Application of Emerging Technologies for Low Acid Liquid Foods

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Abstract

The demand for minimally processed foods has increased in the last few years and gains high acceptability among consumers as it has better nutritional value than highly processed foods. Ultra high temperature (UHT) treatment is carried out to inactivate all types of spores (psychrotrophic, mesophilic, thermophilic) in addition to vegetative cells. However, it has negative impact on vitamins, proteins, colour and flavour. Pasteurization on the other hand is considered as minimally processed but ensues limited shelf life under refrigeration, challenging the transport of pasteurized product to distance markets. Emerging technologies have great potential to process low acid liquid foods at lower temperatures and may lower the energy consumption with better quality compared to conventional technologies. This thesis evaluated the use of ultrasound, ultraviolet and ultra high pressure homogenization with an objective to lower the temperature during processing of low acid liquid foods.

Firstly, combination of ultrasound and thermal treatment was investigated to lower the temperature during sterilization. The effect of pre-treatment with ultrasonication (20 kHz, 750 W) on decimal reduction time (D values) of *B. subtilis* spores ATCC 6633 was evaluated in three different suspending media (water, whole milk and rice porridge) and were compared with thermal treatment. In this study, energy delivered by ultrasound was used to provide the heating needed, which fulfils 55 % of the thermal energy requirement in addition to the effect caused by cavitation. The reduction in D values through the use of combined technology is minimal unless excessive ultrasonication is used, which is commercially not viable. Therefore, the combination was not feasible in terms of energy consumption and only gave marginal benefit towards spore inactivation.

In the second part of this study, Ultraviolet (UV) treatment followed by heat treatment was investigated for inactivation of *B. subtilis and G. stearothermophilus* spores. It was shown that UV treatment in combination with heat is effective in inactivation of these spores in skim milk in comparison to whole cow milk and sheep milk. Therefore, this combined treatment could be a good alternative to sterilization of skim milk at lower temperatures compared to UHT treatment (135 ^{o}C, 3 sec). Moreover, another study investigated the use of UV treatment on commercially pasteurized trim milk to inactivate psychrotrophic spores and thereby extend its shelf life. Microbial study together with physicochemical properties showed that pasteurization followed by UV treatment can enhance the shelf life of trim milk considerably.

There are concerns with the usage of UV treatment as it can result in adverse effects on the quality of milk due to photo oxidative changes. However, photo oxidation in milk that include complex reactions requires oxygen in the media. Limiting dissolved oxygen content within milk can minimize oxidative damage and thus, can result in better product quality. Nitrogen purging is one of the effective methods for removing dissolved oxygen from liquids. Therefore, this study evaluates the effects of nitrogen purging (prior to UV treatment) on milk quality. It was found that nitrogen purged UV treated milk caused minimal changes to physicochemical properties by limiting the oxidative changes in milk arises from light and oxygen.

The benefits of UV were highest in liquids with low absorption coefficient. Whey is the greenish liquid obtained as a by-product during cheese or casein manufacturing. Currently, whey is preserved through thermal pasteurization with treatment temperature that varies depending on the shelf life required. The effect of UV treatment on inactivation of natural microbial load in crude whey and inactivation of inoculated *E. coli* ATCC 25922 in reconstituted whey was studied. The work reported here indicates that UV treatment of whey results in a microbiologically safe product while retaining its physicochemical characteristics and could be an energy efficient alternative to the commonly used thermal pasteurization.

Although, microbial inactivation and physicochemical properties reveal that UV can lower the temperature in comparison to conventional processing, the results suggest that this technology is limited to liquids with low total solid contents. Therefore, liquids with high total solids were analysed with ultra high pressure homogenization (UHPH). The pressure applied during conventional homogenization ranges from 30 MPa to 50 MPa; generally, it has little or no impact on spores. Previously, it was shown that pressure higher than 100 MPa has damaging effects on spores. Therefore, UHPH was investigated for spore inactivation through its combined application of temperature and pressure (higher than 100 MPa) and for its ability to incorporate into a continuous process. In this study, antimicrobial efficacy against *B. subtilis* spores was analysed in whole milk and sheep milk that have high solid contents and compared with inactivation in Skim milk.

This thesis investigated a science based approach for a better understanding of the key principles of the selected emerging technologies, combination of technologies with heat, and associated benefits/drawbacks. The outcome of this work will help in meeting the challenges

for process and product development for targeted food processes delving desirable food properties while maintaining their safety.

Acknowledgements

Thanks and Praise to Almighty God

I would like to express my deepest gratitude towards my supervisors, Prof. Mohammed Farid and Dr. Marliya Ismail for their efforts, help, guidance and continuous support during the course of research. Their critical and constructive comments on my research work are greatly appreciated. It has been an honour for me to work in their world class research teams. Moreover, I want to thank my advisors Dr. Brent Young and Dr. Indrawati Oey for their guidance.

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I also wish to acknowledge the support from all the technicians in the department for their support, training and guidance provided during my research period. Especially, I would like to thank Ms. Laura Liang for training provided to use equipment to analyse particle size distribution, rheology, optical microscopic images and biological samples. Also, I would like to thank Ray Hoffman for assembling the UV unit and for setting up of ultra high pressure homogenization unit as per the requirement of the experiments. Also, I would like to thank Dr. Mathew Sidford for the training provided to use spectrophotometer and multimode plate reader. Moreover, I would like to thank Peter Buchanan to expedite the process of ordering and installation of ultra high pressure homogenization.

Finally, special thanks to my family and friends that supported me spiritually and throughout my life in general.

Thesis structure

The thesis consists of nine chapters followed by conclusions and future recommendations. These chapters have been structured as scientific papers that are either published or will be submitted for publication in international journals. These chapters may have some overlapping information especially in introduction and methodology sections. Design of all experiments and analysis of results were undertaken by myself, and the manuscripts were completed with the assistance of my supervisors, Prof. Mohammed Farid and Dr. Marliya Ismail. All references are listed at the end of thesis.

Chapter 1: Introduction and Objectives

This chapter provides an overview of the basic microbiology, spores and its structure. Also, it discusses thermal processing technologies for microbial inactivation and its relation to shelf life. Moreover, the use of thermal technologies for shelf life extension and their drawbacks were discussed. This chapter also led the path to identify research needs and the project objectives.

