sonication powers is reported in Figure 4.3A. Under the sonication conditions used in this study, Log \((N/N_0)\) decreased linearly with time. Similar
behaviour has been reported in the case of the inactivation of *E. coli* (Patil et al., 2009) by ultrasound. As expected, the inactivation of *E. aerogenes* was found to be dependent on the ultrasound power used, and that the higher the power used the more bacteria cells were inactivated. To quantify the effect of the ultrasound power, the *D*-value, which is equal to the sonication time needed to reduce the number of bacteria by 90%, was calculated. *D*-values of 45, 14 and 11 min were obtained when the *E. aerogenes* suspension with an initial number of $\sim 10^8$ CFU/ml was sonicated at 4 W, 8 W and 12 W, respectively. The *D*-values obtained in this study are relatively high; this is due to the ultrasound power used and the type of microorganism. It is very difficult to compare the results of this study to the published literature since the sonication conditions such as ultrasound power and frequency, ultrasound probe used, temperature of sonication and the volume of culture sonicated are different. However, *D*-values close to the lowest value (11 min) measured in this study were previously reported on different bacteria. For example, a *D*-value of 13.73 min was reported for the ultrasound inactivation of *Escherichia coli* ATCC 25922 at temperatures below 30°C (Spinks et al., 2006).
Figure 4.3 *Enterobacter aerogenes* survival ratio \( \log (N/N_0) \) as a function of sonication time for (A) different sonication powers and for (B) different initial cell numbers (20 kHz). In (A) the initial cell number is \( \sim 10^8 \) CFU/ml and sonication powers are: 4 W (■), 8 W (●), and 12 W (▲). Inset in (A) reports the theoretical number of cavitations \( \mu \) as a function of the sonication powers. In (B) symbols for initial cell numbers are: \( \sim 1.2 \times 10^8 \) CFU/ml (■), \( \sim 1.3 \times 10^6 \) CFU/ml (●), and \( \sim 6.5 \times 10^3 \) CFU/ml (▲). Both in (A) and (B) error bars correspond to standard deviation and solid lines are the result of the fit obtained using the proposed model.
Figure 4.4 *Aureobasidium pullulans* survival ratio $\log \left( \frac{N}{N_0} \right)$ as a function of sonication time for (A) different sonication powers and for (B) different initial cell numbers (20 kHz). In (A) the initial cell number is $\sim 10^7$ CFU/ml and sonication powers are: 4 W (■), 8 W (●), 10 W (▲), and 12 W (▽). Inset in (A) reports the theoretical number of cavitations $\mu$ as a function of the sonication powers. In (B) symbols for initial cell numbers are: $\sim 6.0 \times 10^6$ CFU/ml (■), and $\sim 9.8 \times 10^4$ CFU/ml (●). Both in (A) and (B) error bars correspond to standard deviation and solid lines are the result of the fit obtained using the proposed model.
The experimental data were analysed using the model described in the Experimental section, and the results are reported as solid lines in Figures 4.3 and 4.4. An initial number of $1 \times 10^8$ CFU/ml and a diameter of 1 $\mu$m were used for the calculations. In the case of the effect of the inactivation of *E. aerogenes* as a function of different sonication powers, the model well describes the behaviour of the survival ratio as a function of time (Figure 4.3A). The results of the model yield a value of $R^2$ of 0.994, 0.982 and 0.976 for sonication powers of 4, 8 and 12 W, respectively. The value of the number of cavitations per unit time $\mu$ giving the best fits increase linearly with the ultrasound power $P$ (inset Figure 4.3A). The increase of $\mu$ as a function of $P$ is expected, and several authors reported that the number of cavities initially increases with rising ultrasound pressure, before starting to decrease when high ultrasound pressures are reached. However, the linear variation of $\mu$ as a function of $P$ remains to be elucidated. Although, it is worth mentioning that it was found experimentally that the amount of H$_2$O$_2$ generated during ultrasound treatment is proportional to the ultrasound power $P$ (Kanthale et al., 2008). This might be an indication that in fact at lower values of $P$, the number of cavities generated per unit time is proportional to the ultrasound power.

Figure 4.4 reports the results of the sonication treatment with time for *A. pullulans* at different sonication powers (Figure 4.4A) or as a function of *A. pullulans*’ initial number (Figure 4.4B). Similarly to *E. aerogenes*, the rate of the logarithm of the survival ratio Log ($N/N_0$) as a function of sonication time decreased linearly with time. Calculation from the experimental data yielded $D$-values of 364, 92, 26 and 23 min when the *A. pullulans* suspensions were sonicated at 4 W, 8 W, 10 W and 12 W, respectively. Previous studies on the ultrasound inactivation of different strains of *S. cerevisiae*, a yeast known to contaminate beverages, also reported a linear reduction of the survival ratio with sonication time (Ciccolini et al., 1997; Guerrero et al., 2001; Cameron et al., 2008; Wordon et al., 2012). A 5-log reduction value of 21.85 min was also reported for the ultrasound inactivation of *Pichia fermentans* in tomato juice at 24.4 $\mu$m amplitude with a moderate temperature (Adekunte et al., 2010a). Although
the ultrasound treatment conditions are different from those used in this study, high 
\( D \)-values (20–30 min) were reported for the inactivation of \( S. \) cerevisiae at a moderate 
temperature (35°C) (Guerrero et al., 2001). Fitting of the data using the theoretical 
model and a radius of 6 µm for \( A. \) pullulans also resulted in good agreement between 
experiments and theoretical values, with \( R^2 \) values of 0.868, 0.966, 0.992 and 0.963 
when the \( A. \) pullulans suspensions were sonicated at 4 W, 8 W, 10 W and 12 W, 
respectively. The value of the cavitation number per unit time \( \mu \) was also found to 
increase linearly with the ultrasound power \( P \) (inset Figure 4.4B).

Compared to \( E. \) aerogenes, \( A. \) pullulans was found to be more resistant to ultrasound 
treatment, as shown by the \( D \)-value. This is due to the difference in the strength of the 
microorganism capsule to shear forces. \( E. \) aerogenes is a gram-negative bacterium 
and its cell wall consists mainly of proteins, polysaccharides, lipids and mucopolypeptides 
(Salton, 1963). The cell walls of gram-negative bacteria are known to be much thinner 
and weaker than gram-positive bacteria (Schleifer and Kandler, 1972; Zuber et al., 
2006; Drakopoulou et al., 2009). Gram-negative cells consist of a 2–7 nm 
peptidoglycan layer and a 7–8 nm-thick additional structure called the outer 
membrane (Wang and Chen, 2009). In the case of yeast, the cell wall material is 
mainly composed of mannoproteins and \( \beta \)-linked glucans (Klis, 1994), and \( A. \) 
pullulans is known to produce two different exopolysaccharide layers outside of the 
cells including a pullulan layer and a water-insoluble polysaccharide (Shingel, 2004). 
There are several different cell morphologies for \( A. \) pullulans including yeast-like 
cells, young blastospores, swollen blastospores, chlamydospores and mycelia 
(Guterman and Shabtai, 1996; Ronen et al., 2002). It was reported that the cell walls 
of the blastospores of mutant \( A. \) pullulans B-1 and parent strains were 60–150 nm and 
that of the chlamydospore of mutant \( A. \) pullulans B-1 was in the range of 700–1000 
nm (Gniewosz and Duszkiewicz-Reinhard, 2008). Based on the size of the capsules 
and cell wall, \( A. \) pullulans is expected to be more resistant to ultrasound treatment 
than \( E. \) aerogenes, which would explain the findings of the present study.
Figure 4.5 Viscosity (mPa·s) at 25°C for *Enterobacter aerogenes* (■, □) and *Aureobasidium pullulans* (●, ○) as a function of ultrasound treatment (20 kHz). Error bars correspond to standard deviations. *E. aerogenes’* initial cell number is ~10⁸ CFU/ml (■) or ~10⁴ CFU/ml (□). *A. pullulans’* initial cell number is ~10⁷ CFU/ml (●) or ~10⁵ CFU/ml (○). Ultrasound power was 8 W and error bars correspond to standard deviations.

Dispersions of *E. aerogenes* and *A. pullulans* suspensions with different initial cell numbers were also sonicated for different times but at a constant power (Figure 4.3B and 4.4B). For both microorganisms, the survival ratio was found to decrease with the decrease on the initial number. The experimental results show for *E. aerogenes* D-values of 14, 12 and 8 min for initial numbers of ~1.2×10⁸ CFU/ml, ~1.3×10⁶ CFU/ml and 6.5×10³ CFU/ml, respectively. For *A. pullulans* D-values of 92 and 44 min were experimentally obtained for initial numbers of ~6.0×10⁶ CFU/ml and ~9.8×10⁴ CFU/ml counts, respectively. Note that the theoretical model also fits well with the experimental data (by changing µ), with R² values of 0.989, 0.997 and 0.999 for *E. aerogenes* for initial numbers of ~1.2×10⁸ CFU/ml, ~1.0×10⁶ CFU/ml and ~6.5×10³ CFU/ml, respectively; and 0.966 and 0.988 for *A. pullulans* with initial numbers of ~6.0×10⁶ CFU/ml and ~9.8×10⁴ CFU/ml, respectively. This is in conflict with the proposed model that predicts that the inactivation number of cells present in the
suspensions is independent of the initial number. It is not clear yet why the bacteria and yeast inactivation in this study depends on the initial number of microorganisms. This could be due to different parameters such as viscosity, which should depend on the initial number of microorganisms present. An increase in viscosity is expected to decrease the cavitation threshold (Earnshaw et al., 1995; Salleh-Mack and Roberts, 2007). However, our measurement of the viscosity as a function of sonication time for bacteria and yeast suspensions of different initial numbers of microorganism did not show any marked difference in the viscosity (Figure 4.5). The viscosity of the different microorganism dispersions was close to 0.89 mPa·s, which is the viscosity of water at 25°C. Another possible explanation is the state of the aggregation of the microorganisms with their high concentrations. Microscopy observation showed that the both *A. pullulans* and *E. aerogenes* form large aggregates of microorganisms (Figure 4.2A3 and Figure 4.2B1). The extent of aggregation is expected to be higher with the number of microorganisms present. This will result in more resistance to cell break-up by ultrasound, since the local viscosity of the aggregate is much higher than that of the suspensions. Further studies are needed to confirm the effect of the extent of microorganism aggregation on their inactivation by ultrasound treatment.

### 4.6 Conclusions

The main objective of this study was to understand the mechanisms involved in the inactivation of microorganisms by ultrasound treatment. The forces generated upon collapse of the ultrasound cavitation bubble are considered to be the main cause of the reduction in the number of live microorganism cells. A simple model, which assumes that the shear forces generated on the collapse of an acoustic cavitation bubble near a cell are able to break up the cell, is proposed. To validate the theoretical predictions of the model, ultrasound inactivation experiments on two microorganisms, *E. aerogenes*, a bacterium, and *A. pullulans*, a yeast, were carried out. Experimental results showed that the log of the survival ratio of both *E. aerogenes* and *A. pullulans* decreased linearly with the sonication time and that the rate of decrease (*D*-value) decreased...
with the increase in ultrasound power. The $D$-value was found to increase with the decrease in the initial number. This was not expected and this behaviour is not yet understood. However, we speculate that this could be due to the state of the aggregation of the cells, which is expected to be higher for larger initial numbers. Comparison between the results of the theoretical model and the experiments yielded very good agreement, confirming that the inactivation of the microorganisms is due to the forces generated by collapse of the cavitation bubble. However, the model requires two fitting parameters that take into account the ultrasound power and the microorganism cell shear strength. Further work is needed to obtain these parameters through experimental measurements. The model should be also used on other microorganisms, to further demonstrate its usefulness.

4.7 Acknowledgements

S. Gao is supported by a New Zealand China Food Safety Scholarship, funded by the New Zealand Ministry of Foreign Affair and Trade. This manuscript was finalised during the visit of M. Ashokkumar to the University of Auckland, funded through the University of Auckland Distinguished Visitor Award (Project number 3702372).
CHAPTER 5

Inactivation of bacteria and yeast using high-frequency ultrasound treatment

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The content of this chapter has been published
5.1 Abstract

High-frequency (850 kHz) ultrasound was used to inactivate bacteria and yeast at different growth phases under controlled temperature conditions. Three species of bacteria, *Enterobacter aerogenes*, *Bacillus subtilis* and *Staphylococcus epidermidis* as well as a yeast, *Aureobasidium pullulans* were considered. The microorganisms were sonicated at different powers (9, 15, 50, 62 W) for 2.5 min to 60 min. The study showed that high-frequency ultrasound was highly efficient in inactivating the bacteria in both their exponential and stationary growth phases, and inactivation rates of more than 99% were achieved. TEM observation suggests that the mechanism of bacteria inactivation is mainly due to free radicals and H$_2$O$_2$ generated by acoustic cavitation. The rod-shaped bacterium *B. subtilis* was also found to be sensitive to the mechanical effects of acoustic cavitation. The study showed that the inactivation process continued even after ultrasonic processing ceased due to the presence of H$_2$O$_2$, generated during acoustic cavitation. Compared to bacteria, the yeast *A. pullulans* was found to be more resistant to high-frequency ultrasound treatment.

**Keywords:** High-frequency ultrasound; Bacteria inactivation; Yeast; *Enterobacter aerogenes*; *Bacillus subtilis*; *Staphylococcus epidermidis*; *Aureobasidium pullulans*. 
5.2 Introduction

Water is extensively used in the food industry, ranging from being an ingredient, transport medium to a sanitation tool. Water quality and food safety are inextricably linked and people can suffer from serious illness if water is contaminated by bacteria and other microorganisms. Chlorine disinfection is widely used to eliminate microorganisms in water through chemical oxidation (Cheremisinoff and Cheremisinoff, 1993). However, some bacteria are chlorine tolerant, and need higher chlorine levels or prolonged chlorination time during water treatment. This results in the development of unpleasant flavours due to the formation of chlorophenols (Phull et al., 1997; Drakopoulou et al., 2009). In some cases, certain bacteria are not effectively inactivated by chlorination as they form clusters (Phull et al., 1997). As an alternative result, the use of ultrasound treatment can be considered a potential method for the sterilisation of microorganisms in water, liquid foods and for the treatment of wastewater.

The use of ultrasound in bacteria inactivation was first reported in the 1920s (Harvey and Loomis, 1929), and the fundamental mechanism of bacterial inactivation was explored during the 1950s and 1960s (Davies, 1959; Earnshaw et al., 1995). Biocidal effects of ultrasound treatment are suggested to be due to mechanical effects and sonochemical reactions produced by acoustic cavitation (Mason, 1990; Earnshaw et al., 1995; Sala et al., 1995; Butz and Tauscher, 2002; Russell, 2002; Mason et al., 2005; Wu and Nyborg, 2008; Ashokkumar, 2011). Acoustic cavitation is the formation, growth and collapse of microbubbles in liquid media. When ultrasound passes through liquid media, cavitation bubbles are generated, which collapse when they reach their critical size (Leighton, 1994). Mechanical effects such as shear forces and micro-jettings are produced during the collapse of cavities, and the energy released can damage bacteria (Joyce et al., 2003a; Mason et al., 2003; Mason et al., 2005; Wu and Nyborg, 2008; Collis et al., 2010; O'Donnell et al., 2010; Soria and Villamiel, 2010). Although cavitation can be produced during both low-frequency and
high-frequency ultrasound treatments, the energy released during the collapse of cavities is different at these frequencies. Low-frequency ultrasound generates larger cavitation bubbles and their collapse results in a higher energy release compared to high-frequency ultrasound. Note that for ultrasound treatment, high frequency is defined as a frequency higher than 100 kHz (Mason, 1990; Piyasena et al., 2003; Patist and Bates, 2008). For high-frequency ultrasound, in addition to physical forces, more free radicals are generated compared to low-frequency ultrasound, and this is attributed to the fact that there are more cavitation events occurring (Crum, 1995). Energy released at the moment of the cavitation bubble collapse is sufficient to break chemical bonds, and if the medium is water, then the water molecules are broken into hydrogen atoms and hydroxyl radicals (OH\(^-\)) (Leighton, 1994). Particularly, hydroxyl radicals are markedly produced during high-frequency ultrasonication (He et al., 2006). These hydroxyl radicals can attack the cell wall membrane, and also cause the recombination of the radicals into hydrogen peroxide, a well-known bactericide, also contributes to bacteria inactivation (Al Bsoul et al., 2010; Joyce et al., 2011). Although the inactivation of bacteria by ultrasonic treatment has been widely examined recently, most of the research has focused on low-frequency ultrasonic inactivation. In addition, most studies reported in the literature focused only on one or two bacteria. The main aim of this study is to determine the effects of high-frequency (850 kHz) ultrasound treatment on bacteria and yeast in their different growth phases, and to obtain a deep insight into the mechanism of microbial inactivation by high-frequency ultrasound treatment.