Chapter 2: Literature review-Emerging Technologies

This chapter provides a brief literature review of emerging technologies including ultrasound, ultraviolet and ultra high pressure homogenization. The literature remains focused on mechanism of microbial inactivation, effect on quality, and previous literature on microbial inactivation. This chapter also discusses the gaps in knowledge in the use of emerging technologies in low acid liquid food preservation.

Chapter 3: Investigation of the use of ultrasonication followed by heat for spore inactivation

This chapter examines the use of ultrasound in combination with heat to inactivate spores. The chapter also considers previous literature to find out the possibility of energy efficient application of ultrasound for microbial inactivation. In this study, *B. subtilis* spores were used as heat resistant organism and thereby, can indicate possible sterilization. This study, also compares the decimal reduction time before and after application of ultrasound.

Chapter 4: Investigate the efficacy of UV pretreatment on thermal inactivation of *B. subtilis* spores in different types of milk

This chapter examines the use of ultraviolet treatment for *B. subtilis* spores inactivation in different milk products (skimmed cow milk, whole cow milk, and sheep milk) using a coiled tube reactor. The discussion also looks at optimizing the operating parameters.

Chapter 5: Inactivation of *G. stearothermophilus* spores using Ultraviolet treatment in combination with heat

The results of *B. subtilis* spore inactivation show that ultraviolet treatment can inactivate spores. However, *G. stearothermophilus* spores are the most thermally resistant spores. This chapter examines the possibility of combination of ultraviolet and heat to inactivate *G. stearothermophilus* spores in different milk products. Also, effect of different inlet temperatures were examined to find any sensitization effects (on thermal treatment) from ultraviolet treatment.

Chapter 6: Extension of shelf life of pasteurized trim milk using ultraviolet treatment

Based on the previous studies (chapter 4 and chapter 5), it was found that Ultraviolet treatment can inactivate spores considerably. This chapter examines the application of ultraviolet treatment on pasteurized trim milk to increase shelf life by analysing microbial quality and physicochemical factors under refrigerated storage.

Chapter 7: Effect of nitrogen purging on skim milk quality during UV treatment

With previous literature, it was found that ultraviolet treatment can adversely affect quality of milk. These adverse effects arise from photo oxidation which mainly affects proteins, lipids and nutrients. Also, based on previous results (chapter 4 and chapter 5), it was found that spores inactivation was highest in skim milk using ultraviolet treatment. This chapter examines nitrogen purging prior to ultraviolet treatment to reduce adverse effects in skim milk. To address protein oxidation, protein carbonyls and absorbance at 280 nm were measured with different residence times. Also, other physicochemical properties (pH, titratable acidity, particle size distribution, and rheology) were analysed.

Chapter 8: Ultraviolet treatment: A potential technology for whey preservation

Whey is considered as a valuable by product in the dairy industry and preserved using thermal pasteurization. Further, Chapters 4, 5 & 6 showed that ultraviolet treatment is highly effective for microbial inactivation in high transmittance liquids. Therefore, this chapter evaluates the use of ultraviolet treatment for preservation of whey in terms of microbial inactivation, retention of physicochemical properties and energy usage.

Chapter 9: Investigation of *B. subtilis* spores inactivation in different milk products using ultra high pressure homogenization.

Previous chapters show that, ultraviolet treatment is successful for microbial inactivation in high transmittance products. This chapter evaluated the usage of ultra high pressure homogenization for *B. subtilis* spores inactivation in different milk products. The effect of different inlet temperatures and pressures was analysed on microbial inactivation.

These chapters are followed by conclusions and future work recommendations.

The structure of this thesis complies with the University of Auckland guidelines given in the Doctoral Handbook 2011.

Table of Contents

List of Tables

List of Figures

Abbreviations

A	Absorbance
ATCC	American Type Culture Collection
AOAC	Association of Official Analytical Chemists
ANOVA	Analysis Of Variance
CFU/ml	Colony Forming Units Per Millilitre
CIE	Commission International d'Eclairage
CW	Crude Whey
DNPH	Dinnitrophenylhydrazine
DPA	Dipicolinic Acid
DNA	Deoxyribonucleic acid
EU	European Union
ESL	Extended Shelf Life
FSM	Fresh Skim Milk
HCl	Hydrochloric Acid
HPH	High Pressure Homogenization
HTST	High Temperature Short Time
HPP	High Pressure Processing
NA	Nutrient Agar
NZRM	New Zealand Reference Materials
PEF	Pulse Electric Field
PC	Protein Carbonyls
PSD	Particle Size Distribution
PCA	Plate Count Agar
PFA	Perfluoroalkoxy

PTM	Pasteurized Trim Milk
Re	Reynold Number
RW	Reconstituted Whey
RTD	Ready To Drink
RNA	Ribonucleic Acid
SP	Spore Photoproduct
SPL	Spore Photoproduct Lyase
SM	Sheep Milk
SCM	Skimmed Cow Milk
TCA	Trichloro Acetic Acid
TCD	Total Colour Difference
TYE	Tryptone Yeast Extract
TSA	Tryptic Soy Agar
TVC	Total Viable Count
TSAYE	Tryptone Soy Agar Yeast Extract
UHT	Ultra High Temperature
UV	Ultraviolet
US	Ultrasound Waves
UV-NPSM	Ultraviolet Treated Nitrogen Purged Skim Milk
UHT-TM	Ultra High Temperature Treated Trim Milk
UV-SM	Ultraviolet Treated Skim Milk
UHPH	Ultra High Pressure Homogenization
WCM	Whole Cow Milk

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Extent of contribution by PhD candidate (%)	80%

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Name	Nature of Contribution
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Dr. Marliya Ismail	Advise and manuscript revision

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
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CHAPTER 1

Introduction and objectives

1.1 General introduction

Food safety remains a huge challenge for the food industry as shown by statistics of food outbreaks that resulted in losses to human life (Fung *et al.*, 2018). Thermal treatment has been used for a long time to increase shelf life of foods by inactivating the micro flora present in them. On the other hand, food can also be preserved by employing natural ingredients to alter intrinsic properties (pH, water activity) or by using synthetic preservatives (Tewari *et al.*, 2008). The success of the preservation methods lies in the storage stability obtained while retaining most of the physicochemical properties of food. Thereby, food safety and retention of characteristics of food are equally important in designing food preservation processes. For low acid liquid foods, a preservation process need to inactivate all types of micro flora to attain storage stability as these food products provide more favourable environment for microbial growth than in acidic foods. Thermal technologies offer the possibility of heating low acid liquid foods to very high temperatures to inactivate microorganisms and thereby achieve sterilization (Tucker *et al.*, 2011). However, high temperature operations can also adversely affect the nutrition value and physicochemical properties of the food (Burton, 2012). Thus, combination of thermal and emerging technologies may provide the opportunity to lower the processing temperature while providing the possibility of retaining physicochemical properties of low acidic liquid foods in comparison to thermal treatment alone.