Microorganisms with different sizes, shapes, and types of cell wall (Gram nature) were selected, namely *Enterobacter aerogenes*, *Bacillus subtilis* and *Staphylococcus epidermidis* and a yeast, *Aureobasidium pullulans*. *E. aerogenes* exists widely in water, dairy products, soil, as well as human and animal faeces (Grimont and Grimont, 2006). *B. subtilis* was chosen as one of the indicators of water treatment efficiency in a water treatment plant (Huertas et al., 2003). *S. epidermidis* as a common skin organism (Schleifer, 2009) is known to easily adhere to surfaces to form biofilms
(Katsikogianni et al., 2006), this happens on water pipe surfaces which can cause potential microbiological contamination (Wingender and Flemming, 2004). *A. pullulans* is found ubiquitously in various environments including water, soil, wood, rocks with high humidity and plant materials (Urzi et al., 1999; Chi et al., 2009). *E. aerogenes* is a gram-negative rod-shaped bacterium from the family *Enterobacteriaceae* and the size range of these bacteria are 0.3–1.0 µm×1.0–6.0 µm (Imhoff, 2005). *B. subtilis* is a gram-positive rod-shaped bacterium that has a size range of 0.7–0.8 µm×2.0–3.0 µm (Schleifer, 2009). *S. epidermidis* is a gram-positive coccus and its diameter is 0.8–1.0 µm (Schleifer, 2009). *A. pullulans* is a yeast-like fungus, also popularly known as black yeast (Cooke, 1959; Hoog, 1993), and is 2–13 µm wide (Zalar et al., 2008). Low-frequency ultrasound has been studied widely for the disinfection of wastewater, involving microbes such as *Escherichia coli* XL-1 Blue (24 kHz) (Antoniadis et al., 2007), *E. coli* K12 (24–80 kHz) (Paleologou et al., 2007), total coliforms, faecal coliforms, *Pseudomonas* spp. faecal streptococci and *Clostridium perfringens* species (24 kHz) (Drakopoulou et al., 2009). The bacteria and yeast considered in this study were previously investigated for their behaviour under low-frequency ultrasound treatment (Singer et al., 1999; Joyce et al., 2003b; Gao et al., 2014a; Gao et al., 2014b). *B. subtilis* spores were disrupted by high-frequency ultrasonication using dual transducers (Warner et al., 2009). The synergic effect of high-frequency ultrasound and vancomycin on *S. epidermidis* biofilm was also reported in a study that focused mainly on the mechanical effects induced by ultrasound such as the enhancing transportation of vancomycin (Dong et al., 2013). However, to the best of our knowledge, there are no reports about high-frequency ultrasonication of *E. aerogenes* and *A. pullulans*. 
5.3 Materials and methods

5.3.1 Materials

Hydrogen peroxide (30%) and t-butanol were obtained from AR ECP Ltd, New Zealand. Glucono-δ-lactone (GDL) was purchased from Sigma Aldrich (USA). GDL was used to adjust the acidic pH of the bacteria cultures, and t-butanol was used as a hydroxyl radical scavenger. All other chemicals were of analytical grade and were purchased from Sigma Aldrich.

5.3.2 Preparation of microbial suspensions

*E. aerogenes*, *B. subtilis*, *S. epidermidis*, and *A. pullulans* were obtained from the Microbiology Laboratory of the School of Biological Sciences at the University of Auckland, New Zealand. 1 ml of bacterial stock stored at −80°C freezer was incubated overnight on Nutrient agar at 37°C by spread method. Then a loop of the bacterial colony was inoculated into 50 ml of Nutrient broth to be incubated at 37°C overnight while shaking (200 rev min⁻¹). After incubation, a working bacterial culture was prepared. 4 ml of working culture was then transferred into 100 ml of fresh Nutrient broth to be incubated under the same conditions. The bacteria suspensions at the exponential phase were obtained after 2 h, 5 h and 3 h incubation for *E. aerogenes*, *B. subtilis* and *S. epidermidis*, respectively, and the bacteria suspensions at the stationary phase were obtained after 20 h incubation. *A. pullulans* was incubated at 28°C, and the yeast suspension to be ultrasonicated was obtained after 72 h growth.

A physiological salt solution (0.9% NaCl, PSS) was used to wash microbial suspensions in order to remove excess nutrients while keeping the bacteria and yeast alive. Bacterial suspensions were centrifuged at 10,000 g for 10 min at 4°C using a Biofuge Stratos centrifuge (Heraeus, Thermo Electron Corporation, Germany). The supernatant was discarded and the pellet was resuspended in PSS. *A. pullulans* was centrifuged at 3,000 g for 10 min at 4°C, and the pellet of each sample was washed
twice. After washing, the initial number of each species was estimated by measuring
the optical density at 600 nm using a HeλIOS β UV-visible spectrophotometer
(Thermo Electron Corporation, UK).

5.3.3 Ultrasound treatment

Bacterial and yeast suspensions were ultrasonicated by a K80 ultrasound generator
(Meinhardt Ultraschalltechnik, Germany) at 850 kHz, which was connected to an
ultrasonic transducer E/805/T and a double-walled cylindrical glass vessel. The vessel
was connected to a water bath (PolyScience SD07R-20-A12E, USA). The glass vessel
was filled with 250 ml of Milli-Q water. 5 ml of microbial suspensions were
transferred into a 15 ml glass tube. The tube was then inserted through a PVC cover
of the vessel and positioned in the centre of the vessel. The lids of the tubes were held
tightly by the PVC cover in order to keep the tubes in the same position. Circulating
water baths (2°C) were used to control the temperature during ultrasonication. All
ultrasound treatments were performed at least in duplicate.

The ultrasound power $P$ was determined using the calorimetric method (Koda et al.,
2003; Kikuchi and Uchida, 2011):

$$P = mC_p(\Delta T/\Delta t)$$  \hspace{1cm} (5.1)

where $C_p (=4.18 \text{ J/(g K)})$ is the specific heat capacity of water, $m$ is the mass of
ultrasonicated water, $\Delta T$ is the increase in the temperature, and $\Delta t$ is the applied
ultrasound time.

5.3.4 Microbial enumeration

A slightly modified Miles-Misra method (Miles et al., 1938) was used to count the
viable microbial cells. Unless specified, bacteria and yeast counts were performed 2 h
after ultrasound treatment. For each sample, serial 1:10 dilutions from $10^0$ to $10^6$
times depending on the initial number of microorganisms were made in a 96 Well Tissue Culture plate (Cellstar, Greiner bio-one, Germany) by mixing PSS with bacterial or yeast samples. Nutrient agar plates were marked into six sections in advance. Each diluted sample (50 µl × 3) was dropped onto three sectors. After that, the plates were incubated at 37°C overnight for the bacteria and at 28°C for 24 to 36 h for the yeast. The countable number (15 to 150) of colonies for each section was counted under a light microscope. The original bacterial number was calculated using:

\[ N = \bar{n} \times d_i \times 20 \]  

(5.2)

where \( N \) is the number of total colonies (CFU/ml), \( \bar{n} \) is the average number of colonies for a dilution and \( d_i \) is dilution factor. Note that a large volume of sample (100 µl × 3) was used when the number of bacteria was low.

5.3.5 Hydrogen peroxide measurement

A colorimetric method (Awtrey and Connick, 1951; Weissler, 1959; Alegria et al., 1989) was used for the measurement of the yield of \( \text{H}_2\text{O}_2 \) by using a UV-Vis absorption spectrophotometer (UV mini 1240, Shimadzu, Japan) at 353 nm wavelength. The potassium iodide (KI) used in this method was obtained from ECP Ltd, New Zealand, and all other chemicals were purchased from Sigma-Aldrich, USA. The iodide reagent was made by mixing 1 ml of Reagent A (3.32 g potassium iodide, 0.10 g sodium hydroxide, 0.01 g ammonium molybdate tetrahydrate in 50 ml of water) and 1 ml of Reagent B (1.021 g potassium hydrogen phthalate). 1 ml of ultrasonicated sample was transferred into 2 ml of iodide reagents before measuring the absorbance. The concentration of \( \text{H}_2\text{O}_2 \) (\( C, \text{M} \)) can be calculated by the following equation:

\[ C = A/(\varepsilon l) \times 3 \]  

(5.3)

where \( A \) is the absorbance value at 353 nm, \( \varepsilon \) is the molar extinction coefficient (26400 M\(^{-1}\)cm\(^{-1}\)), \( l \) is the path length of the cuvette (1 cm), and 3 is dilution times.
5.3.6 TEM

Bacteria samples were stained by 0.2% or 2% aqueous uranyl acetate before examining by transmission electron microscope (TEM) (CM12 TEM, Philips, Netherlands). In order to maintain the hydrophilic surface of carbon-coated copper TEM grids, the grids were glow discharged at 500 V for 15 s. The discharged grids were immersed into the bacteria suspensions for 30 s then washed twice by quickly dipping them into Milli-Q water and drying by filter paper. The dried grids with E. aerogenes and B. subtilis were then immersed into a drop of 2% uranyl acetate for 30 s, and the grid with S. epidermidis was stained by 0.2% uranyl acetate for 10 s. After that the stained grids were dried with fresh filter paper before microscope observation.

5.4 Results and discussion

5.4.1 Generation of OH• and H• radicals and H2O2

As mentioned previously, acoustic cavitation induces mechanical effects and sonochemical reactions such as the formation of hydroxyl radicals during high-frequency ultrasonication. For low-frequency high-power ultrasound treatment of bacteria, inactivation was mainly due to mechanical effects induced by acoustic cavitation (Gao et al., 2014a; Gao et al., 2014b). However in the case of high-frequency ultrasonication, sonochemical effects (Koda et al., 2009) and the combination of both mechanical and sonochemical effects (Hua and Thompson, 2000) were suggested as the main mechanism for the inactivation of bacteria. Thus, it is important to obtain an indication about the amount of hydroxyl radicals generated by the ultrasound unit. This can be indirectly quantified by measuring the H2O2 yield (Weissler, 1959; Alegria et al., 1989). The primary sonochemical reactions in aqueous solutions involve the generation of OH\(^-\) and H\(^-\) radicals and H2O2 (Reactions 5.4 and 5.5) (Dai et al., 2006; Olvera et al., 2008; Thangavadivel et al., 2009):

\[ \text{H}_2\text{O} + \rightarrow \text{OH}^- + \text{H}^+ \] (5.4)
The iodide method is commonly used to quantify the concentration of hydrogen peroxide produced during ultrasonication (Weissler, 1959; Alegria et al., 1989). Iodide ions are oxidised by hydrogen peroxide to form molecular iodine (Reaction 5.6). The molecular iodine can react with the excess iodide ions to generate an $I_3^-$ complex (Reaction 5.7), which can be detected by UV measurements at 353 nm.

\[
2 \text{OH}^* \rightarrow \text{H}_2\text{O}_2 \quad (5.5)
\]

\[
\text{H}_2\text{O}_2 + 2 \text{I}^- \rightarrow \text{I}_2 + 2 \text{OH}^- \quad (5.6)
\]

\[
\text{I}_2 + \text{I}^- \rightarrow \text{I}_3^- \quad (5.7)
\]

Figure 5.1 reports the amounts of H$_2$O$_2$ generated in water during ultrasonication at different ultrasound power at a constant sonication time of 20 min. A marked increase in the amount of generated H$_2$O$_2$ can be clearly seen for ultrasound power higher than 10 W. These results are in agreement with previous studies that showed that high-frequency ultrasonication results in an increase of H$_2$O$_2$ with increasing ultrasonication power (Kanthale et al., 2008). It should be noted that at post ultrasonication, the amount of the H$_2$O$_2$ slowly decreased with time (results not shown). The increase in H$_2$O$_2$ with an increase in acoustic power was also mirrored by a decrease in the pH of the ultrasonicated PSS aqueous solution, which decreased from a value of 6.0 before sonication to a value of 3.7 after sonication at 62 W for 20 min (Figure 5.1). The pH was measured because acidic conditions are known to contribute to the inactivation of some microorganisms such as *Escherichia coli* (Salleh-Mack and Roberts, 2007). The pH decrease during ultrasonication can be explained by the formation of nitrous and nitric acid (Mead et al., 1976; Supeno and Kruus, 2000), and the formation of carbonic acid by the dissolution of carbon dioxide (Semenov et al., 2011) by ultrasonication.
Figure 5.1 Hydrogen peroxide (■) produced by high-frequency sonication of Milli-Q water and pH (●) of PSS, as a function of the ultrasound power. Ultrasound frequency was 850 kHz and sonication time was 20 min. Error bars correspond to standard deviations.

5.4.2 Effect of sampling time on bacteria counts

Since the production of OH⁻ and H⁺ radicals and H₂O₂ are known to inactivate microorganisms and that the amount of the H₂O₂ decrease with time of post ultrasonication as indicated above, it is important to study the effect of sampling time on the bacteria counts. Sampling time here corresponds to the time after ultrasonication at which the ultrasound-treated bacteria samples were cultured on agar gels in order to determine the survival rate. A preliminary experiment was performed where *E. aerogenes* was ultrasonicated under different ultrasound powers and sonication time conditions. These conditions corresponded to ultrasonication at 50 W for 20 min, 62 W for 10 min and 62 W for 20 min. A non-ultrasonicated *E. aerogenes* suspension was also used as a control. The ultrasound-treated *E. aerogenes* suspensions were kept on ice after ultrasonication and aliquots were drawn at regular time intervals to be grown on agar plates for viable bacteria numbering.
Figure 5.2 shows the effect of sampling time on the Log ($N/N_0$) of the survival ratio of the non-sonicated and sonicated *E. aerogenes* dispersions. In the case of the non-sonicated dispersion there was no effect of the sampling time for up to 12 h (only the first 4 h are shown in Figure 5.2). This was expected since the non-sonicated suspensions were kept on ice and their viable number was not expected to increase nor decrease during that time. For the bacteria suspension treated at 50 W for 20 min, there was no obvious difference after 4 h storage. However, in the case of bacteria suspensions treated at higher acoustic powers, there was clearly an effect of sampling time. Under these conditions, the Log ($N/N_0$) of the ultrasonicated dispersions decreased linearly with an increase in the sampling time. The decrease in viable bacteria was also noticeable for suspensions sonicated at 62 W for 20 min, the decrease in Log ($N/N_0$) was two folds higher in magnitude when the suspension was left for 200 min after sonication. This was also expected since the longer the bacteria suspensions remain in an acidic environment in the presence of H$_2$O$_2$, the higher the number of bacteria was inactivated. This result is very important as it demonstrates clearly that the sampling time has a direct consequence on the counting of the number of viable bacteria. For this reason, it was decided that in the present work all bacteria counts would be performed at a constant time (2 h) after sonication, to ensure reproducibility of the bacteria counting results.
Figure 5.2 Log of survival ratio (Log \( \frac{N}{N_0} \)) of *Enterobacter aerogenes* as a function of storage time (different sampling time after ultrasonication) for bacterial suspensions sonicated under different conditions (850 kHz). Control (without ultrasound treatment) (■); ultrasonicated at 50 W for 20 min (●); ultrasonicated at 62 W for 10 min (▲); and ultrasonicated at 62 W for 20 min (▼). Error bars correspond to standard deviations.

5.4.3 Ultrasound treatment of bacteria

The effects of high-frequency (850 kHz) ultrasound treatments of different bacteria at different growth phases were investigated. The initial numbers of the bacteria were approximately \( \sim 10^8 \) CFU/ml. Figure 5.3 shows the survival ratio Log \( \frac{N}{N_0} \) of *E. aerogenes, B. subtilis* and *S. epidermidis* at both exponential and stationary phases as a function of ultrasound power. All results indicated that the bacteria inactivation had been enhanced with the increasing ultrasound power at both the exponential and stationary phase. There were \( \sim 4.2 \), \( \sim 2.5 \) and \( \sim 4.4 \) log reductions achieved at 62 W for 20 min ultrasonication in the stationary phase for *E. aerogenes, B. subtilis* and *S. epidermidis*, respectively. Moreover, there was no marked difference in the inactivation ratio between the bacteria in the exponential phase and stationary phase. In addition, there was no obvious effect on the bacterial viability at lower power ultrasound treatment (at 9 W or 15 W), which is likely due to the lower concentration
of hydroxyl radicals and hydrogen peroxide produced at lower powers as shown in Figure 5.1.