1.2 Food microbiology

Microbial flora of food is quite diverse and consists of pathogenic as well as non-pathogenic microorganisms. Numerous food borne diseases may arose through consumption of contaminated food or unprocessed food. Food borne diseases are addressed by personal hygiene and food safety programs during food processing. These diseases are caused by different microorganisms including *Salmonella* spp., *Staphylococcus aureus, Listeria monocytogenes, Clostridium perfringens, B. cereus, Bacillus* spp. and *Escherichia coli* (De Buyser *et al.,* 2001). Microorganisms are mainly classified into Gram positive and Gram negative based on the difference during staining procedures due to different cell wall composition. Gram positive organisms retain crystal violet dye while Gram negative organisms retain safranin during gram staining procedure (Hucker *et al.,* 1923).

Microorganisms are also classified based on the temperature requirement for their growth. According to temperature requirement, microbial sources are mainly classified into three categories as given below (Donnelly, 2014):

- Psychrophiles usually require 15 to 20 ^{o}C for their growth but able to grow under refrigerated conditions (0 to 15 ^{o}C). They are mainly responsible for spoilage of dairy foods under refrigerated conditions.
- Mesophiles requires 30 to 37 ^{o}C for their growth
- Thermophiles requires 50 to 60 ^{o}C for their growth
- Extreme thermophiles can grow up to 122 ^{o}C.

1.3 Spore forming microorganisms

Microorganisms require specific environmental conditions to survive and multiply. These conditions include intrinsic factors such as composition of matrix, moisture content, and acidity; and extrinsic factors such as oxygen availability and storage temperature are important (Sun, 2011). Under unfavourable conditions, some microorganisms possess the capability to convert themselves to dormant form which is referred as spores. This dormant form of microorganism can survive for longer duration and can be difficult to inactivate during processing. Upon availability of favourable conditions, these spores can convert to vegetative cells and can spoil food. Considering *B. subtilis* after reaching the stationary phase, they must enter into one of the survival systems that include endospore formation, nutrient scavenging or transformation. The first choice after reaching stationary phase is to go towards nutrient scavenging which involves the expression of genes towards nutrient sources with secretion of antibiotics and secretion of enzymes to hydrolyse environmental macromolecules to use as nutrient sources. If the adverse conditions persist, cells start to transform. Transformation involves cell differentiating and results in recombinant cells that could increase survival rate. Endospore formation is complex and an energy intensive process. Therefore, microbial cells consider this as a last option (Juodeikiene *et al.*, 2012; Setlow, 2014). The endospore formation can be defined into seven different stages (*Bacillus* spp) as given below (Figure 1-1).

Stage 0: Vegetative cells

Stage I: Spore development starts when DNA coils

Stage II: Separation of DNA to form protoplast

Stage III: The resulted protoplast then converts to intermediate structure forespore.

Stage IV: Core (cell) wall, cortex, and spores coats are generated

Stage V: Reduction in water content within spores lead to increased resistance to heat

Stage VI: Spore separation from the mother cell

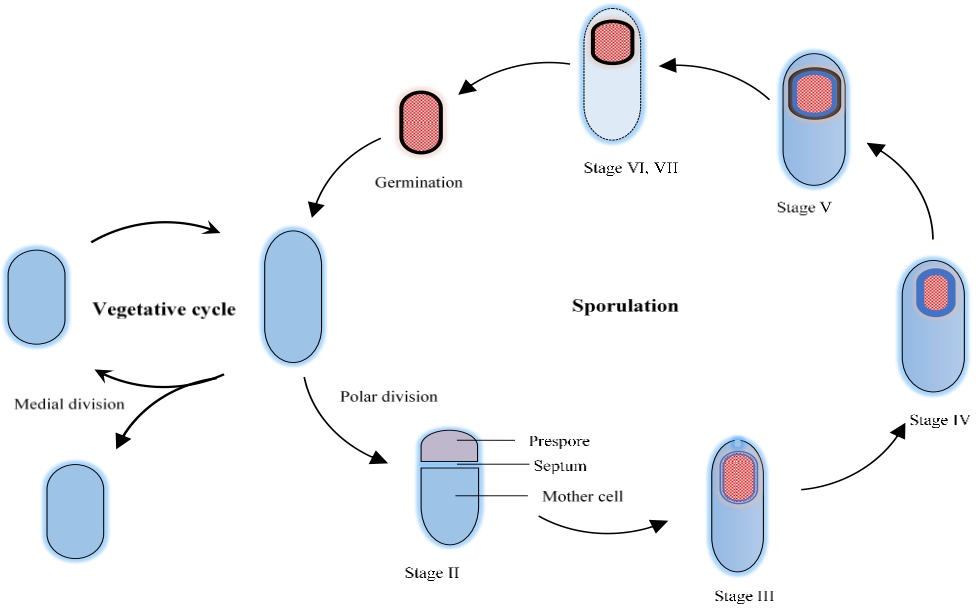


Figure 1-1: Stages during spore formation (Adapted from Juodeikiene *et al.* (2012))

1.3.1 *B. subtilis*

B. subtilis is a rod shaped (4-10 µm long and 0.25-1.0 µm in diameter), Gram positive bacteria, usually found in soil and can produce spores (Allen *et al.*, 2014). The spores from *B. subtilis* are quite resistant to heat and UV treatment (Datta *et al.*, 2015). It is mostly investigated in laboratory studies including sporulation process, validation of sterilization process and to asses effectiveness of antibiotics (Pharmacopeia., 2016). *B. subtilis* is considered an obligate aerobe and possesses the capability to produce enzymes (Gopal *et al.*, 2015).