**Figure 5.3** Log of survival ratio (Log ($N/N_0$)) for ultrasonicated bacteria for 20 min as a function of different ultrasound powers (850 kHz). (A) *Enterobacter aerogenes*, (B) *Bacillus subtilis* and (C) *Staphylococcus epidermidis*. Symbols are: bacteria at stationary phase (■); and bacteria at exponential phase (●). Error bars correspond to standard deviations.
It is likely that the inactivation of the bacteria is due to the generated hydroxyl radicals and H$_2$O$_2$ directly attacking bacterial cells. The formation of free radicals during ultrasonication was demonstrated previously by electron spin resonance spectroscopy and spin trapping techniques (Riesz et al., 1985). It is also known that the effects of free radicals on cells include damage to DNA, enzymes, liposomes and membranes (Riesz and Kondo, 1992). For instance, hydroxyl radicals are known to oxidise macromolecules present in cells, including DNA, lipids and proteins (Cabiscol et al., 2010; Liu et al., 2011). It is also known that the chemical structure of bacteria cell walls can be attacked, weakened and then disintegrated by free radicals produced during cavitation (Joyce et al., 2003b). For example, OH$^-$ radicals were reported to play an important role in ozone inactivation of \textit{B. subtilis} spores (Cho et al., 2002). In addition to the direct effects of OH$^-$ radicals on bacteria cells, it was suggested that hydroxyl radicals and H$_2$O$_2$ might react with peroxidase released from the bacterial cells as a result of the mechanical damage due to ultrasound cavitation (Jyoti and Pandit, 2003). These reactions might result in the formation of oxygen radicals, which can further affect the viability of cells by attacking the cell membranes (Jyoti and Pandit, 2003).

TEM observations were performed to investigate the physical changes induced by high-frequency sonication on the bacteria cells (Figure 5.4). It can be seen that the structure of these three bacterial species was significantly affected by sonication. Prior to sonication, the surfaces of bacteria remained stable, intact and smooth. After sonication, \textit{E. aerogenes} was significantly damaged and the cells were misshapen and malformed, but remained as whole cells, although the cells lost their turgor pressure (Figures 5.4A2-4). The effect of high-frequency sonication on \textit{E. aerogenes} cells was similar to the results from the addition of H$_2$O$_2$ (Figures 5.4B2-4) confirming again that the main mechanism of inactivation is the attack of hydroxyl radicals and H$_2$O$_2$ during ultrasound treatment. It should be noted that the physical effect of sonication on \textit{E. aerogenes} cell structure (Figure 5.4 A2-4) seems far greater than addition of H$_2$O$_2$ (Figure 5.4 B2-4). It is possible that pH and physical turbulence might have contributed during cavitation. For \textit{S. epidermidis}, similar results were obtained, with most of the sonicated cells remaining whole, and in some cases leakage of inner contents was also observed (Figures 5.4D2-4). However, in the case of \textit{B. subtilis} both
whole cells and broken parts were observed after sonication (Figures 5.4C2-4). This is an indication that *B. subtilis* can be inactivated through breakage due to the mechanical effects of ultrasound cavitation. This is not surprising as this bacterium was found to be very sensitive to acoustic cavitation compared to *E. aerogenes* and *S. epidermidis*. This is due to the fact that *B. subtilis* cells are rod-shaped and these are known to be more susceptible to breaking under acoustic cavitation compared to cocci-shaped ones (Ahmed and Russell, 1975; Alliger, 1975), and *S. epidermidis* is also known to have a thick capsule layer, which protects it against mechanical damage by cavitation (Gao et al., 2014a).

**Figure 5.4** Transmission electron micrographs of (A) *Enterobacter aerogenes*, (C) *Bacillus subtilis* and (D) *Staphylococcus epidermidis* before (1) and after (2, 3 and 4) ultrasound treatments (850 kHz, 62 W, 20 min). (B) *Enterobacter aerogenes*, before (1) and after (2, 3 and 4) the addition of H$_2$O$_2$ (200 mM).
In order to further demonstrate the biocidal effects of hydroxyl radicals and H$_2$O$_2$ on bacterial cells and estimate the contribution of mechanical effects on bacterial inactivation by high-frequency ultrasound treatment, $t$-butanol, an excellent scavenger of hydroxyl radicals, was added to the bacteria culture prior to ultrasonication. $t$-butanol scavenges hydroxyl radicals according to the following the reaction (Kumar et al., 2003):

$$\cdot{\text{OH}} + (\text{CH}_3)_3\text{COH} \rightarrow \text{H}_2\text{O} + \cdot\text{CH}_2\text{C(Ch}_3)_2\text{OH}$$

In addition, it is also known that the cavitation bubble temperature is lowered by the presence of $t$-butanol at high frequencies. This is due to the evaporation of $t$-butanol into the bubble followed by the accumulation of pyrolysis products within the bubble (Ashokkumar, 2011). A lower bubble temperature and the presence of pyrolysis products within the bubble also decreases the amount of OH’ radicals generated within cavitation bubbles.

Figure 5.5 shows the Log of survival ratio (Log ($N/N_0$)) of different bacteria ultrasonicated in the presence of different amounts of $t$-butanol. The addition of up to 100 mM of $t$-butanol alone to bacteria cultures did not result in their inactivation (result not shown). Additionally, the ultrasonication of E. aerogenes and S. epidermidis in the presence of 10 mM of $t$-butanol did not result in the inactivation of these two bacteria (Figure 5.5, square and triangle symbols). For E. aerogenes, four various concentrations of $t$-butanol were added, 0.7 mM, 3 mM, 10 mM, 30 mM and 100 mM. It was found that E. aerogenes was almost not inactivated when a power of 50 W was used, and the log reduction increased with the decreasing concentrations of $t$-butanol at 62 W. A 2.3-log reduction was achieved when 0.7 mM of $t$-butanol was added, and only 0.04 to 0.2 log reductions were achieved for higher $t$-butanol concentrations. For S. epidermidis sonicated at 62 W, a 0.3-log reduction was achieved when 10 mM of $t$-butanol was added. However, for B. subtilis, log reductions of 1.6 and 2.0 were achieved in the presence of 10 mM of $t$-butanol and
ultrasounds at 50 W and 62 W, respectively. These values of log reduction remained smaller than the 2.8 and 2.9-log reductions achieved under similar sonication condition but with no added t-butanol (Figure 5.3.B). This would indicate that although most of the free radicals were scavenged in the presence of 10 mM of t-butanol, the mechanical effects due to cavitation still played an important role in the inactivation of *B. subtilis*. This also confirms TEM observation clearly showed that *B. subtilis* cells break under high-frequency ultrasonication.

![Figure 5.5](image)

**Figure 5.5** Log of survival ratio (Log \((\frac{N}{N_0})\)) of bacteria as a function of the concentration of t-butanol. *Enterobacter aerogenes* at 50 W (□); *Enterobacter aerogenes* at 62 W (■); *Bacillus subtilis* at 50 W (○); *Bacillus subtilis* at 62 W (●); *Staphylococcus epidermidis* at 50 W (▲); *Staphylococcus epidermidis* at 62 W (▲). Ultrasound frequency was 850 kHz and error bars correspond to standard deviations.

**5.4.4 Post ultrasonication effects**

As stressed earlier, care needs to be taken in the sampling time before bacteria counting (Figure 5.2) since the effect of high-frequency ultrasound continues to affect bacteria survival after the ultrasonication treatment ceases. Several experiments on *E.*
*aerogenes* were performed in the present study in order to investigate the origin of these effects, and the results are summarised in Table 5.1. Firstly as shown in Figure 5.1, high-frequency sonication results in a marked decrease in the pH of the bacteria solution from 6.0 to pH 3.7. To investigate the effect of pH alone, the pH of PSS solution was adjusted to pH 3.7 by the addition of HCl or Glucono-δ-lactone (GDL). These two acidification methods were used to determine if the inactivation by HCl addition is due to chloride. Two *E. aerogenes* suspensions with different initial numbers (∼1.49×10^8 CFU/ml and ∼1.73×10^6 CFU/ml) were added to the acidified PSS solutions. Experimental results showed that no change in the bacteria number was observed when the bacteria were placed in acidic conditions (pH 3.7) for 2 h (Table 5.1). This experiment allows excluding the contribution of the acidic environment alone to the inactivation of *E. aerogenes*.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Initial Number (CFU/ml)</th>
<th>Log Reduction</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic PSS (by HCl)</td>
<td>3.7</td>
<td>1.49×10^8</td>
<td>0.00</td>
<td>0.05</td>
</tr>
<tr>
<td>Acidic PSS (by HCl)</td>
<td>3.7</td>
<td>1.73×10^6</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Acidic PSS (by GDL)</td>
<td>3.7</td>
<td>1.49×10^8</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>Acidic PSS (by GDL)</td>
<td>3.7</td>
<td>1.73×10^6</td>
<td>0.07</td>
<td>0.03</td>
</tr>
<tr>
<td>Adding H₂O₂ in Acidic PSS</td>
<td>3.7</td>
<td>1.46×10^8</td>
<td>0.00</td>
<td>0.04</td>
</tr>
<tr>
<td>Adding H₂O₂ in Acidic PSS</td>
<td>3.7</td>
<td>1.26×10^6</td>
<td>0.08</td>
<td>0.06</td>
</tr>
<tr>
<td>Adding H₂O₂ in PSS*</td>
<td>6.0</td>
<td>1.41×10^8</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>Adding H₂O₂ in Acidic PSS*</td>
<td>3.7</td>
<td>1.41×10^8</td>
<td>1.66</td>
<td>0.84</td>
</tr>
<tr>
<td>Ultrasonicated PSS</td>
<td>3.7</td>
<td>1.39×10^8</td>
<td>2.59</td>
<td>0.23</td>
</tr>
<tr>
<td>Ultrasonicated PSS</td>
<td>3.7</td>
<td>1.40×10^6</td>
<td>2.75</td>
<td>1.13</td>
</tr>
<tr>
<td>Ultrasonicated PSS + t-butanol</td>
<td>6.3</td>
<td>1.55×10^8</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Ultrasonicated PSS + t-butanol</td>
<td>6.3</td>
<td>1.18×10^6</td>
<td>0.06</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Note: (1) The reaction time without sonication of bacteria in buffers was 2 h except where labelled (*: 24 h). (2) GDL stands for Glucono-δ-lactone. (3) Added H₂O₂: 5.88 mM. (4) Ultrasonicaton conditions: 850 kHz, 62 W, 20 min.
The second experiment performed was through the addition of *E. aerogenes* into pH-adjusted PSS (3.7 or 6.0) containing H$_2$O$_2$. The amount of H$_2$O$_2$ added was 5.88 mM (200 mg/l) which is 27 times higher than the amount (0.214 mM) measured in sonicated PSS solution for 20 min at 62 W (Figure 5.1). This amount was chosen as it was previously reported that 150 mg/l H$_2$O$_2$ is effective in the inactivation of bacteria (Jyoti and Pandit, 2003). The PSS solution was also adjusted to pH 3.7 using HCl to take into account the effect of the acidic environment similar to the results from sonication. Two *E. aerogenes* cultures with different initial numbers of $\sim 1.49 \times 10^8$ CFU/ml and $\sim 1.73 \times 10^6$ CFU/ml cultures were added to this PSS solution and left for 2 h at 4°C. A very slight decrease in bacteria number (log reduction <0.1) was observed (Table 5.1). Note that when the bacteria was left for 2 h in a PSS solution containing the same amount of H$_2$O$_2$ but with a pH of 6.0, similar results on the number of bacteria inactivated were obtained. However, when the bacteria culture was added to the PSS solution with 5.88 mM of H$_2$O$_2$ with a pH of 3.7 and left for 24 h, the number of bacteria decreased markedly and a log reduction of 1.66 was obtained. This shows that the effect of H$_2$O$_2$ is time dependent and that the longer the residence time of the bacteria in a medium containing H$_2$O$_2$ the higher the extent of their inactivation. A possible way to increase the efficiency of H$_2$O$_2$ at inactivating bacteria is by increasing the amount of H$_2$O$_2$ added rather than increasing the residence time of the bacteria culture. Figure 5.6 shows the Log of survival ratio (Log ($N/N_0$)) for *E. aerogenes* as a function of residence time of H$_2$O$_2$ in a PSS solution (pH 6.0). It can be seen that for 0.214 mM and 5.88 mM of H$_2$O$_2$ there was no marked change in the Log ($N/N_0$) for a residence times of up to 2 h. This confirms the results reported in Table 5.1 for PSS solutions containing 5.88 mM under acidic and non-acidic conditions. However, when the H$_2$O$_2$ is increased to 200 mM, Log ($N/N_0$) decreases markedly with the increase in residence time to reach a value of 3.5 after incubation for 2 h.
Figure 5.6 Log reduction of *Enterobacter aerogenes* as a function of reaction time after addition of different H$_2$O$_2$ amounts: 0.214 mM (■), 5.88 mM (●), 200 mM (▲). Note that ultrasonication at 62 W for 20 min produces 0.214 mM H$_2$O$_2$. Error bars correspond to standard deviations.

As a well-known powerful oxidant, H$_2$O$_2$ is widely used as a biocide and it is believed that the core of the oxidation effect of hydrogen peroxide is due to hydroxyl radicals generated during the decomposition of H$_2$O$_2$ (Wolfe et al., 1989; Jyoti and Pandit, 2003; Petri et al., 2011). Previous studies showed that 1% (294 mM) to 3% (882 mM) H$_2$O$_2$ have been used as dental and contact lens disinfectant solutions (Hughes and Kilvington, 2001; Walker et al., 2003), and 1% (294 mM) H$_2$O$_2$ was used to decontaminate apples (Sapers and Sites, 2003). It was observed that approximately a 3-log reduction was achieved when *Escherichia coli* K12 was exposed to 50 mM of H$_2$O$_2$ at 37°C for 15 min (Imlay and Linn, 1986). It was also reported that 5 mg/l of H$_2$O$_2$ (0.147 mM) treatment for 15 min caused 13%, 9% and 9% reductions in bore well water for total coliforms, fecal coliforms and fecal streptococci individually, while there was a 95%, 96% and 88% reduction achieved, respectively when a combination of ultrasonic bath and 5 mg/l H$_2$O$_2$ (0.147 mM) treatments were used.
(Jyoti and Pandit, 2003). Clearly, the minimum amount of H$_2$O$_2$ required to inactivate bacteria is still not well known, and it might depend on the bacteria species. Further, it needs to be clarified that temperature and other conditions used in the published literature are different. Thus, in light of the different results reported in the literature on the effect of H$_2$O$_2$ on the inactivation of bacteria, the results obtained in the present study are not unexpected. For instance, it was observed that 100 mg/l H$_2$O$_2$ treatment alone without catalyst stored in the dark conditions for 20 min did not inactivate $E.\text{coli}$ K12 (Paleologou et al., 2007).

Clearly, while H$_2$O$_2$ can inactivate $E.\text{aerogenes}$, the amounts of H$_2$O$_2$ required to achieve this within 2 h are much higher than that generated during ultrasonication. It was decided to perform further experiments as follows. First the PSS solution alone was ultrasonicated at 62 W for 20 min, and then the ultrasonication treatment was stopped. Subsequently, a bacteria culture was added to the ultrasonicated solution and left at 4°C for 2 h. Bacteria counts showed a log reduction of $2.59 \pm 0.23$ and $2.75 \pm 1.13$, for $E.\text{aerogenes}$ cultures with an initial number of $\sim 1.39 \times 10^8$ CFU/ml and $\sim 1.40 \times 10^6$ CFU/ml, respectively (Table 5.1). When the same experiments were repeated by adding bacteria cultures into sonicated PSS solutions containing 10 mM of $t$-butanol, only a very slight log reduction ($\sim 0.02$) was achieved (Table 5.1). This finding, in addition to the experiments involving the addition of H$_2$O$_2$ described above, shows that the most important effect in bacteria inactivation by high-frequency sonication is due to OH’ radicals. However, these OH’ radicals are expected to undergo a reaction to form H$_2$O$_2$, and while H$_2$O$_2$ can contribute to the inactivation of $E.\text{aerogenes}$, its efficiency remains lower than that of the OH’ radicals.

5.4.5 Ultrasound treatment of yeast

High-frequency ultrasound inactivation of yeast, $A.\text{pullulans}$, was also considered in this study. Two different initial concentrations ($\sim 10^7$ CFU/ml and $\sim 10^5$ CFU/ml) of A.
pullulans were prepared. These suspensions were ultrasonicated for 5, 10, 20, 40, and 60 min at 50 W. Figure 5.7 shows the results of the inactivation of A. pullulans at different initial numbers. The inactivation of E. aerogenes suspensions at different concentrations (~10^8 CFU/ml, ~10^6 CFU/ml, and ~10^4 CFU/ml) is also reported in Figure 5.7 for comparison. The Log (N/N_0) of A. pullulans decreased with a decrease in the initial numbers. This dependence on the initial number was previously reported for this yeast when it was inactivated by low-frequency ultrasound treatment (Gao et al., 2014b). Similarly, the inactivation for E. aerogenes was also found to be dependent on the initial number (Figure 5.7). However, the results showed clearly that the yeast A. pullulans was more resistant to high-frequency sonication than the bacterium E. aerogenes. For instance, one hour of ultrasonication of A. pullulans at 50 W resulted in a less than 1-log reduction for an initial concentration of 4.2×10^7 CFU/ml, and less than a 2-log reduction for an initial concentration of 3.1×10^5 CFU/ml. However in the case of E. aerogenes, under the same sonication conditions, there was a 3.2-log reduction for an initial number of ~10^8 CFU/ml and a 4.4-log reduction for an initial number of ~10^6 CFU/ml. Note that for the initial number of ~10^4 CFU/ml, no bacterial colonies were found after 40 min ultrasonication. It is also worth noting that in the case of the E. aerogenes, most bacterial inactivation was achieved during the first 10 min ultrasound treatment. Increasing ultrasonication time resulted only in a slight increase in the reduction of Log (N/N_0). For example, for the initial number of ~10^8 CFU/ml, Log (N/N_0) was 2.4 at 10 min and decreased to 3.2 after 60 min ultrasonication, which was probably due to the presence of two groups of E. aerogenes cells from same strain, with one group showing more resistance to ultrasonication than the other.
Figure 5.7 Log of survival ratio (Log (\(N/N_0\))) for ultrasonicated yeast and bacteria as a function of ultrasonication time in different initial numbers at 850 kHz. The initial cell numbers of *Aureobasidium pullulans* are \(\sim 4.2 \times 10^7\) CFU/ml (■) and \(\sim 3.1 \times 10^5\) CFU/ml (●). The initial cell numbers of *Enterobacter aerogenes* are \(\sim 1.5 \times 10^8\) CFU/ml (□), \(\sim 1.7 \times 10^6\) CFU/ml (○), and \(\sim 1.5 \times 10^4\) CFU/ml (△). Error bars correspond to standard deviation.

The resistance of *A. pullulans* to ultrasound treatment compared to *E. aerogenes* is mainly due to the difference in cell wall characteristics of these two microorganisms. *E. aerogenes* is a gram-negative bacterium and the main components of its cell wall are proteins, polysaccharides, lipids and mucopolypeptides (Salton, 1963). The cell wall of gram-negative bacteria is thin and is mainly composed of a 2 to 7 nm peptidoglycan layer and a 7 to 8 nm outer membrane (Wang and Chen, 2009). The cell wall of yeast consists mainly of mannoproteins and β-linked glucans (Klis, 1994). It was reported that the cell wall of the blastospores of mutant *A. pullulans* B-1 was 60–150 nm (Gniewosz and Duszkiewicz-Reinhard, 2008). Therefore, based on the thickness of the cell wall, *A. pullulans* was expected to be more resistant to ultrasound treatment than *E. aerogenes*. The light micrographs of *A. pullulans* for both non-ultrasonicated and ultrasonicated cells are shown in Figure 5.8. It can be seen that some cell
envelopes (see arrows) were present in both sonicated and non-sonicated samples, and this could be a result of the damage to the yeast cells resulting in the leaking of their inner contents.

![Figure 5.8](attachment:image1.png)

**Figure 5.8** Light micrographs of *Aureobasidium pullulans* (A) before ultrasound treatment and (B) after ultrasound treatment at 850 kHz and 62 W for 60 min.

### 5.5 Conclusions

The effects of high-frequency ultrasound on three species of bacteria, *E. aerogenes*, *B. subtilis* and *S. epidermidis* and a yeast strain, *A. pullulans*, were investigated. These bacteria were chosen as they have different cell wall types, sizes and shapes. These bacteria were also sonicated at different growth phases, namely the exponential and stationary phases. The study found that high-frequency ultrasound efficiently inactivated bacteria in both growth phases. Ultrasonication resulted in the inactivation
of more than 99% of the bacteria. In contrast, the yeast *A. pullulans* was found to be very resistant to high-frequency ultrasound treatment. High frequency ultrasound was efficient in bacterial inactivation at higher applied energy densities or longer times treatments, while it was not efficient at lower energy densities or shorter processing times although these were already practically high.

It was found in this study that the main mechanism of the high-frequency ultrasonic inactivation of bacteria was due to sonochemical effects related to the generation of hydroxyl radicals and hydrogen peroxide. This was confirmed by TEM observation, which showed that most of the bacteria cells retained their physical integrity. However, TEM observation also showed that the rod-shaped *B. subtilis* cells were susceptible to mechanical damage due to cavitation. It was also found that there was a post-sonication effect on the bacteria cultures. In other words, the longer the residence time of the bacteria in the ultrasonicated medium, the higher the number of bacteria cells that was inactivated. Overall this study indicates that high-frequency ultrasound presents a potential method for the inactivation of microbes in contaminated water, and thus can be used in wastewater treatment.

### 5.6 Acknowledgements

S. Gao is supported by a New Zealand China Food Safety Scholarship, funded by the New Zealand Ministry of Foreign Affairs and Trade. Funding for the purchase of the high-frequency unit was covered by a CMI-DB breweries grant.
CHAPTER 6

Inactivation of *Enterobacter aerogenes* in reconstituted skim milk by high- and low-frequency ultrasound

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The content of this chapter has been published

6.1 Abstract

The inactivation of *Enterobacter aerogenes* in skim milk using low-frequency (20 kHz) and high-frequency (850 kHz) ultrasonication was investigated. Bacterial suspensions in water and in skim milks (5, 10 and 15 wt%) were treated by both low-frequency (20 kHz) at different powers of ~8–9 W and high-frequency (850 kHz) at a power of 50 W for 0, 5, 10, 20, 40, and 60 min. It was found that low-frequency acoustic cavitation resulted in lethal damage to *E. aerogenes* (8–9 W, >5 min). The bacteria were more sensitive to ultrasound in water than in reconstituted skim milk with different protein concentrations. However, high-frequency ultrasound was not able to inactivate *E. aerogenes* in milk even when powers as high as 50 W for 60 min were used. This study also showed that high-frequency ultrasonication had no influence on the viscosity and particle size of skim milk, whereas low-frequency ultrasonication resulted in a decrease in viscosity and particle size of the milk. The decrease in particle size is believed to be due to the breakup of the fat globules, and possibly to the cleavage of the κ-casein present at the surface of the casein micelles. Whey proteins were also found to be slightly affected by low-frequency ultrasound, with the amounts of α-lactalbumin and β-lactoglobulin slightly decreased.

**Key words:** Low-frequency ultrasound; High-frequency; Bacteria inactivation; *Enterobacter aerogenes*; Skim milk
6.2 Introduction

Milk is considered as the most complete food for human beings as it can provide a complex mixture of all macronutrients including proteins, carbohydrates and fat, as well as micronutrients such as minerals, vitamins, and other nutrients (Ronsivalli et al., 1992; Martino et al., 2001). To extend the shelf life of milk, thermal treatment is usually used to inactive microorganisms in milk. However, thermal treatment is known to cause loss of nutrients, affect the flavours of milk (Martín et al., 1997; Aronsson et al., 2001), and induce protein denaturation (Corzo et al., 1994; Lan et al., 2010). As an alternative method, ultrasound technology can be used for microbial inactivation, while avoiding the undesirable effects caused by conventional heat treatments under controlled sonication conditions (Cameron et al., 2008).

Ultrasound refers to a frequency of 20 kHz or above (Mason, 1990), and ultrasound instruments operate with frequencies ranging from 20 kHz to 10 MHz (Piyasena et al., 2003). Power ultrasound refers to high-intensity low-frequency (20–100 kHz) systems, whereas frequencies higher than 100 kHz are normally associated with high-frequency low power ultrasound (Mason, 1990; Piyasena et al., 2003). Microbial inactivation by ultrasound treatment is mainly due to the acoustic cavitation (Mason et al., 2003; Mason et al., 2005). Cavitation means “the formation and the subsequent dynamic life of bubbles in liquids” (Suslick, 1988). When ultrasound passes through a liquid medium, microbubbles grow due to the compression and decompression of the ultrasound waves, until they reach a critical size at which they collapse violently. The collapse of cavitation bubbles produces extreme temperatures and pressures (Mason, 1990). There are two types of cavitation, transient cavitation, which causes large-scale variations in bubble size leading to a violent bubble collapse and stable cavitation, which generates relatively small amplitude, weak and symmetrically oscillating bubbles (Suslick, 1988; Scherba et al., 1991; Ashokkumar, 2011). The mechanical effects induced by acoustic cavitation include shear forces and micro-jettings. Shear forces caused by microbubbles collapse near a surface can generate shear stresses higher than 100 kPa (Maisonhaute et al., 2002a; Maisonhaute et al., 2002b). When
cavitation bubbles collapse near a surface (or within a cloud of bubbles), the collapse is no longer symmetrical and micro-jets are formed (Lamminen, 2004). The speed of the liquid jet acting on a solid surface can exceed 100 m/s (Zuo et al., 2012). Shock waves can also be generated during cavitation collapse (Virot et al., 2010), and they can induce pressures of 40–60 kbar at 20 kHz (Pecha and Gompf, 2000). Inactivation of bacteria by ultrasound treatment may be due to both the mechanical effects and the free radicals generated by cavitation. The shear forces cause the disruption of cell membranes of bacteria (Gao et al., 2014a; Gao et al., 2014b). Particularly in the case of high-frequency ultrasound, free radicals are produced in addition to the mechanical effects (Earnshaw et al., 1995; Hua and Thompson, 2000; Joyce et al., 2011). These free radicals are a result of the dissociation of the water molecules due to the extreme conditions resulting from the collapse of microbubbles (Sochard et al., 1997). The free radicals produced in aqueous solutions, which include hydroxyl radicals, attack the chemical structure of cell walls and can cause DNA damage, destroy enzymatic activity, and damage liposomes and bacteria membranes (Riesz and Kondo, 1992; Andreassi, 2004; Hunter et al., 2008; Cabiscol et al., 2010; Liu et al., 2011).

Bovine milk contains 3.0-3.5% (w/v) of proteins, which mainly consist of caseins and whey proteins (Wang et al., 2009). Caseins, namely, α_{s1}-casein (α_{s1}-CN), α_{s2}-casein (α_{s2}-CN), β-casein (β-CN) and κ-casein (κ-CN), are present in a ratio of 4:1:4:1, and exist in milk as small aggregates (60 to 400 nm diameter) termed casein micelles. Whey proteins include α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lg) with a ratio of 1:3 (Visser et al., 1991; Bonfatti et al., 2008; Wang et al., 2009), and some minor proteins such as bovine serum albumin (BSA) and immunoglobulins (Igs) (Singh and Creamer, 1991; Farrell et al., 2004). Whey proteins are prone to denaturation during heating. Although whey protein denaturation can be exploited to modify their functional properties and obtain desired modifications in final products (e.g. achieving high gel strength of yoghurt), it can cause some undesired effects including deposit formation, milk instability and gelling (Kessler and Beyer, 1991; Law and Leaver, 1999; Anema, 2008a).
The main aim of this study is to determine the effects of both low-frequency (20 kHz) and high-frequency (850 kHz) ultrasound treatment on *E. aerogenes* suspended in skim milk. Further, the effect of ultrasound on the state of milk proteins is also considered. Ultrasound inactivation of microorganisms in milk was reported previously on bacteria including *Salmonella* (35–40 kHz) (Wrigley and Llorca, 1992); *Escherichia coli*, *Saccharomyces cerevisiae* and *Lactobacillus acidophilus* (20 kHz, 750W) (Cameron et al., 2008); and *Listeria innocua* ATCC 51742 (24 kHz, 120 μm amplitude) (Bermúdez-Aguirre and Barbosa-Cánovas, 2008). However, there are no reports on the effect of ultrasound treatment on *E. aerogenes* in milk. Moreover, there are only few published papers on the effect of ultrasound on the physical and chemical properties of milk proteins (Villamiel and de Jong, 2000b; Stathopulos et al., 2004; Chouliara et al., 2010; Gordon and Pilosof, 2010; Shanmugam et al., 2012). The findings of these studies will be discussed in the light of the results obtained in the present paper.

### 6.3 Material and methods

#### 6.3.1 Material, bacterial and milk samples preparation

The following chemicals were purchased from Sigma Aldrich (St. Louis, Missouri, USA): Calcium chloride (CaCl₂), ethylenediaminetetraacetic acid (EDTA), trifluoroacetic acid (TFA), dithiothreitol (DTT), BisTris and sodium citrate. The following chemicals were purchased from Merck KGaA (Darmstadt, Germany): Guanidine hydrochloride (GdnHCl) and acetonitrile (gradient grade). For reversed-phase high-performance liquid chromatography (RP-HPLC), α-casein (α-CN), β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin standards were purchased from Sigma Aldrich (USA).

Skim milk powder (SMP) was purchased from Westland Co-operative Dairy Company Limited, New Zealand. The compositions of the SMP consist of mainly protein (≥ 32.4 wt%), fat (≤ 1.25 wt%) and moisture (≤ 4.0 wt%). The reconstituted
milk samples were made by mixing the appropriate amount of SMP with Milli-Q water using a magnetic stirrer at room temperature for 2 h. The milk samples were then left overnight in a fridge (4°C) to ensure full hydration. Samples with SMP concentrations of 0, 5, 10 or 15% were made. Bacteria inoculation of these samples was achieved by adding 1 ml of bacteria culture to 9 ml of the prepared skim milk. Similarly for the control samples, 1 ml of tap sterilized water was added to 9 ml of the prepared skim milks. These skim milks were stirred for 30 min prior to ultrasound treatment.

_E. aerogenes_ culture was obtained from a stock kept in a −80°C freezer in the Environmental Microbiology Laboratory, School of Biological Sciences, the University of Auckland. The bacteria suspension was made by incubating the bacteria stock (100 µl stock into 100 ml Nutrient broth) at 37°C overnight under shaking (200 rev min⁻¹) to reach a stationary phase. The bacterial suspensions were then transferred into 50 ml tubes, and centrifuged (Biofuge Stratos, Heraeus, Germany) (10,000 g, 4°C, 10 min) to obtain a bacterial cell pellet. After quickly moving the supernatant, the pellet was washed twice with sterilised water. Then the washed bacteria suspensions were diluted to ∼10⁹ CFU/ml by measuring the optical density (OD) using a Helios β UV Visible Spectrophotometer (Thermo Electron Corporation, UK) at an absorbance of 600 nm. 1 ml of the washed _E. aerogenes_ suspension was added into 9 ml of water or 9 ml of skim milk to make bacteria in water or bacteria in skim milk samples. The final bacteria count, both in water or different skim milks, was ∼10⁸ CFU/ml. Bacteria counts were performed using a modified Miles-Misra method (Miles et al., 1938) as described in our previous work (Gao et al., 2014a; Gao et al., 2014b).

6.3.2 Ultrasonication

Low-frequency ultrasonication of the bacterial suspensions was carried out using a 20 kHz ultrasound homogeniser (Sonic Ruptor 250, Omni International, USA) fitted with an ultrasonic horn (processing tip diameter 12.7 mm). For low-frequency ultrasonication, 15 ml of samples were transferred in 20 ml columniform glass vials,
then the vials were placed into an ice bath in order to maintain the solution temperature below 30°C, and the ultrasonic horn was immersed 1 cm below the surface of the sample.

High-frequency ultrasonication of the bacterial suspensions was carried out using an 850 kHz ultrasound generator K80 (Meinhardt Ultraschalltechnik, Germany). In this setup, an ultrasound generator was connected to a Transducer E/805/T, on which a double-walled cylindrical glass vessel connected to a water bath (PolyScience SD07R-20-A12E, USA) was mounted. The temperature of the water bath was set to 2°C, resulting in the temperature in the vessel not exceeding 20°C under the sonication condition used in this study. The glass vessel was filled with 250 ml of Milli-Q water. 5 ml of sample was transferred into a 15 ml glass tube. The tube was inserted through the centre of a PVC cover sitting on top of the glass vessel. The tube containing the sample was positioned in the centre of the chilled water.