1.3.2 *G. stearothermophilus*

G. stearothermophilus is also a rod shaped, Gram positive, aerobic and spore forming bacterium. *G. stearothermophilus* spores are quite resistant to heat and can survive harsh conditions (Donk, 1920). It is usually found from soil and hot springs. Spores of this organism are usually used in validation studies as challenge organism for sterilization (Pharmacopeia., 2016). *G. stearothermophilus* can cause a flat sour defect in milk by producing acid and is

responsible for reduced shelf life of milk powders (Kakagianni *et al.*, 2016; Ledenbach *et al.*, 2009)

1.3.3 *B. cereus*

B. cereus is a Gram positive, rod shaped and spore forming organism. Some of the strains of this organism considered as pathogenic and can cause food borne illness (Bottone, 2010). It can also produce toxins therefore, safety programs consider preventive measures to control it during food processing (Christiansson *et al.*, 1989). It is commonly found in soil and has high possibility to contaminate milk. As a spore former, *B. cereus* spores is one of the factor for limited shelf life of pasteurized milk (Rysstad *et al.*, 2006). Thus, considerable measures (animal and farm hygiene) are required to minimize its count.

1.4 Spores

Structure of spores possesses high importance and ensures its ability to survive for long time. Vreeland *et al.* (2000) isolated spore forming bacterium (*Bacillus* species) from 250 million years old salt crystals after careful sampling, which shows its survival over long period. Thus, structure of spore can help in determining the resistivity to different treatments and designing an effective process. It consists of different layers including exosporium, coat, outer membrane, cortex, germ cell wall, inner membrane, and core respectively (Figure 1-2)

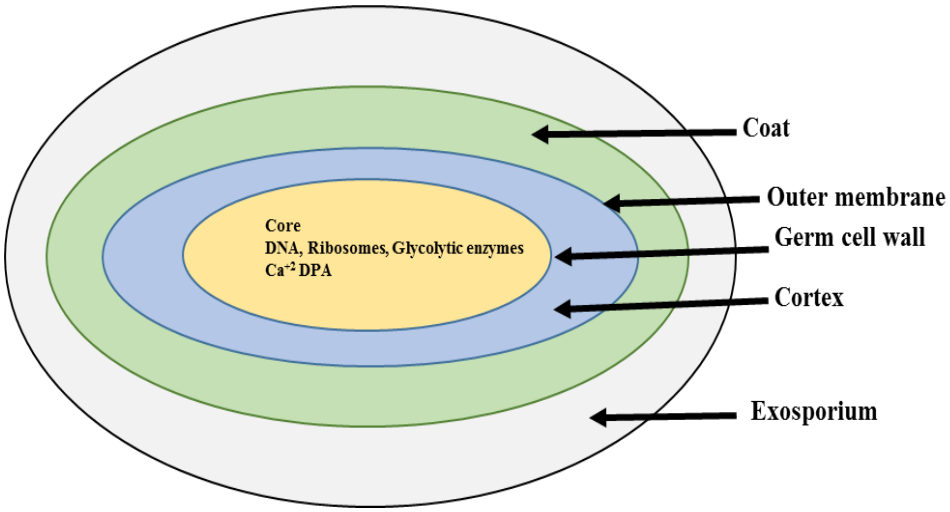


Figure 1-2: Structure of spore (not to scale)

1.4.1 Spore structure

1.4.1.1 *Exosporium*

Exosporium is the outer most layer of spore mainly comprised of proteins. It is found only in *B. cereus* and *B. subtilis* spores (Setlow, 2006a).

1.4.1.2 Coat

Spore coat is a structure composed of several layers containing more than 50 proteins as in the case of *B. subtilis* and mainly responsible for providing resistance to enzymes but not much important in providing resistance to heat, radiation, and chemicals (Setlow, 2006a).

1.4.1.3 Outer membrane

Outer membrane possesses no importance in providing heat resistance and also not contributing as a permeability barrier. As outer membrane does not have any specific role, the removal of outer membrane does not have any effect on spore resistance (Setlow, 2006b).

1.4.1.4 Cortex

Spore cortex is composed of peptidoglycan similar to that found in vegetative cells but with modifications. It is found to be vital in formation of dormant spore and responsible for reduced water content of spore core. During spore germination, it degrades, and this degradation is important in cell outgrowth (Setlow, 2006b).

1.4.1.5 Germ cell wall

Germ cell wall is also composed of peptidoglycan and similar to vegetative cell peptidoglycan. It plays no role in spore resistance and becomes the cell wall of the bacterial cell after germination (Setlow, 2006b).

1.4.1.6 Inner membrane

Inner membrane of spore is important in providing resistance to chemicals due to its limited permeability. Lipid molecules present in inner membrane are largely immobile, and it particularly protects DNA (Setlow, 2006b).

1.4.1.7 Core

The last layer of spore is called as core. The core contains most spore enzymes as well as DNA, ribosomes, and tRNA. There are three main components present, considered to be important in the functionality of spores. First one is water, which comprised of 27-55 % of spore core weight. The lower water content is responsible for spore enzyme dormancy. In the initial stages of germination, increase in water content restores enzymes functionality. The second core small molecule important is dipicolinic acid chelated with calcium. Lastly, it also contains small acid soluble proteins that comprised of 3-6 % of spore core and provides resistance to heat and UV (Setlow, 2006a).

1.4.2 Spore heat resistance

Water content of spore core tends to play an important role in its heat resistance. It also depends on sporulation temperature as higher sporulation temperature results in lower core water content and thus, have high heat resistance. DPA content plays a significant role in resistance of spores to heat. Spores without DPA contains higher water content making them sensitive to heat. Wet heat induces inactivation of enzymes in spore core and damage membrane permeability. Thus, affecting outgrowth of spores and causing death. For wet heat inactivation of spores, most important factors include sporulation temperature, spore core mineralization, and presence of small acid soluble proteins (Setlow, 2006b)

1.4.3 Spore germination

The spores can remain in dormant form for years, but availability to favourable conditions, return them back to life within short time. Spore germination is brought by nutrient or non-nutrient agents. Nutrient germinants work by interacting with receptors. It includes amino acids, sugars, purine nucleotides, asparagine, glucose, fructose, lysozyme and salt.