The calorimetric method was used for determination of ultrasound power (Koda et al., 2003; Kikuchi and Uchida, 2011):

\[ P = mC_p(\Delta T/\Delta t) \]  

(6.1)

where \( C_p \) is the specific heat capacity of the sample, \( m \) is its mass, \( \Delta T \) is the increase in the temperature, and \( \Delta t \) is the applied ultrasound time. For water, \( C_p = 4.18 \text{ J/(g·K)} \) was used (Ashokkumar et al., 2008), and for skim milk a value of \( 3.98 \text{ J/(g·K)} \) for \( C_p \) was used (Hui et al., 2007). Heat capacity is expressed as J/(g·K) in SI units, which equates to \( 1/4186 \text{ cal/g/°C} \) in c.g.s. units (Hui, 1993).

6.3.3 RP-HPLC analysis

Milk proteins were separated by reversed-phase high-performance liquid chromatography (RP-HPLC) and the method was adapted with slight modification from previous publications (Parris and Baginski, 1991; Bobe et al., 1998; Tolkach and Kulozik, 2005; Wang et al., 2009). Two solvents were used to carry out gradient
elution. Acetonitrile (HPLC grade), Milli-Q water, and trifluoroacetic acid (TFA) in a ratio of 100:900:1 (v:v:v) were mixed to make up Solvent A, and in a ratio of 900:100:1 to make up Solvent B. Solvent A and Solvent B were ultrasonicated in a Soniclean sonication bath (Soniclean Pty.Ltd., Thebarton, S.A., Australia) to get rid of micro air bubbles before operating the HPLC instrument. A reversed-phase analytical column Jupiter 5µm C18 300Å (250mm length × 4.6mm i.d., Phenomenex ) and a Jupiter C4 column (250 × 4.6 mm, Phenomenex) were used in an Agilent 1260 Infinity HPLC system (Agilent Technologies, USA) to separate caseins and whey proteins, respectively. RP-HPLC was performed at room temperature under the following chromatographic conditions: injection volume of final solution, 50 µl; flow rate, 1.0 ml/min; detection wavelength: 220 nm for caseins and 205 nm for whey proteins. The solvent gradient program for caseins was set to 0-40 min, 30-50% B; 40-42 min, 50-100% B; 42-43 min, 100-100% B; 43-46 min, 100-30% B; 46-51 min, 30-30% B; 51-55 min, re-equilibration. The gradient program for whey proteins was set as followings: 0-1 min, 35-35% B; 1-8 min, 35-38% B; 8-16 min, 38-42% B; 16-22 min, 42-46% B; 22-24 min, 46-90% B; 24-25 min, 90-90% B; 25-30 min, 90-35% B; 30-35 min, 35-35% B; 35-40 min, re-equilibration.

For the quantification of casein, 200 µl of milk samples were mixed with 200 µl of digestion reagents (0.1 M BisTris buffer, 6 M guanidine hydrochloride, 5.37 mM sodium citrate). Then 5 µl of 19.5 mM dithiothreitol (DTT) were added into the mixture and left at room temperature for 1 h to ensure that the casein micelles were fully dissociated. The mixtures were then centrifuged at 14,000 g for 5 min and the fat layer was removed. 200 µl of the remaining solubilised sample were transferred into an Eppendorf tube to which 400 µl of 4.5 M guanidine hydrochloride was added. This diluted sample was transferred into a fresh Eppendorf tube and kept frozen until HPLC analysis.

For the analysis of the whey proteins, 200 µl of 0.2 M sodium acetate buffer (pH 3.95) were added into 200 µl of milk samples which were left for 1 h at room temperature to allow the caseins to precipitate. The mixture was centrifuged at 14,000 g for 5 min.
200 µl of the supernatants were then transferred into a fresh Eppendorf tube and kept frozen until analysis.

6.3.4 Viscosity measurement

Viscosity measurements were performed using an SI Analytics Ubbelohde capillary (diameter 1.50 ± 0.01 mm, viscometer constant K=0.3 mm²/s) fitted into the viscosity Measuring Unit Visco-Clock (Schott, SI Analytics, Mainz, Germany). A temperature controller (SD07R-20-A12E, Ply Science, USA) was used to control a constant temperature of 25°C. The viscosity (η) was calculated using (Young, 1981):

\[
\eta = \eta_0 \frac{t}{t_0} \frac{\rho}{\rho_0}
\]

(6.2)

where \(\eta_0\) (=0.890 mPa·s) is the viscosity of water at 25°C, \(\rho_0\) (=1.00 g/ml) is its density, \(t_0\) and \(t\) are the flow-through times in the capillary of the water and the sample respectively. All measurements were performed on duplicate samples and the flow-through time was measured six times for each sample.

6.3.5 TEM and Cryo-TEM observation

Transmission electron microscopy observation was carried out in a Philips CM12 TEM (Netherlands). Carbon-coated copper TEM grids (3.05 mm) were glow discharged (500 Volts, 15 s) to keep the surface of the grids hydrophilic. A grid was immersed into the bacterial suspension for 30 s, then quickly washed twice with Milli-Q water. The washed grid was dried using a filter paper, and then was immersed into 2% uranyl acetate for 30 s. The stained grids were kept on a fresh filter paper until observation by TEM. The grids were examined at 120 kV in the TEM.

Casein micelles were observed by Cryo-TEM. 2 µl of milk samples were dropped on a C-flat Holey Carbon-coated Grid and then prepared in a Vitrobot™ automated vitrification device unit (FEI, Netherlands) at controlled temperature and humidity conditions (27°C, 100% RH). The grids were examined by a FEI Tecnai 12 TEM.
(Netherlands), and they were always kept into liquid nitrogen during the sample preparation and TEM observation.

6.3.6 Particle size measurement

Particle size measurements were performed using the Malvern Zetasizer Nano ZSP (Malvern Instruments Ltd., UK). A calcium-imidazole buffer (pH 6.7) made of 5 mM CaCl₂, 20 mM imidazole, and 30 mM NaCl, was used as a dispersant (Anema, 2008b). All measurements were performed at least in duplicate at 25°C, and each sample was measured 10 times.

Milk samples were prepared by dispersing 10 µl of milk in 1.5 ml of the imydazole buffer. The refractive index of imydazole buffer was set as 1.330, and the viscosity was 0.8872 mPa·s. Measurements of the milk fat droplets were obtained by mixing the milk sample with 0.1 M EDTA (pH 6.7) at a ratio of 1:100 (v:v). Addition of milk to EDTA results in the full dissociation of casein micelles but does not affect the milk fat droplets.

6.4 Results and Discussion

6.4.1 Inactivation of bacteria in water and skim milk

Initial populations of *E. aerogenes* in water or milk samples were about $\sim1 \times 10^8$ CFU/ml. Bacterial suspensions in water and in skim milks (5, 10 and 15 wt%) were treated by both low-frequency (20 kHz) at different powers of $\sim8–9$ W and high-frequency (850 kHz) at a power of 50 W for 0, 5, 10, 20, 40, and 60 min. Power values used in this study correspond to power densities of $\sim0.5–0.6$ W/ml for low-frequency and 0.2 W/ml for the high-frequency treatment. The log reduction of *E. aerogenes* as a function of sonication time is shown in Figure 6.1. For the low-frequency ultrasound treatment, the log reduction was found to decrease linearly with an increase in sonication time. The bacteria were more sensitive to ultrasonication when dispersed in water than in milk. The behaviour of *E. aerogenes*
in water when treated by low-frequency ultrasound treatment was previously reported (Gao et al., 2014a; Gao et al., 2014b), and the inactivation is mainly due to the mechanical effects induced by acoustic cavitation (Mason et al., 2003; Mason et al., 2005; Gao et al., 2014b). The log reductions were \(-3.64 (\pm 0.04)\), \(-2.73 (\pm 0.23)\), \(-2.31 (\pm 0.41)\) and \(-2.21 (\pm 0.03)\) for the bacteria in water, 5% milk, 10% milk and 15% milk, respectively, after ultrasonicated for 60 min. Clearly the milk concentration has an effect on the bacteria inactivation, as the higher the milk concentration the lower the number of bacteria inactivated. This is likely due to the increase in viscosity of the milk samples with the increase in concentration. Viscosity measurements showed that the viscosities were 1.072 (±0.002), 1.293 (±0.007), and 1.735 (±0.009) mPa·s, for 5, 10 and 15 wt% skim milks, respectively. The occurrence of cavitation decreases in high viscous media, due to acoustic impedance by high viscous liquids (Salleh-Mack and Roberts, 2007).

**Figure 6.1** Log of survival ratio (Log \(N/N_0\)) of *Enterobacter aerogenes* in skim milk and water as a function of sonication time. In water, 20 kHz, 8.0 W (■); In 5% skim milk, 20 kHz, 8.2W (●); In 10% skim milk, 20 kHz, 8.5W (▲); In 15% skim milk, 20 kHz, 9.2W (▼); In 5% skim milk, 850 kHz, 50 W (○); In 10% skim milk, 850 kHz, 50 W (△). Error bars correspond to standard deviation.
Other studies on the inactivation of bacteria in milk systems also reported a decrease in the log reduction when compared to the inactivation of bacteria in aqueous solutions. For instance, *S. cerevisiae* was found to be more resistant in UHT milk than in saline solution, where there was a 2.10 and 3.62 log-reduction, respectively, when ultrasonicated for 10 min at 20 kHz with power of 750 W (Cameron et al., 2008). *L. innocua* ATCC 51742 was also found to be more resistant when sonicated (24 kHz, 120 μm amplitude) for 30 min in milks with different fat contents (Bermúdez-Aguirre and Barbosa-Cánovas, 2008). The rate of inactivation increased with the decrease in fat contents, and 2.5, 3.2, 4.5 and 4.9 log-reductions were obtained for whole milk (3.47% fat content), 2% and 1% butter fat content milks, and fat free milk, respectively. However, it was reported that the inactivation of *E. coli* ultrasonicated at 20 kHz at a power of 750 W for 10 min was similar when the bacteria were presented in saline solution (3.88-log reduction) and UHT milk (4.42-log reduction) at 20 kHz and 750 W for 10 min (Cameron et al., 2008). It is worth pointing out that the ultrasound conditions, especially power and time used, and the bacteria suspensions were different in all these studies which they can all potentially affect the final inactivation result.

In the case of high-frequency ultrasound treatment of *E. aerogenes* in milk (5 and 10 wt%) there was no change for the viable cells after ultrasonication up to 60 min at 50 W (Figure 6.1, open symbols). This is not surprising, since the inactivation of bacteria by high-frequency ultrasound is mainly due to the generation of free radicals including hydroxyl radicals (OH•) and hydrogen peroxide (Leighton, 1994; He et al., 2006). In fact, a ∼3-log reduction was achieved when the bacteria were sonicated in water at 850 kHz and 50 W for 60 min (Figure 5.3A). Because milk is an excellent free radicals scavenger, as a result, the free radicals produced during high-frequency ultrasonication were consumed by the milk. This is due to the presence of several antioxidants in milk including vitamins and enzymes which scavenge radicals or hydrogen peroxide (Lindmark-Månsson and Åkesson, 2000). In addition, milk proteins and hydrolysates are also reported to have antioxidative activities (Pihlanto,
2006) and both caseins and whey proteins in skim milk showed antioxidant activities (Taylor and Richardson, 1980a). It should be noted that high-frequency ultrasonication also results in mechanical effects that could inactivate bacteria in water, however at a much less extent to that generated by low-frequency ultrasonication. To the best of our knowledge, there is only one report on the inactivation of bacteria in milk by high-frequency ultrasound. Munkacsi and Elhami (Munkacsi and Elhami, 1976) applied ultrasound treatment (800 kHz, 8.4 W/cm² for 1 min) followed by UV treatment. While the combination of the two treatments resulted in the inactivation of total bacteria and coliform, ultrasound treatment alone resulted in a 100% survival.

To confirm the effect of ultrasonication on *E. aerogenes* suspensions TEM observations were carried out (Figure 6.2). Before sonication, the bacterial cells remained stable and intact although it can be clearly seen that they are surrounded by the casein micelles of milk (Figure 6.2A2). After low-frequency ultrasonication, the bacterial cells were disrupted into misshapen structures made of cell debris (indicated by circles in Figures 6.2B1 and B2). However, treatment by high-frequency ultrasound did not affect the bacteria cells, which remained intact and whole (Figures 6.2C1 and C2).
Figure 6.2 Transmission electron micrographs of *Enterobacter aerogenes* in water and 5% skim milk before and after ultrasonication. A1: Before ultrasonication in water; A2: Before ultrasonication in milk, the cells remained stable and intact; B1 and B2: After ultrasonication at 20 kHz for 20 min (8.5W), the bacteria had misshapen structures which were highlighted by circles; C1 and C2: After ultrasonication at 850 kHz for 60 min (50 W), the cells remained stable and intact.
6.4.2 Effects of ultrasonication on viscosity and particle size of skim milk

To investigate the effect of ultrasound treatment on skim milk (10 wt%) without bacteria, viscosity measurements and particle size determination were carried out under different sonication times. High-frequency ultrasound treatment of milk for up to 60 min did not result in any change in the viscosity of the milk (Figure 6.3). However, low-frequency ultrasound treatment resulted in a slight decrease in viscosity after 5 min treatment. The initial viscosity of skim milk was 1.293 (±0.007) mPa·s at 25°C, and decreased to 1.231 (±0.001) mPa·s after 5 min ultrasound treatment. Longer time treatment by low-frequency did not result in a marked decrease in the viscosity. The values of the viscosity measured were 1.228 (±0.005), 1.220 (±0.001), 1.213 (±0.005) and 1.203 (±0.007) mPa·s individually for 10, 20, 40 and 60 min ultrasonication, respectively (Figure 6.3).

**Figure 6.3** Viscosity change of milk and bacteria (*Enterobacter aerogenes*) suspensions. Water in 10% milk, 20 kHz, 8.5 W (■); Bacteria suspension in 10% milk, 20 kHz, 8.5 W (○); Water in 10% milk, 850 kHz, 50 W (□); Error bars correspond to standard deviation.
To investigate the observed changes in viscosity, particle size measurements were performed on the same milk samples treated by low and high-frequency ultrasound. The intensity mean-average diameter as a function of sonication time is reported in Figure 6.4. In the case of high-frequency ultrasound treatment, similar to viscosity, there are no changes in the mean diameter as a function of sonication time. While in the case of low-frequency ultrasound treatment, the particle size did decrease from 182.4 (±1.2) nm before ultrasound treatment to 168.6 (±1.2) nm after 5 min ultrasonication. The mean-size diameter also further decreased slightly when the low-frequency sonication time was increased, to reach 152.8 (±1.2) nm after 60 min sonication. Milk is a complex colloidal dispersion, and the dispersed phase is made of casein micelles and fat globules while the dispersion medium is an aqueous solution that includes whey proteins and lactose (Bienvenue et al., 2003). Although the milk used in this study is reconstituted from skim milk, it still contains some fat droplets. Thus it was important to investigate the effect of ultrasonication on their size, particularly because ultrasound is well known to substantially reduce the size of fat droplets (Villamiel and de Jong, 2000b; Wu et al., 2001; Chandrapala et al., 2012; Shanmugam et al., 2012). To do so, non-sonicated and sonicated milk samples were diluted in 0.1 M EDTA solution to dissociate casein micelles, prior to particle size measurement. The particle size measurement showed clearly that the average size did decrease from 104.0 (±0.8) nm to 90.4 (±0.0) nm after low-frequency ultrasonication for 60 min.
Figure 6.4 The particle size diameter of milk (10% skim milk) and fat globules samples as a function of sonication time: milk, 20 kHz, 8.5 W (■); milk, 850 kHz, 50 W (●); Fat globules, milk: EDTA=1:100 (v/v), 20 kHz, 8.5 W (▲). Error bars correspond to standard deviation.