Germination cycle occurs in two stages. In stage I, ions are released from spore causing increase in pH (6.5 to 7.7) which is necessary for enzyme activity. Also, release of spore core DPA causes increase in water content within spore. In stage II, Peptidoglycan layer hydrolysed and results in swelling by water intake. This water intake results in expansion of germ cell wall and increased enzyme activity. On the other side, non-nutrient germinants includes lysozyme, cationic surfactants, high pressures and salts (Setlow, 2003, 2014).

1.5 Thermal treatment

Thermal treatment has long being used and have been effective in food preservation. It is a process that inactivates different types of microorganisms which includes bacteria, fungi, viruses, and spores. Thermal treatments are used more frequently than any other preservation methods (chemicals, radiation) due to its adaptability, scalability, cost effectiveness, adequate inactivation rate of microorganisms and consumer acceptability (Richardson, 2001).

Thermal treatment of milk usually done by using direct or indirect plate heat exchangers. These heat exchangers use steam/ hot water as heating medium. Direct /indirect heating systems differs in come up time to achieve target temperature. Direct heating usually provides shorter come up time in comparison to indirect heating. Holding tube is another important component in thermal treatment systems as most of the legislative requirements address holding time/temperatures (Burton, 2012).

Kinetics of thermal inactivation

On exposure of microorganisms to heat treatment, microorgansims reduces in number and described by general model as given below (Singh *et al.*, 2001)

Equation 1-1

$$\frac{dN}{dt} = -kN^n$$

Where *k* is the rate constant and n is the order of the model.

Exposure of microbial suspension to specific temperature range results in the reduction of microbial count. In microbial kinetics, decimal reduction time (D value) is defined as the time required to reduce number of microorganisms by one log factor at constant temperature and also represented as D = 2.303/*k* (Berk, 2009). D values vary with the nature of microorganism

and medium characteristics. It is a known fact that heating a microbial suspension at constant lethal temperature results in logarithmic reduction of microorganisms or spores. This logarithmic reduction is described in terms of first order kinetics (Figure 1-3) (Singh *et al.*, 2001).

Equation 1-2

$$D = \frac{t}{\log N_o - \log N}$$

If the initial population is N_o and the final population is N at time t, the equation can be solved to

Equation 1-3

$$\frac{N}{N_o} = e^{-kt}$$

Hence, the relation between rate constant (*k*) and D value can be described as

Equation 1-4

$$k = \frac{2.303}{D}$$

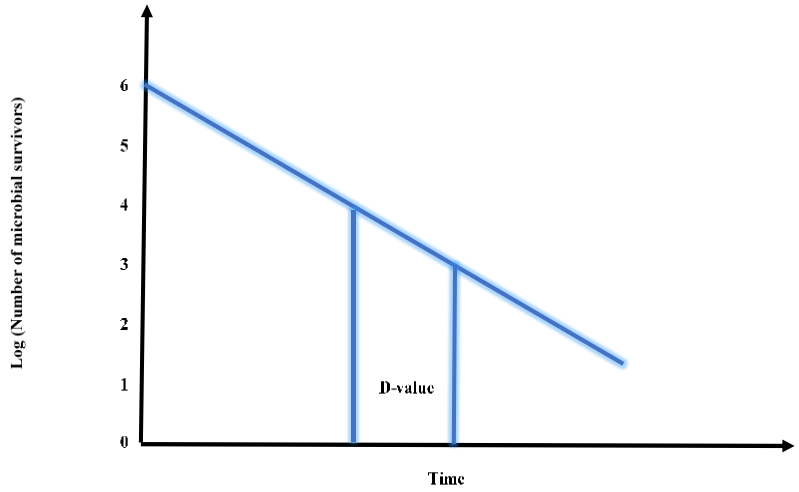


Figure 1-3: Decimal reduction time curve

1.5.1 Thermal preservation technologies

Low acid liquid foods are thermally processed to inactivate microorganisms and thereby to achieve preservation. Milk is considered as one of the best examples to study thermal processing of low acid liquid foods. Thermal preservation of milk vary in processing conditions, extent of microbial inactivation, quality attributes, storage conditions, packing requirements and shelf life. Preserved milk is mainly categorized as pasteurized, extended shelf life (ESL), and ultra high temperature (UHT) treated products (Table 1-1). UHT milk fulfils the requirement of increased shelf life up to 180 days under ambient conditions (Cappozzo *et al*., 2015; Efigênia *et al*., 1997; Kessler *et al.,* 1991; Kilshaw *et al.,* 1982; Patton, 1955; Shimamura *et al.,* 2012; Van Boekel, 1998). Pasteurization treatment is effective as it can retain fresh milk taste in comparison to UHT milk but it cannot retain its quality beyond 14-21 days under refrigeration. ESL milk provides a solution with fresh milk taste similar to pasteurized milk with extended shelf life up to 60 days under refrigeration depending on different parameters including initial microbial count and packaging conditions (Rysstad *et al.*, 2006).

Table 1-1: Thermal technologies for an increase in shelf life (Rysstad *et al.,* 2006)

Process	Treatment conditions applied	Log reduction in aerobic psychrotrophic spores	Expected shelf life	Drawbacks
Pasteurization	72 ºC for 15 sec	Negligible	Up to 14 days at 4 ºC	No spore reduction and thereby shelf life of only 14 days
UHT treatment	135 ºC for a few seconds	Greater than 8	180 days at ambient temperature	High temperature treatment results in undesirable quality changes.
ESL	130 ºC for a second	Greater than 8	Up to 45 days at 4 ºC (depending on filling solution)	Asceptic packaging is required to obtain extension of shelf life

1.5.2 Drawbacks of thermal technologies

Table 1-1 show that intense thermal treatment like UHT (135-142 ºC for 3-4 sec) is capable of giving extended shelf life at ambient conditions. However, these intense processing conditions compromise quality by adversely affecting the natural characteristics of milk in terms of nutrition, colour and sensory properties as discussed below.