The decrease in viscosity and in particle size of skim milk after treatment with low-frequency ultrasound was previously reported. It was reported that the size of particles in skim milk decreased (Nguyen and Anema, 2010; Chandrapala et al., 2012) and the decrease was related to the breakup of fat globules (Chandrapala et al., 2012). The decrease in viscosity was also previously reported for skim milk (Chandrapala et al., 2012) and concentrated milks (Zisu et al., 2013). However, the reduction in particle size of the fat droplets without a change in the volume fraction should not affect the viscosity. In fact, for dilute suspension of non-interacting spherical particles, the viscosity \( \eta_{\text{dis}} \) is given by Einstein formula \( \eta_{\text{dis}} = \eta_{\text{sol}}(1 + 2.5\phi) \) (Larson, 1999). This equation involves only the viscosity of the continuous phase \( \eta_{\text{sol}} \) and the volume fraction \( \phi \) occupied the particles. Further investigations of the state of the individual proteins in milk after ultrasound treatment were performed using RP-HPLC.
6.4.3 Effect of ultrasound treatment on milk proteins

Reversed-phase high-performance liquid chromatography (RP-HPLC), which separates proteins based on their molecular hydrophobicity (Aguilar, 2004), is considered an accurate, rapid, and repeatable method to identify and quantify the concentration of milk proteins (Bonfatti et al., 2008; Wang et al., 2009). In this study, the caseins, namely κ-CN, α-CN (both αs1-CN and αs2-CN) and β-CN, and the whey proteins, α-Lac and β-Lg were considered. Typical RP-HPLC chromatograms of caseins and whey proteins are shown in Figure 6.5A and Figure 6.6A, respectively. The amount of each individual protein is obtained by integrating the corresponding RP-HPLC peak. The results are reported as the difference in area between the ultrasonicated milk and the non-ultrasonicated skim milk. The results of this exercise are reported in Figure 6.5B and Figure 6.6B for the caseins and whey proteins, respectively. In the case of caseins, the amounts of α-CN and β-CN remained nearly constant within experimental errors. However, the amount of κ-CN decreased by ~13% when the milk was sonicated for 10 min with a power of 8.5 W. Increasing further the treatment time did not result in a further markedly decrease for the amount of κ-CN. In the case of whey proteins, the amount of both α-Lac and β-Lg decreased after 5 min sonication, and increasing the sonication time did not result in a further decrease in the amount of α-Lac and β-Lg. Amongst the whey proteins, approximately 8 to 15% and approximately 7 to 11% reductions were observed for α-Lac and β-Lg, respectively (Figure 6.6B).
Figure 6.5 (A) Chromatograms of caseins from skim milk by reversed-phase high-performance liquid chromatography (RP-HPLC): Control skim milk (solid line); Ultrasonicated skim milk, 30 min, 20 kHz, 8.5 W (Dotted line). (B) The relative reductions of concentration of caseins from RP-HPLC: κ-casein (■), α-casein (●), and β-casein (▲); Error bars correspond to standard deviation.
Figure 6.6 (A) Chromatograms of whey proteins from skim milk by reversed-phase high-performance liquid chromatography (RP-HPLC): Control skim milk (solid line); Ultrasonicated skim milk, 30 min, 20 kHz, 8.5 W (Dotted line). (B) The relative reductions of concentration of whey proteins from RP-HPLC: α-lactalbumin (■) and β-lactoglobulin (●); Error bars correspond to standard deviation.

The results obtained in this study on the effect of ultrasound treatment on the individual milk proteins is in very good agreement with Shanmugam et al.
(Shanmugam et al., 2012), who also reported a decrease in whey proteins and κ-CN using 20 kHz, 20 W and 41 W. In the case of the decrease of whey proteins, these authors suggested that transient cavitation can easily damage the proteins causing denaturation of soluble whey proteins followed by their self-aggregation. Note however that aggregation of the whey proteins will lead to an increase in viscosity, while a reduction in viscosity was observed in this work. A decrease in the amount of κ-CN would lead to a decrease in viscosity. κ-CN is believed to be present mainly at the surface of the casein micelle (Horne, 1998). If the cavitation generated by ultrasound is high enough to cleave the κ-CN, then the radius of the casein micelle would decrease slightly. The small decrease in the casein micelle size, and thus in their overall volume fraction, will lead to a reduction in viscosity. To confirm the decrease in casein micelle particle size, TEM micrographs obtained from both non-sonicated and sonicated milk (20 min, 8.5 W) were analysed using GIMP 2.8 software. After analysing 200 casein micelles under each condition, the results showed that the average diameter was changed from 111 ± 53 nm for the non-sonicated milk compared to 104 ± 57 nm for the sonicated sample. While these results should be taken with caution since particle size determination by microscopy is extremely difficult, they are nonetheless very encouraging since the decrease in the casein micelle reported by Shanmugam et al. (Shanmugam et al., 2012) is in the order of magnitude (approximately 5 nm at the highest power they used). While this value seems small, it is certainly non-negligible and would explain the slight decrease in viscosity.

6.5 Conclusions

Inactivation of *E. aerogenes* in skim milks with different protein concentrations and the subsequent effects on some physical properties of milk and individual milk proteins were studied. *E. aerogenes* suspensions were treated by both low-frequency (20 kHz) and high-frequency (850 kHz) ultrasonication. It was found that low-frequency ultrasound cavitation resulted in lethal damage to *E. aerogenes*, and
this was confirmed by direct observation using TEM. The logarithm of the survival ratio of the bacteria decreased linearly with an increase in sonication time. However the extent of bacteria inactivation decreased with an increase in the skim milk concentrations. This is likely due to an increase in the viscosity of the solution with an increase in the concentration of the milk an increase in solution viscosity is known to affect acoustic cavitation. However, *E. aerogenes* was not inactivated by high-frequency ultrasound treatment. This is mainly due to the radical scavenging properties of milk.

This work also showed that while high-frequency ultrasound treatment of milk did not result in physical changes, low-frequency ultrasonication treatment decreased the particle size and the viscosity of the milk especially at higher powers. The main decrease in particle size is due to the reduction of the fat globule size by ultrasound treatment. Analysis of the individual milk proteins showed that the amount of the main whey protein, α-Lac and β-Lg, slightly decreased, while in the case of caseins only the amount of κ-CN did decrease. If acoustic cavitation, did indeed cleave the κ-CN, which is known to be present at the surface of the casein micelles, this would explain the decrease in milk viscosity. Clearly, more work is needed to confirm if high-power ultrasound is able to break the integrity of casein micelles.

**6.6 Acknowledgements**

S. Gao is supported by a New Zealand China Food Safety Scholarship, funded by the New Zealand Ministry of Foreign Affair and Trade. YH and MA thank the University of Auckland for the award of the Distinguished Visitor Award (Project number 3702372). Funding for the purchase of the high-frequency unit is covered by a CMI-DB breweries grant.
CHAPTER 7

General discussion, overall conclusions and future work
7.1 General discussion

7.1.1 Introduction

As an underutilised processing technology, ultrasound treatment has the potential for inactivation of microorganisms in foods, as it reduces loss of nutrients and flavours compared to the thermal treatment of food when applied at appropriately low energy density (Chemat et al., 2011). Although it is known that the main reasons for ultrasonic inactivation are due to physical damage caused by mechanical effects and the contribution of free radicals generated during cavitation, in most cases, the exact mechanism of the process of inactivation is still being debated. Moreover, there has not been a parallel and systematical study of both low-frequency and high-frequency systems for the same species of microorganisms.

In this thesis the effects of both low-frequency and high-frequency ultrasounds were used to inactivate bacteria and yeast in media with different viscosities. The effects of ultrasound conditions, such as intensity and time, on a group of bacteria and yeast were considered. A simple theoretical model based on ultrasonic cavitation was established in a low-frequency system. This allowed, on one hand, the determination of the important parameters that influence bacterial inactivation and alternatively, at the fundamental level, the understanding of the exact mechanism involved in cell breakage.

The overall objectives of this study were:

- To determine the effects of low-frequency (20 kHz) ultrasound treatment on different bacteria based on the properties of bacterial cells at different growth phases (Chapter 3).
- To obtain a fundamental understanding of the mechanism of microbial inactivation by ultrasonication through establishing a theoretical model based on cavitation in a low-frequency system (Chapter 4).
• To investigate the effects of high-frequency (850 kHz) ultrasound treatment on different bacteria and yeast at different growth phases (Chapter 5).

• To investigate the inactivation of bacteria in skim milk by both low-frequency and high-frequency ultrasound treatment (Chapter 6).

• To investigate the physical and chemical effects of ultrasonication on skim milk (Chapter 6).

7.1.2 Review of experimental design

• Microorganisms used.

Three species of bacteria \textit{E. aerogenes}, \textit{B. subtilis} and \textit{S. epidermidis} as well as a species of yeast \textit{A. pullulans} were the main targeted microorganisms for ultrasound treatment in this study. Two key aspects were considered for their selection. The first was their cell properties, including the type of cell wall, size and shape. In addition, they are significant to food safety, although the bacteria are not pathogenic. These three bacteria vary in their properties. \textit{E. aerogenes} is a gram-negative rod-shaped bacterium (Imhoff, 2005), and it tends to be a cocal form at the stationary phase (about 0.8–1.0 µm in diameter), it is an important species classified as a coliform bacterium (Bej et al., 1991; Camper et al., 1991; Raghubeer et al., 1995). \textit{B. subtilis} is a gram-positive rod-shaped bacterium (Schleifer, 2009) that may produce spores which are highly resistant to heat treatment (Jagannath et al., 2005). \textit{S. epidermidis} is a gram-positive coccus (Schleifer, 2009) that has a similar size with \textit{E. aerogenes} in the stationary phase. \textit{S. epidermidis} is a common skin microorganism (Schleifer, 2009) that easily adheres to surfaces to form biofilms (Katsikogianni et al., 2006) and can pose a challenge to food processing. \textit{A. pullulans} is a yeast-like fungus and is well-known as black yeast (Cooke, 1959; Hoog, 1993) that has a much larger size than these bacteria. \textit{A. pullulans} is a ubiquitous spoilage microorganism (Mills et al., 2008) that can cause food to spoil.
Experimental conditions.

Control of temperature is significant for ultrasound inactivation experiments of microorganisms to manage the integrating effects of heat and ultrasound cavitation at the targeted microorganisms. The collapse of acoustic cavitation bubbles during sonication can produce extremely high local temperatures that can reach as high as 5000 K and pressures of 1000 atm (Suslick and Price, 1999). Therefore, the experiment systems required cooling to control heat generated from cavitation. For the low-frequency (20 kHz) ultrasound system in this study, the samples were always surrounded by an ice bath. The ice was replaced every 5 min to make sure the temperature of the samples during ultrasonication was below 30°C. The high-frequency (850 kHz) ultrasound system was connected to a 2°C water bath to control temperature. Clearly, high temperatures will also make contribution to microbial inactivation. For instance, low-temperature long-time (LTLT) pasteurisation only needs 63°C for 30 min to inactivate bacteria (Jay, 2000). Bacteria are also known to be more sensitive to ultrasound treatment combined with heat treatment (Earnshaw et al., 1995).

7.1.3 Comparisons of low- and high-frequency ultrasonication of microorganisms

*E. aerogenes, B. subtilis, S. epidermidis* and *A. pullulans* were all sonicated at both low frequency (20 kHz) and high frequency (850 kHz). *S. epidermidis* SK and *S. pseudintermedius* were also treated at 20 kHz.

7.1.3.1 Effects

Firstly, both low-frequency and high-frequency ultrasound treatment had a lethal impact on 2 species of bacteria *E. aerogenes* and *B. subtilis*. The inactivation rate increased with higher ultrasonic power, and also increased with longer treatment times. However, low-frequency ultrasonication could not inactivate *S. epidermidis, S. epidermidis* SK and *S. pseudintermedius*. High-frequency ultrasound treatment, in
contrast, inactivated *S. epidermidis*. As a gram-positive bacterium, *S. epidermidis* has a thicker cell wall than the gram-negative bacterium *E. aerogenes*. Gram-positive bacteria have been reported to be more resistant than gram-negative to ultrasound (Drakopoulou et al., 2009). *B. subtilis* and *S. epidermidis* are both gram-positive, but they are differently shaped. Rod-shaped cells are known to be more sensitive to breaking under acoustic cavitation compared to cocci-shaped ones (Ahmed and Russell, 1975; Alliger, 1975). More importantly, *S. epidermidis* and other strains of *Staphylococcus* in this study have a thick capsule layer assemblage outside the bacterial cell wall (~100 nm from TEM observations) that may also contribute to their comparative resistance.

The bacterial capsule can be considered a “soft” material similar to aqueous polysaccharide solutions, as it mainly consists of homogeneous polysaccharides and proteins (Schleifer and Kandler, 1972; Tuson et al., 2012), and it is highly hydrated with 99.9% water (Sutherland, 1979). The shear forces produced by ultrasound cavitation acting on the bacterial cell are reduced due to the presence of capsules. A dampening of the mechanical effects is also caused during the collapse of the cavitation bubbles because of the “soft” layer. Therefore the bacteria with a thicker soft capsule showed more resistance to ultrasound treatment as observed at low-frequency ultrasound. Capsules protect against mechanical but not chemical damage. This study showed that the main effectors of bacterial survival at high-frequency ultrasound are hydroxyl radicals and hydrogen peroxide. *S. epidermidis* was inactivated up to a 4.4-log reduction at 62 W for 20 min by high-frequency ultrasound treatment. This is because the main components of the bacterial capsules, polysaccharides, are rapidly oxidised by hydrogen peroxide (Miller, 1986; Fry, 1998; Schweikert et al., 2000). The production of hydroxyl radicals and hydrogen peroxide were much greater in high-frequency ultrasound systems than in low-frequency ones (Ashokkumar et al., 2008).
Physical damage caused by low-frequency and high-frequency ultrasounds was not the same for all treated microorganisms. The physical change of these bacteria and yeast by ultrasonication were shown from TEM and light micrographs as well as particle size measurements. For low-frequency ultrasonication, the structural integrity of cells of *E. aerogenes* and *B. subtilis* and the yeast, *A. pullulans*, was destroyed, the intracellular contents were leaked, and the cells were broken into uncountable smaller fragments. However the cells of *S. epidermidis* remained intact after the low-frequency treatment. For the high-frequency ultrasonication, the cells of *E. aerogenes* were misshapen and malformed but remained as whole cells. Most of the sonicated *S. epidermidis* remained as whole cells, and some leakage of cell content was also found. Broken fragments were only found from the *B. subtilis* cells, which showed that rod-shaped cells were more sensitive to mechanical damage from low- and high-frequency ultrasound.

This was the first time that the effects of both low-frequency and high-frequency ultrasounds on bacteria in different growth phases were comprehensively and systematically investigated. In most cases, there was no marked difference in the effects of inactivation on these bacteria in the stationary phase or the log phase in both frequencies. The only exception was *E. aerogenes*, which was more sensitive in the log phase than in the stationary phase. The main reason for this occurrence was that *E. aerogenes* changes from a rod-like shape in the exponential phase to a more resistant coccus shape in the stationary phase (Figure 1.1A and inset Figure 1.1a).

### 7.1.3.2 Efficiency

It is difficult to compare whether low-frequency or high-frequency ultrasound is more efficient for the inactivation of bacteria. Although shear forces produced from high-frequency ultrasound are less violent than low-frequency ultrasound, there are more hydroxyl radicals generated in high-frequency ultrasound, especially at high powers. We converted absorbed acoustic powers (W) into volumetric powers (W/ml)
in order to compare between the two different frequencies used in this thesis, and the efficiency of inactivation was compared in aqueous solutions. For *E. aerogenes*, a 3.7-log reduction was achieved at 20 kHz and 0.85 W/ml (12.7 W) for 20 min (Figure 3.3A), while 2.7 and 4.2-log reductions were obtained at 850 kHz after 20 min with 0.20 W/ml (50 W) and 0.25 W/ml (62 W) respectively (Figure 5.3A). For *B. subtilis*, there was a 0.9-log reduction for low-frequency ultrasonication at 0.33 W/ml (5.0 W) and up to a 4.2-log reduction at the highest power density (20 kHz, 0.85 W/ml, 20 min), while there was a 2.5-log reduction for high-frequency ultrasonication at a much less power density (850 kHz, 0.25 W/ml, 20 min). Similarly, for the yeast *A. pullulans*, a 0.65 log reduction was obtained at 20 kHz and 0.53 W/ml (8 W) for 1 h, while a 0.77 log reduction was achieved at 850 kHz and 0.20 W/ml for 1 h. It can be concluded that at low frequency it is necessary to apply a high power density to achieve a similar reduction for these two bacteria and the yeast, than the level of reduction achieved by high-frequency ultrasonication. Moreover, the results for *S. epidermidis* were drastically different. This bacterium suffered almost no damage under low-frequency ultrasonication, while it had a 4.4-log reduction under high-frequency ultrasonication (0.25 W/ml, 20 min). Overall, both bacteria and yeast were all susceptible to inactivation by high-frequency ultrasonication at high ultrasound power, and the high-frequency ultrasonication was more efficient for inactivating the bacteria and yeast in aqueous solutions. Note that under both low-frequency and high-frequency ultrasonication, the yeast *A. pullulans* was more resistant to ultrasound treatment than *E. aerogenes* and *B. subtilis* at both low- and high-frequencies. This is not surprising, since the mechanisms involved in the inactivation of bacteria under low (mechanical damage) and high (chemical effects) frequencies are different.

### 7.1.3.3 Mechanisms

The mechanisms of bacterial inactivation by low-frequency and high-frequency ultrasounds are vastly different. Ultrasound inactivation of microorganisms is
suggested to be due to mechanical effects and sonochemical reactions produced by acoustic cavitation (Mason, 1990; Earnshaw et al., 1995; Sala et al., 1995; Butz and Tauscher, 2002; Russell, 2002; Mason et al., 2005; Wu and Nyborg, 2008; Ashokkumar, 2011). A model for the mechanical effects for low-frequency ultrasonication was discussed in Chapter 4. The shear forces produced during the collapse of cavitation bubbles are the main cause of microbial inactivation through breakage of cell walls for low-frequency ultrasound treatment.

The inactivation mechanism of high-frequency ultrasound is different. In addition to the mechanical effects generated by cavitation, more free radicals are produced with high-frequency ultrasound than in low-frequency due to more cavitation events occurring during sonication (Crum, 1995). Energy released upon the collapse of cavitation bubbles makes water molecules break into hydrogen atoms and hydroxyl radicals (OH•) (Leighton, 1994). These hydroxyl radicals attack the cell wall membrane, or recombine to hydrogen peroxide to further oxidise bacterial cells (Al Bsoul et al., 2010; Joyce et al., 2011). The biocidal effects of free radicals on bacteria were confirmed by adding t-butanol, a free radicals scavenger, to the bacteria *E. aerogenes* and *S. epidermidis*. They were not inactivated when more than 10 mM of t-butanol was added into the bacterial suspensions even in high ultrasound power (Figure 5.5), which indicated that mechanical effects alone did not destroy the bacteria. Even with the presence of t-butanol, *B. subtilis* was still broken by cavitation (Figure 5.4 and Figure 5.5), which demonstrated that the combination of sonochemical and physical effects were the reason for its inactivation. More importantly, the effect of post-ultrasonication was discovered in high-frequency ultrasonication, which indicated the bacteria were still being inactivated by hydrogen peroxide generation even after sonication was complete. Therefore, overall the main mechanism of bacterial inactivation in high-frequency ultrasonication is the sonochemical effects based on the production of hydroxyl radicals and hydrogen peroxide, which carry on the inactivation during and after ultrasonication.
7.1.4 Media

In order to fully understand the bacterial inactivation potential of ultrasound treatment and its application in food systems, bacteria were suspended in several media, mainly including sterile tap water, fresh Nutrient broth, incubated Nutrient broth, 0.9% NaCl (physiological salt solution, PSS), and 5−15 wt% skim milk.

Tap water, Nutrient broth and PSS have similar viscosities, and the log reduction of *E. aerogenes* in these media in stationary phases is similar in identical ultrasonic conditions. Log reductions were 3.7 and 3.9 for the bacteria in fresh and incubated Nutrient broth respectively (20 kHz, 12.7 W, 20 min, Figure 3.3). There were 1.6 and 1.3-log reductions for the bacteria in PSS (Figure 4.3, initial bacterial number $1.2 \times 10^8$ CFU/ml) and tap water (Figure 6.1, initial bacterial number $1.9 \times 10^8$ CFU/ml) respectively at 20 kHz and 8 W for 20 min individually. *E. aerogenes* became increasingly resistant to ultrasound treatment as protein concentrations increased from tap water to 5, 10 and 15 wt% skim milk (Figure 6.1), and the viscosity also increased. The occurrence of cavitation decreased in highly viscous media because the vibration of ultrasound waves are hindered in such media and require increasing power to be dispersed (Salleh-Mack and Roberts, 2007). Significant cavitation may occur even in high viscosity solutions but the efficiency of cavitation and the ability to inactivate microorganisms is reduced.

As the main application in food systems for ultrasonic inactivation, skim milk was chosen as the typical medium for both low-frequency and high-frequency ultrasound treatment of bacteria. The viable number of *E. aerogenes* decreased markedly in 5, 10 and 15 wt% skim milk by low-frequency ultrasonication as discussed above while it did not change in either 5% or 10% skim milk with high-frequency treatment. The reasons are mainly because the energy released in high-frequency ultrasound systems is much lower than in low-frequency ones and it becomes less violent in highly viscous media (Mason and Lorimer, 1988). In addition, skim milk itself consumes
hydroxyl radicals produced by cavitation, which in theory is the main factor in inactivating bacteria by high-frequency ultrasound. Therefore, low-frequency ultrasound treatment has more applications in microbial inactivation in food such as skim milk than high-frequency ultrasound. The amount of κ-CN decreased by ~13% when skim milk was treated by low-frequency ultrasound (20 kHz, 8.5 W, 10 min). κ-CN is present mainly at the surface of the casein micelle (Horne, 1998). If the cavitation generated by ultrasound is high enough to cleave the κ-CN, then the radius of the casein micelle would decrease slightly. The decrease in the casein micelle size would also lead to a decrease in the viscosity of the milk.

7.2 Overall conclusions

In this thesis, several species of bacteria and yeast were treated using either low-frequency (20 kHz) or high-frequency (850 kHz) ultrasounds in different matrices. These microorganisms included *E. aerogenes*, *B. subtilis*, *S. epidermidis*, *S. epidermidis* SK, *S. pseudintermedius* and *A. pullulans*. The relationship between the effects of ultrasonic inactivation and physic-chemical properties of bacteria were investigated in a low-frequency ultrasound system. Lethal damage of *E. aerogenes* and *B. subtilis* (up to a 4.5-log reduction) was caused by ultrasonication, and the bacterial cells were broken into numerous smaller fragments that were observed by TEM and the Mastersizer. *E. aerogenes* was also found to be more sensitive to ultrasound in the exponential phase than in the stationary phase. However, *S. epidermidis* was not markedly affected, which was mainly because the gram-positive cells were protected by a thick soft capsule from mechanical damage induced by ultrasound cavitation.

A theoretical model was established based on shear forces produced by low-frequency ultrasound cavitation to explain how ultrasound affects microbial cells. In this model, it was assumed that shear forces generated by the collapse of cavitating microbubbles were able to break up cells. It is proposed that the shear forces are the main causes for
the microbial inactivation. Experimental results indicated that the survival number of both *E. aerogenes* and *A. pullulans* decreased linearly with increasing ultrasound time, which fits well with the model.

*E. aerogenes*, *B. subtilis* and *S. epidermidis* were all measurably inactivated by high-frequency ultrasound treatment. It was demonstrated that the main reason for the inactivation was the production of hydroxyl radicals and hydrogen peroxide. Some *E. aerogenes* and *S. epidermidis* were malformed and misshapen, but most of them maintained whole cells after sonication and displayed a vastly different appearance with the damage from low-frequency ultrasonication. However, rod-shaped bacterium *B. subtilis* was sensitive to mechanical damage induced by ultrasonic cavitation. This research also found that post-ultrasonication effects still inactivated bacteria even after ultrasonication ceased.

*E. aerogenes* was treated in skim milk having different protein concentrations (5-15 wt% skim milk). It was found that the bacterial cells were inactivated significantly in the low-frequency system, and they were more susceptible to ultrasound in water than in milks, which is mainly because of the effect of different viscosities on cavitation. However, *E. aerogenes* was not inactivated by high-frequency ultrasound treatment in milk, which was mainly a result of less energy released in the high-frequency system, and the milk scavenged free radicals produced by cavitation. This study also showed that ultrasonication had no marked influence on the total milk proteins as well as on the structure and the size of the casein micelles, whereas it inconsiderably denatured the whey proteins in skim milk. *αs1-casein*, *αs2-casein* and *β-casein* were not affected by ultrasound treatment, while some *κ-casein* proteins were damaged by shear forces induced by ultrasonic cavitation because they are located on the outer surface of casein micelles.
7.3 Future work

Relevant to the present thesis, several lines of research could be considered in the future.

- To further examine encapsulated bacteria in low-frequency ultrasound systems.

This study demonstrated that the encapsulated bacterium *S. epidermidis* was resistant to low-frequency ultrasound treatment, while the bacteria without capsules were sensitive to ultrasound. The ideal scenario is to isolate non-encapsulated bacteria from the same strain of *S. epidermidis* rather than comparing different species of bacteria, and then to compare the ultrasonicated effects between non-capsulated and encapsulated bacteria from the same strain. Extracellular capsules help protect bacteria against detrimental environments and the colonies of *S. epidermidis* are glistening. As a result, a series of experiments were conducted to isolate non-encapsulated *S. epidermidis*. Firstly, an encapsulated *S. epidermidis* was placed in optimal growth conditions and was subcultured for several generations in various media including Nutrient broth, (low-salt) LB broth and BHI broth. After that, the final subculture was diluted and dropped on Nutrient agar petri dishes (20-30 plates) to incubate at 37°C for 24 h. These plates were placed into a plastic box with pieces of wet paper towels in order to maintain humid surroundings for suitable growth of the bacteria. After incubation, the colonies of bacteria were examined to note any exceptional change in shape, size or surface, and especially to check whether there were any non-glistening colonies. Then these exceptional colonies were stained with India ink to check whether they had capsules. Control samples were also stained and examined. However, non-encapsulated *S. epidermidis* could not be isolated using these three media. Thus, to find a method of isolating non-encapsulated or encapsulated strains would be fundamental and strong evidence for ultrasonically treating encapsulated bacteria. In addition, it would be worthwhile to develop a method to

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22 The components of LB broth/agar and BHI broth are shown in Appendix A1.3.
measure the exact size of bacteria and then to sonicate the isolates with different sizes of capsules.

- To sonicate a large number of different types of bacteria to support the main finding of this study.

It would be beneficial to sonicate more bacteria and find additional evidence to support the results from this study. One of the targeted bacteria would be gram-positive bacteria with the same size and similar shape to *S. epidermidis* but without capsules. This would support the main viewpoint of Chapter 3, which described the significant role of the capsule during sonication. One of the bacterium recommended is *Enterococcus faecalis*, which has no or significantly smaller capsules compared to *S. epidermidis*. A few experiments using *E. faecalis* have been conducted and showed it was less resistant to ultrasound treatment than *S. epidermidis* but more resistant than *E. aerogenes*. Moreover, a mixed group containing 2 or more species of bacteria is worth to be further treated by ultrasound. In addition, other microorganisms should be introduced into ultrasound systems to demonstrate the usefulness of the theoretical model established in Chapter 4.

- To sonicate bacteria in a low-frequency ultrasound system with the presence of a free radicals scavenger.

This study demonstrated that bacterial inactivation by low-frequency ultrasound systems was mainly due to mechanical effects induced by ultrasonic cavitation as discussed in Chapters 3 and 4. In contrast, it was mainly because the sonochemical reaction included the effects of hydroxyl radicals and hydrogen peroxide in the high-frequency system. Although it is well known that the production of hydroxyl radicals is much lower in low-frequency ultrasound treatment than high-frequency ones, it would still be worthwhile to examine the effects of pure mechanical processes in low-frequency systems by adding free radical scavengers into it. This was not considered in this study since the overall inactivation of bacteria by low-frequency
ultrasound was considered and not specifically mechanical effects versus chemical effects.

- To design a less expensive and more efficient ultrasound machine for application in the food industry.

Both the low-frequency and high-frequency ultrasound instruments have advantages and drawbacks for bacterial inactivation. It is more efficient to use high-frequency ultrasound to inactivate bacteria, but sonochemical effects are generated that may affect the quality of some foods. In addition, although it has been widely reported that ultrasonic inactivation can eliminate or minimise the quality degradation of food compared to thermal treatment, it still has limitations to its applications in the food industry. For example, the cost of an ultrasound machine is still extremely expensive; only a small amount of sample can be treated at one time for batch processes; when titanium probe is used, it can easily erode at high amplitude thereby contaminate the direct contacted food samples; and the free radicals produced by high-frequency ultrasound could oxidise the food itself. As a result, efforts are also needed to focus on the design of less cost and high-efficiency for ultrasound machine.

- To develop a theoretical framework to fully understand the mechanical effects of cavitation on bacteria.

While a simple theoretical model was developed in this thesis, it still requires two unknown parameters. The first parameter that is related the elasticity of the bacteria capsule, this could be measured using atomic force microscopy (AFM), for example. The second deals with the exact number of cavitations generated in the presence of bacteria. This is a more difficult task that will require developing imaging techniques, when the bubbles are not microscopic. Other theoretical calculations should be also performed. For instance, it will be interesting to carry out analytical calculations and numerical simulations to investigate why rod-shaped microorganism tend to break more easily than cocci-shaped microorganisms.
• *Elucidate the occurrence of micro-jetting near the micro-organism surface.*

Micro-jetting which has been described in 2.3.2 is due to the asymmetrical collapse of the ultrasound cavitation. Particularly in the case of high-frequency ultrasound, micro-jetting is known to generate pits on solid surfaces. It is the case of high-frequency ultrasound treatment of starch granules suspended in water treated with high-frequency ultrasound (Gallant et al., 1972; Degrois et al., 1974). However, direct observation on the bacteria is extremely difficult since this might require the use of electron microscopy, and thus additional sample preparation which might affect the final images of the sonicated bacteria. It will be interesting to use optical microscopy on yeast or electron microscopy on colloidal particles with physical characteristics (particularly the interfacial properties) close to bacteria.
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REFERENCES


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Appendix 1 Microbiological methods

A1.1 General methods

Sterile and deionised Milli-Q water (18.2 MΩ.cm, Millipore, USA) was used to prepare all media and solutions, except where stated, which were sterilised by autoclaving at 121°C at 15 psi for 20 min (Burns and Ferral Model 94890, Auckland, New Zealand). All the weights including chemicals, media and solutions were measured on a Sartorius balance (Model Basic, two decimal place, 10 mg sensitivity, Germany) or a Mettler Toledo balance (Delta® Range PC440, to 3 decimal places and 1 mg sensitivity, Switzerland). Petri dishes (Labserv) were purchased from Thermo Fisher Scientific New Zealand Ltd. Cuvettes (Polystrene, the Sarstedt Group, Germany) were purchased from Global Science, New Zealand.

A1.2 Microbial culture, isolation and storage

Short term. Bacterial and yeast isolates were stored in broth for up to 1 week at 4°C, and they were stored on solid agar plates for 4 to 6 weeks.

Long term. A colony of bacteria or yeast was transferred into 5 ml of broth and then was incubated overnight in an optimal temperature with shaking. Then the microbial suspension and 80% Glycerol (volume, Glycerol with Milli-Q water, autoclaved) were mixed to obtain 20% Glycerol (volume). 0.5 ml of the mixture was transferred to a 1.5 ml Eppendorf tube and then stored in a −80°C freezer.
## A1.3 Microbial growth media

Table A1.1 Microbial growth media.