1.5.2.1 Nutrition

Raw milk is considered as nutritionally rich food and contains carbohydrates (lactose), proteins (casein and whey), fats (long chain saturated and unsaturated fatty acid, short chain fatty acids), vitamins and minerals (Meurant, 1995). Nutritional value of raw milk is higher in comparison to UHT processed milk and consumers increasingly prefer raw milk or minimally processed milk. Numerous studies have been done to analyse the effect of heat treatment on milk and milk proteins. Cappozzo *et al.* (2015) observed no change in proximate composition of milk in UHT treatment (138 ºC for 2 sec); while Vitamin A and D were lost during treatment. In another study, Kilshaw *et al.* (1982) found reduction in vitamins on heat treatment (121 ºC for 20 min) of skim milk. It reduced all of the vitamin B_{12}, 60 % of vitamin B_6 and thiamine, 70 % of ascorbic acid, and about 30 % of the folate. Considering protein loss, Efigênia *et al.* (1997) found that consumption of UHT treated milk by rats resulted in reduced growth due to the loss of lysine and denaturation of proteins.

1.5.2.2 Colour

Colour of milk serves as an important parameter that impacts consumer perception. Heat treatment results in change of colour due to specific chemical changes. Maillard reaction possesses a significant effect on colour changes that get initiated at high temperatures and thereby autocatalytic in nature (Patton, 1955). Many studies were done to evaluate the effect of heat treatment on changes during processing and storage (Cappozzo *et al.*, 2015; Hewedy *et al.*, 1994; Nangpal *et al.*, 1990; Renner, 1988; Van Boekel, 1998). Particularly lactose and lysine in milk, significantly contributed to Maillard reaction and resulted in the formation of lactulosyllysine, which further breakdown to products including lysylpyrraline, pentosidine, hydroxymethylfurfural, (iso) maltol, furfurals and formic acid (Nangpal *et al.*, 1990). However, quantity of browning products vary between direct and indirect heating. UHT treatment by

direct heating produce 0.1-0.2 mmol/l and indirect heating 0.6-0.9 mmol/l of amadori products (calculated from furosine content). Lower amounts of amadori products in direct heating could be due to dilution effects provided by addition of steam in milk (Nangpal *et al.*, 1990) whereas in bottled sterilized milk, it was around 1-2 mmol/l. Also, Hewedy *et al.* (1994) found increase in lactulose content, furosine content and hydroxylmethylfurfural on heat treatment at 142-148 ^{o}C for varying duration. Similarly, Van Boekel (1998) stated a direct relationship between heat treatment and heat indicators. They noticed an increase in lactulosyllysine and Maillard reaction products (hydroxymethyl furfural, iso maltol, furfurals and formic acid). They also found reduction in lactose content and suggested that lactose could have been reduced due to isomerization and participation in Maillard reaction. In addition to colour changes during processing, Maillard reaction also possesses an influence during storage and can continue to effect colour of milk depending on the storage temperature. Renner (1988) reported loss of lysine and formation of HMF during storage of milk powders that depends on storage temperature. Likewise, Popov-Raljić *et al.* (2008) studied colour changes in UHT milk (with fat content of 1.6 % and 3.2 %) over storage up to 90 days. Darkening in colour was observed after 60 days of storage in both UHT samples (1.6 % and 3.2 % fat).

1.5.2.3 Effects on milk proteins

Proteins are quite diverse in milk and some of the proteins are quite sensitive to heat treatment. On heat treatment, the size of casein micelles tends to increase due to denaturation and aggregation of casein micelle. Serum proteins in milk mainly found in two variants (α-lactalbumin and β-lactoglobulin). These proteins are quite sensitive and become denatured on heat treatment and cause deposition within heat exchangers. The reaction kinetics shows significant changes at treatment even below 100 ^{o}C. This is less severe in high temperature treatment (above 100 ^{o}C) due to very short treatment time. In general, β-lactoglobulin is more sensitive than α-lactalbumin). It could be explained that at higher temperatures serum protein associate with other proteins in milk (Burton, 2012).

1.5.2.4 Effects on vitamins

Milk contains water soluble vitamins that includes vitamin B1 (Thiamine), vitamin B2 (Riboflavin), vitamin B3 (Niacin), vitamin B5 (Pantothenic acid), vitamin B6 (Pyridoxine) and vitamin C (Ascorbic acid) in addition to fat soluble vitamins (Vitamin A, D, E and K) (Burton,

2012). These vitamins are reduced on heat treatment and the mechanism differs for these vitamins.

Many of the vitamins are considered as heat stable to pasteurization conditions and thereby are not affected by heat treatment. Ascorbic acid is considered as heat labile and results in losses with mild heat treatments. However, Vitamin C is present as oxidized form as dehydroascorbic acid in milk (heat labile and destroyed by pasteurization conditions). Thereby, its degradation is attributed towards oxidation more than thermal degradation. Vitamin B_{12} and folic acid results in losses due to complex interactions with each other, with –SH groups and with oxygen. Vitamin B_6 is slightly affected by heat and during storage. Vitamin B_1 is largely damaged in UHT treatment conditions with temperatures between 120-150 ^oC with longer treatment times (Burton, 2012).

1.5.2.5 Other changes

Flavour and texture are also important factors on consumer perception and greatly affected by the time and temperature of heat treatment. Liem *et al.* (2016) mentioned that consumer taste preference is for less heat treated milk. Moreover, the aroma of cooked flavour in milk was found to be due to formation of H_2S. Other aroma compounds produced during Maillard reaction include aldehydes, reductones and furfurals (Renner, 1988). Further, an increase in FFA (free fatty acid) was observed in UHT milk in comparison to raw milk on storage for 14 days under refrigeration (Cappozzo et al., 2015).

1.6 Research needs

The temperate climatic conditions together with abundance of lush green pastures favour livestock farming in New Zealand. Thereby dairy and related industries are a thriving business that drives the country's economy contributing ~ $ 7.8 billion to its GDP (Ballingall *et al.*, 2017). As a result, New Zealand exports 95 % of its milk production which accounts for 3 % of global milk production (MBIE, 2017).

As discussed earlier, heat treatment can have adverse effects on the quality of low acid liquid foods. These adverse changes affect mainly the organoleptic properties, colour, and nutrition of the product. However, high temperature during processing is required to assure food safety by inactivating the microbial load. Besides, some milk products (like sheep milk and whey)

cannot be treated at very high temperatures as their functionality, and nutritional value are lost considerably.