<table>
<thead>
<tr>
<th>Media</th>
<th>Components (contents/litre)</th>
<th>Manufacturer</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient broth</td>
<td>Beef Extract 3.0 g, Peptone 5.0 g</td>
<td>BD Difco™, USA</td>
<td>Autoclaved</td>
</tr>
<tr>
<td>Nutrient agar</td>
<td>Beef Extract 3.0 g, Peptone 5.0 g, Agar 15.0 g</td>
<td>BD Difco™, USA</td>
<td>Autoclaved</td>
</tr>
<tr>
<td>Marine broth 2216</td>
<td>Peptone 5.0 g, Yeast Extract 1.0 g, Ferric Citrate 0.1 g, Sodium Chloride 19.45 g, Magnesium Chloride 5.9 g, Magnesium Sulphate 3.24 g, Calcium Chloride 1.8 g, Potassium Chloride 0.55 g, Sodium Bicarbonate 0.16 g, Potassium Bromide 0.08 g, Strontium Chloride 34.0 mg, Boric Acid 22.0 mg, Sodium Silicate 4.0 mg, Sodium Fluoride 2.4 mg, Ammonium Nitrate 1.6 mg, Disodium Phosphate 8.0 mg.</td>
<td>BD Difco™, USA</td>
<td>Autoclaved</td>
</tr>
<tr>
<td>Marine agar 2216</td>
<td>As for Marine broth with addition of 15.0 g agar</td>
<td>BD Difco™, USA</td>
<td>Autoclaved</td>
</tr>
<tr>
<td>YPD broth</td>
<td>Yeast Extract 6.0 g, Peptone 3.0 g</td>
<td>Bacto® Difco Laboratories, USA</td>
<td>pH was adjusted to 5.50; Autoclaved</td>
</tr>
<tr>
<td></td>
<td>Dextrose 8.0 g</td>
<td>J.T. Baker Chemical Co., Phillipsburg, USA</td>
<td></td>
</tr>
<tr>
<td>YPD agar</td>
<td>Yeast Extract 6.0 g, Peptone 3.0 g</td>
<td>Bacto® Difco Laboratories, USA</td>
<td>pH was adjusted to 5.50 before adding agar; Autoclaved</td>
</tr>
<tr>
<td></td>
<td>Dextrose 8.0 g</td>
<td>J.T. Baker Chemical Co., USA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Agar 15.0 g</td>
<td>Sigma, USA</td>
<td></td>
</tr>
<tr>
<td>LB broth</td>
<td>Tryptone 10.0 g, Yeast Extract 5.0 g, Sodium Chloride 10.0 g</td>
<td>BD Difco™, USA</td>
<td>Autoclaved</td>
</tr>
<tr>
<td>LB broth with low salt</td>
<td>Tryptone 10.0 g</td>
<td>BD Difco™, USA</td>
<td>Autoclaved</td>
</tr>
<tr>
<td></td>
<td>Yeast Extract 5.0 g</td>
<td>Bacto® Difco Laboratories, USA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sodium Chloride 5.0 g</td>
<td>Sigma Aldrich, USA</td>
<td></td>
</tr>
<tr>
<td>LB agar with low salt</td>
<td>Same to “LB broth with low salt” with addition of 15.0 g agar</td>
<td>Agar: Sigma, USA</td>
<td>Autoclaved</td>
</tr>
<tr>
<td>BHI broth</td>
<td>Brain Heat Infusion. Calf brain infusion solids 12.5 g, Beef heat infusion solids 50.0 g, Proteose peptone 10.0 g, Glucose 2.0 g, Sodium chloride 5.0 g, Di-sodium phosphate 2.5 g.</td>
<td>Oxoid Ltd., England</td>
<td>Autoclaved</td>
</tr>
</tbody>
</table>
A1.4 Bacteria and yeasts staining

A1.4.1 Bacterial smear preparation

A Microscope Slide (Fronie, Lomb Scientific Pty Ltd, Taren Point NSW, Australia) was labelled with a permanent mark pen. A drop of tap water (about 20 µl) was placed on the slide, and then a small amount of microbes from an agar plate was transferred into the water and mixed thoroughly using an inoculating loop, and then spread on the slide over a large area. After that the mixture was air dried at room temperature and then the slide was burned several times through a Bunsen burner flame. For liquid cultures, about 20 µl microbial suspension was dropped on a slide without extra water.

A1.4.2 Gram staining

The Gram Stain Kit (Difco BBL, Becton, Dickinson and Company, USA) was used to gram stain. The kit included one 250 ml bottle each of Gram Crystal Violet, Stabilized Gram Iodine, Gram Decolorizer, and Gram Safranin.

A1.4.3 Spore staining

5% malachite green was used to soak bacterial smears by gentle steaming with boiling water. Safrain was used as a counter stain, and the smears were rinsed with water and blot dried with paper towels. Then the smears were examined under oil immersion by a light microscope. Spores were shown as green in colour and the other parts of the cell were stained to pink.

A1.4.4 Yeasts staining

Simple crystal violet staining. A yeast smear was made by using the same method to prepare a bacterial smear. 0.1% crystal violet was covered on the smear for 30 s, and

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then was rinsed by tap water and air dried. The smear was examined under oil immersion.

*Wet mount.* A small amount of yeast from agar was placed on a slide or a drop of yeast suspension from growth media was mixed with a drop of Milli-Q water on a slide. Then the mixture/suspension was covered by a Coverslip (LabServ, New Zealand) without spreading. After that, the sample was examined under oil immersion.

**A1.4.5 Capsule and slime staining**

*Anthony’s staining.* The method of capsule and slime staining was adopted from Anthony’s staining. 1% crystal violet, as the primary stain, was used to stain both cells and capsules. 20% copper sulphate, as the decoloriser and counterstain agent, was used to remove the crystal violet from capsules. Capsules appeared as white halos while the cells remained purple. A drop of skim milk broth or other proteinaceous material was added on a slide to provide a background for contrast. Then a small amount of bacteria was transferred into the broth then mixed and spread gently. The smear was air dried at room temperature. After that, the smear was covered by 1% crystal violet for 1 min, and then rinsed gently with a 20% copper sulphate solution. The slide was air dried and then observed by microscope under an oil immersion lens.

*India ink staining.* India ink staining method was used for observe capsules. A drop of India ink (Pébéo Graphic, China) was added on a slide. A small amount of bacteria was transferred on the ink, then mixed and spread gently by gliding a new slide from one side to the end. Then the smear was air dried and covered with 1% crystal violet for 1 min. After that the smear was gently rinsed with tap water. After air drying, it was observed by microscope under an oil immersion lens. Capsules appeared as white halos while the cells remained purple. Capsules can also be examined by using the method of Anthony’s staining, however slime cannot be examined by India ink stains.

A1.5 Molecular methods

A1.5.1 Extraction of bacterial DNA: Bead Beating DNA extraction

This method was revised from a previous report\(^2\). 1.5 ml of overnight culture in broth was transferred in a 1.5 ml microfuge tube and then centrifuged at 13,200 rpm in an Eppendorf Centrifuge 5417C (Hamburg, Germany) for 2 min to obtain a pellet. Then the pellet was washed twice with 1 ml of sterile water at 13,200 rpm for 10 min. The supernatant (about 980 µl) was discarded and the pellet was suspended in 270 µl of phosphate buffer, and 300 µl of SDS buffer was added in and mixed gently. After that the mixture was transferred into a 2 ml polypropylene bead beater vial, and then 300 µl of chloroform-isoamyl alcohol was quickly added. The vial was closed tightly to avoid contamination and put in a box of ice.

Then the vial was placed in a rack of Tissue Lyser II (Retsch, Germany) and was tightened with balance. The frequency was set to 30 for 40 s, and then the vial was shaken twice. Then the vial was cooled in the box of ice. After that the tube was centrifuged at 13,200 rpm for 5 min to pellet down cell debris. Then the supernatant (about 650 µl) was transferred to a clean microfuge tube. 360 µl of 7M ammonium acetate was added and mixed by hand to achieve a final concentration of 2.5 M (about 360 µl). Then the mixture was spun at a full speed of 13,200 rpm for 5 min to produce a clear supernatant.

Then the supernatant (about 580 µl) was transferred to a new tube. A 0.54 volume (315 µl) of isopropanol were added and incubated at room temperature for 15 min. Then the tube was spun at 13,200 rpm for 5 min. The supernatant was removed carefully and quickly, and the tiny pellet (DNA) was washed with 1 ml of 70% ethanol at 13,200 rpm for 7 min. After that the supernatant was removed and the pellet was dried in a desiccator chamber for 10 min. After drying, the DNA pellet was

resuspended in 40 µl of ultrapure water. Then the solution was vortexed and centrifuged with a short spin. Finally, the solution was stored at −20°C before operating the PCR.

A1.5.2 Check the concentrations of the extracted DNA

The NanoDrop (Biolab) was used to measure concentrations of the extracted DNA. The purity of extracted DNA is sufficient if the value of 260/280 is more than 1.8.

A1.5.3 DNA amplification by PCR

A master mix (totalling 50 µl) was prepared in a 250 µl tube including 25 µl Go Taq, 2 µl of Primer PB36, 2 µl of Primer PB38 (Table A1.2), 19 µl of Ultrapure H2O and 2 µl of Extracted DNA (Template). The preparation procedure was conducted on ice. The mixture was centrifuged with quick spinning.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primers</th>
<th>Primer Sequence</th>
<th>Position in gene</th>
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</thead>
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<tr>
<td>16S rDNA</td>
<td>PB36</td>
<td>5'-AGR GTT TGA TCM TGG CTC AG-3'</td>
<td>8..27</td>
</tr>
<tr>
<td></td>
<td>PB38</td>
<td>5'-GKT ACC TTG TTA CGA CTT-3’</td>
<td>1492..1509</td>
</tr>
</tbody>
</table>

Note: Concentration of the Primers: 10 µM.

Then the tube held by a DNA rack was placed in to a PCR Sequencing Facility Machine, GeneAmp® PCR System 9700 (PE Applied Biosystems). PCR conditions were set as in Table A1.3.27

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
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<tbody>
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<td>94°C</td>
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<tr>
<td>94°C</td>
<td>45 s</td>
</tr>
<tr>
<td>55°C</td>
<td>45 s</td>
</tr>
<tr>
<td>72°C</td>
<td>90 s</td>
</tr>
<tr>
<td>72°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Hold at 15°C</td>
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</tbody>
</table>

27 Ibid.
A1.5.4 Agarose electrophoresis

50 ml of 1.2% Agarose (Invitrogen) was made by using a TBE Buffer after microwaving for about 2 min. Then the agarose was cooled to about 50°C, and then 5 µl of Sybr Safe DNA Gel Strain (10,000 × concentrate in DMSO) was added and mixed. After that the agarose solution was transferred into a gel container with an eight well comb. When it coagulated into gel, the comb was taken off and the gel was placed into an Electrophoresis System (Enduro™, Labnet, USA), and aTBE buffer was added to cover the gel.

Making the ladder. 5 µl of 1kb ladder (1kb+1:10 oil) and 1 µl of loading dye were mixed on a Parafilm, and then carefully transferred into a well. Two ladders were made. Control (water) and 5 µl each of the PCR products were added into wells. Then the Electrophoresis Systems were connected to PowerPack Basic (Bio-Rad, USA), and set to 90 V and for 45 min.

Capturing the image. The gel was placed into a Universal Hood II (Bio-Rad, Segrate, Milan, Italy). Quantity One 4.51 software was used to capture the image, and it was printed by a Mitsubishi P93D printer (Malaysia).

A1.5.5 Purification to PCR product

A DNA Clean & Concentrator™-5 (Zymo Research) was used to purify the PCR product. 40 µl of product was mixed with 200 µl of DNA Binding Buffer. Then the mixture was transferred into a Zymo-Spin™ Column in a collection tube. The tube was centrifuged at 10,000g for 30 s, and then the flow-through was discarded. 200 µl of Wash Buffer (with ethanol) was added into the column and then centrifuged two times at 10,000g for 30 s. The column was placed into a 1.5 ml tube to collect DNA. 22 µl of ultrapure water at 50°C was added to the column, and then was centrifuged at 10,000 g for 30 s to elute the DNA.
The concentrations of the purified PCR product were measured. Then the concentration of the PCR product was diluted to 5–20 ng/µl. The concentrations of primers were diluted from 10 µM/l to 5 µM/l. The Volume Primer per primed reaction was 5 µl. Both forward and reverse reactions were conducted for each PCR product.

A1.5.6 DNA sequencing analysis

The sample was analysed by the Centre for Genomics and Proteomics at the University of Auckland, and DNA sequencing was carried out by Dr. Kristine Boxen. Geneious Pro and Chromas Lite were used to analyse sequence results.

A1.5.7 Bacteria identification

In this study, Staphylococcus epidermidis and the two unknown Staphylococcus spp. were obtained, including Staphylococcus spp. (A) borrowed from another laboratory and Staphylococcus spp. (SK) isolated from human skin. The PCR method was used to identify the unknown bacteria. The DNA of three Staphylococcus species were extracted, analysed by gel Electrophoresis (Figure A1.1), amplified by the PCR machine, purified using the DNA Clean & Concentrator TM-5, and DNA sequencing was analysed. After sequence search and analysis, it was found that Staphylococcus spp. (A) was Staphylococcus pseudintermedius and Staphylococcus spp. (SK) was another strain of S. epidermidis. We named Staphylococcus spp. (SK) as S. epidermidis SK for the convenience of this research.
Figure A1.1 Gel electrophoresis of DNA. Two lines of L are 1 kb ladders, Line 1 is the control (H₂O), Line 2 is *Staphylococcus* spp. (SK), Line 3 is *Staphylococcus epidermidis*, Line 4 is *Staphylococcus* spp. (A).

A1.6 Enumeration of bacteria and yeasts

The Miles-Misra method\(^2\) was adapted for the viable cell counting. Serial dilutions from \(10^0\) to \(10^8\) times were made in a working buffer (broth, 0.9% NaCl solution or sterile tap water) with bacterial or yeast samples. The dilution was conducted on a 96 Well Cell Culture Plate (Cellstar, Greiner bio-one, Germany). Nutrient agar plates were dried overnight to period of one day at room temperature in advance after pouring sterile Nutrient agar (50°C) into disposable plastic petri dishes. The outside surface of the bottom of these petri dishes was pre-marked in six sections with a permanent marker pen, and dilution factors were labelled. Diluted samples were dropped on the plates and then incubated at 37°C overnight for bacteria and at 28°C for 24–36 h for yeast. The microbial colonies were counted by using a low magnification light microscope. The procedure for making one series of dilution and enumeration for the initial number of about \(10^9\) CFU/ml is detailed below.

(1) Choose the first line of a “96 Well Cell Culture Plate” to make dilution (8 wells in total).

(2) Dispense 180 μl of working buffer into each well with a 200 μl tip.

(3) Add 20 μl of bacterial suspension to the first well with a 20 μl tip, and mix it with the buffer thoroughly by using a micropipettor with a 1000 μl tip. Adjust the volume of the pipettor to 250-350 μl in order to gather all the suspension each time. Choose a small volume for the foaming sample like milk. Mix them gently to avoid generating bubbles and splashing inside the tip to avoid contaminating the pipette.

(4) With a new tip transfer 20 μl of mixed suspensions from the first well to the second well, until the eighth well is finished. Discard the tips after each use.

(5) With a fresh tip drop 50 μl of dilution with a 200 μl tip from each well into one section of a Nutrient agar plate. Several small drops should be dispensed on each section for each 50 μl of dilution. Each diluted suspension will be dropped in triplicate. Use a fresh tip for each dilution and start at the highest dilution (i.e. lowest number of bacteria) and work down, which prevents the risk of cross contamination.

(6) Incubate the plates in suitable conditions. In order to observe separate colonies, bacteria normally only are needed to be incubated overnight so they do not grow too big. Yeast like *A. pullulans* needs to be counted after incubating for 24–36 h. However, it cannot grow in a very short time because some microbes still do not become visible. A sample of *E. aerogenes* incubated overnight is shown in Figure A1.2.
Figure A1.2 Colonies of *Enterobacter aerogenes* on Nutrient agar (overnight, 37°C).

(7) Count the colonies for the sector where the colonies are countable (15-150 colonies), and calculate the total CFU for the original microbial suspension using:

\[ N = \bar{n} \times d_i \times 20 \]  \hspace{1cm} (A2.1)

where \( N \) is the number of total colonies (CFU/ml), \( \bar{n} \) is the average number of colonies for a dilution and \( d_i \) is the dilution factor. It should be noted that a large volume of sample (i.e. 100 µl × 3) should be used when the initial number of bacteria is low.
Appendix 2 List of publications and presentations

A2.1 Publications


A2.2 Seminar presentations

Gao, S., Hemar, Y., Lewis, G. D. (2011), Bacterial inactivation using high-power low-frequency ultrasound treatment, provisional year seminar, School of Chemical Sciences, the University of Auckland, 20th October 2011.

Gao, S., Hemar, Y., Lewis, G. D. (2012), Bacterial inactivation using high-power low-frequency ultrasound treatment, Microbiology Seminar, School of Biological Sciences, the University of Auckland, 14th August 2012.

Gao, S., Hemar, Y., Lewis, G. D. (2013), Bacterial inactivation using low-frequency and high-frequency ultrasound treatment, Microbiology Seminar, School of Biological Sciences, the University of Auckland, 30th April 2013.

A2.3 Poster presentations

Gao, S., Hemar, Y., Lewis, G. D. (2013), Bacterial inactivation using ultrasound treatment and application in skim milk, the 1st Meeting of the Asia-Oceania Sonochemical Society, 10-12th July 2013, the University of Melbourne, Australia. (The poster won the Best Poster Award).

Gao, S., Hemar, Y., Lewis, G. D. (2013), Bacterial inactivation using ultrasound treatment and application in skim milk, Research Showcase 2013, 12th June 2013, School of Chemical Sciences, the University of Auckland, Auckland, New Zealand.

Gao, S., Hemar, Y., Lewis, G. D. (2012), Bacterial inactivation using high-power low-frequency ultrasound treatment, Research Showcase 2012, 6th June 2012, School of Chemical Sciences, the University of Auckland, Auckland, New Zealand.