Consequently, there is a need to investigate new technology/ies that could inactivate microbial load at a lower temperature. The use of emerging technologies can provide the possibility to lower the required temperature for processing and thereby, extend shelf life of milk. However, it is essential that these technologies are evaluated for their technical feasibility in a number of potential products. Therefore, this study focuses on the use of ultrasound, ultraviolet and ultra high pressure homogenization to process liquid whey, skimmed cow milk, whole cow milk and sheep milk that has diverse composition to evaluate food safety and technological effectivity.

Thesis objectives

The foundation of this study was built on the use of emerging technologies for spore inactivation. The primary aim was to reduce temperature during conventional thermal processing by a combination of thermal and emerging technologies. For this purpose, three different technologies (ultrasound, ultraviolet, ultra high pressure homogenization) were assessed for their effect on spore inactivation. Microbial spores were selected depending on their severity for thermal resistance and also that are commonly used for validation of sterilization studies. The specific objectives of this research were as follows:

1. To measure the thermal resistance of microorganisms (*B. subtilis* spores, *G. stearothermophilus* spores and *E. coli*) with respect to the different media used in this study.
2. To study the use of ultrasonication, ultraviolet and ultra high pressure homogenization in combination with heat for spore inactivation.
3. To assess the extension of shelf life of milk using a combination of technologies
4. To evaluate the effect of Nitrogen purging prior to ultraviolet treatment on the quality of milk
5. To evaluate the potential of ultraviolet for pasteurization of liquid whey

Thesis framework

Chapter 1- Introduction and objectives	
Chapter 2- Potential of emerging technologies	
Chapter 3- Investigation of the use of ultrasonication followed by heat for spore inactivation	**Objectives 1, 2**
Chapter 4- Investigate the efficacy of UV pretreatment on thermal inactivation of *B. subtilis* spores in different types of milk	**Objectives 1, 2**
Chapter 5- Inactivation of *G. stearothermophilus* spores using UV treatment in combination with heat	**Objectives 1, 2**
Chapter 6- Extension of shelf life of pasteurized trim milk using UV treatment	**Objectives 2, 3**
Chapter 7- Investigation of nitrogen purging prior to UV treatment on quality of skim milk	**Objective 4**
Chapter 8- UV treatment: A potential technology for whey preservation	**Objectives 1, 5**
Chapter 9- Investigation of *B. subtilis* spores inactivation in different milk products using ultra high pressure homogenization	**Objectives 1, 2**
Conclusion and future work recommendations	

CHAPTER 2

Literature review-Emerging Technologies

Emerging technologies have an immense potential to contribute towards the concept of minimally processed foods. Emerging technologies differ in their mechanism of action for microbial inactivation and thereby the temperature required could be lower than conventional thermal processing. However, safety of food demands investigation of a particular emerging technology against a range of microorganisms and intrinsic properties of food. At the same time, it is important to consider its application in terms of energy consumption, suitability to continuous production and possible flexibility to integrate with current thermal facilities. Thereby this chapter focuses on a brief literature review on the use of ultrasound, ultraviolet and ultra high pressure homogenization as presented below. Also, a more detailed literature review is discussed with latter chapters accordingly.

2.1. Ultrasound

Ultrasound waves (US) can be defined as sound waves having frequency above 20 kHz, higher than the audible limit of human hearing. The food industry has been exploring US for the extraction of different food components (Chavan *et al.*, 2013; Duba *et al.*, 2015; Kadam *et al.*, 2015; Vinatoru, 2001), homogenization (Wu *et al.*, 2000), mixing, defoaming and emulsification (Delmas *et al.*, 2015; Feng *et al.*, 2010). Moreover, US is well known for its antimicrobial effects in combination with antibiotics, pressure, heat and chemical agents (Bevilacqua *et al.*, 2015; Raso *et al.*, 1998a; Yu *et al.*, 2012).

Potential of US as antimicrobial waves was discovered by its use in anti-submarine warfare that resulted in detrimental effects on fish life (Earnshaw *et al.*, 1995). Long before that, *Harvey et al.* (1929) found the effect of luminous high frequency ultrasound on bacterial life. These effects were further explored on different microorganisms. However, antimicrobial effects of US were ignored initially due to some of its negative effects on quality. In order to improve antimicrobial effects, US was combined with pressure and termed as Manosonication (MS). Also, a combination of heat treatment and US was used and termed as Thermosonication (TS). While simultaneous application of pressure (up to 600 kPa), heat treatment and US termed as Manothermosonication (MTS) (Pagán *et al.*, 1999; Vercet *et al.*, 1999).

2.1.1. Mechanism of action

Exposure of liquid samples to US results in the formation of microbubbles. These microbubbles release high amount of energy on collapse and may result in localised heating, causing

inactivation of microorganisms. This phenomenon refers as cavitation (Figure 2-1) (Earnshaw *et al.*, 1995; Scherba *et al.*, 1991). Another theory suggested that US results in the formation of radicals that have the ability to damage microbial DNA (Earnshaw *et al.*, 1995).

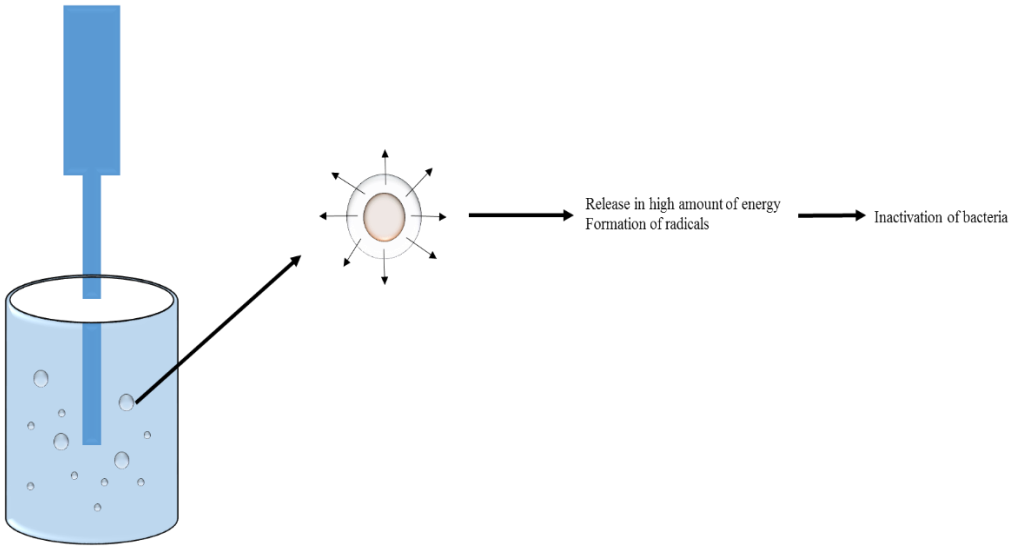


Figure 2-1: Schematic representation of cavitation and its effects

2.1.2. Microbial inactivation

The use of US was studied for microbial inactivation in different conditions to optimize its effectivity. The combination of ultrasound with heat, pressure and chemical agents were studied to obtain higher microbial reduction than US alone (Raso *et al.*, 2003). For inactivation of vegetative cells, US can be combined with either temperature and/or pressure. Pagán *et al.* (1999) analysed the effect of combination of pressure up to 400 kPa, temperature up to 40 ºC and US with amplitude up to 117 µm on inactivation of *Streptococcus faecium*, *Listeria monocytogenes*, *Salmonella enteritidis*, and *Aeromonas hydrophila* and obtained lower D values in comparison to thermal treatment.

Moreover, combinations of US with other emerging technologies have been tested for improved inactivation. In this sense, Palgan *et al.* (2012) studied combination of MTS and pulsed electric field (PEF) to obtain antimicrobial effects similar to pasteurization in a milk based smoothie. They found that MTS followed by PEF provided 5.6 log reduction in *L. innocua* compared to PEF followed by MTS (4.2 log reduction). This could be due to the cavitation effects from MTS that resulted in sensitizing the microbial cells to subsequent electroporation from PEF treatment. Also, Halpin *et al.* (2013) found that MTS treated milk followed by PEF treatment produced microbiologically stable milk for up to 14 days of storage.

However, after 14 days, conventionally pasteurized milk maintained lower microbial count than MTS + PEF treated milk. This might be due to survival of certain organisms in ultra sonicated samples showing the need for further investigation.

In addition to vegetative cells, US can inactivate spores in combination with heat (Table 2-1) where heat was found to be a driving factor in terms of spore inactivation. In a study, Burgos *et al.* (1972) found that ultrasonic treatment (20 kc, 1.2 A) prior to heat treatment resulted in marked reduction in *B. cereus* and *B. licheniformis* spores. After ultrasonic treatment, $D_{110}{}^{o}{}_{C}$ decreased from 11.5 to 1.5 min for *B. cereus* spores and $D_{99}{}^{o}{}_{C}$ decreased from 5.5 min to 3 min for *B. licheniformis* spores. A decreasing trend of heat resistance after sonication was also observed by Ordoñez *et al.* (1976). Based on the studies (Table 2-1), it is important to consider energy consumption during ultrasonication treatment. As treatment with ultrasonication may consume immense energy which is not commercially feasible.

Table 2-1: Overview of literature on Ultrasonic inactivation of bacterial spores

Equipment	**Matrix**	**Spore strain**	**Conditions**	**Effect on spores**	**Source**
Ultrasonication (at controlled temperature) pre-treatment followed by heat					
Not given	Ringer solution	*B. cereus*	Ultrasonication (20 kHz, 10-12 ^{o}C for 12 min with 4 ml suspension) followed by heat treatment at 110 ^{o}C for *B. cereus* and 99 ^{o}C for *B. licheniformis*	87 % reduction in $D_{110\,C}$	Burgos *et al.* (1972)
		B. licheniformis		45.45% reduction in $D_{99\,C}$	
Ultrasonic disintegrator MSE 60W	Ringer solution	*B. subtilis* 189	Ultrasonication (20 kHz, 0 ^{o}C for 10 min with 5 ml suspension) followed by heat treatment at 105 ^{o}C, 110 ^{o}C and 112 ^{o}C	Approx. 20 % reduction in $D_{105\,C}$, 20 % reduction in $D_{110\,C}$, 23% reduction in $D_{112\,C}$ No effect on z-values	Ordoñez *et al.* (1976)

Vibra cell ultrasonic processor VC 505 (Sonics & materials) 500W	Non-fat milk	*B. licheniformis* (ATCC) 6634	Ultrasonication (20 kHz, 3.679 W/ ml , 91.2 µm, 0 ºC-33 ºC for 10 min with 20 ml suspension) followed by heat treatment 63 ºC for 30 min	0.42 log reduction	Khanal *et al.* (2014)
		B. coagulans (ATCC) 12245		0.56 log reduction	
		G. stearothermophilus ATCC (15952)		0.73 log reduction	
Thermosonication (Ultrasound + Heat Simultaneously)					
UP 200S Hielscher 200W	Beef slurry	*C. perfringens* (NZRM 2621 and NZRM 898)	24 kHz 0.33 W/g, 75 ºC for 60 min with 100 ml suspension, 210 µm	Less than 1.5 log reduction in both strains	Evelyn *et al.* (2015c)
UP 200S Hielscher 200W	Skim milk	Psychotrophic *B. cereus* NZRM 984	24 kHz 0.33 W/g or W/ml 70 ºC for 1.5 min with100 ml suspension, 210 µm	0.3-0.4 log reduction (Approx. 66 % reduction in $D_{70\,C}$)	Evelyn *et al.* (2015b)
	Beef slurry			Greater than 4 log reduction (Approx. 84 % reduction in $D_{70\,C}$)	
	Cheese slurry			Greater than 3 log reduction (Approx. 72 % reduction in $D_{70\,C}$)	
	Rice porridge			Greater than 4 log reduction (Approx. 85	

				reduction in $D_{70\,C}$)	
Heat system Ultrasonic wave generator mod (W-220 F) 150 W	Whole milk	*B. subtilis* var *niger*-40	Thermosonication 20 kHz, 100 °C, 30 ml	79 % reduction in $D_{100\,C}$	Garcia *et al.* (1989)
		B. subtilis ATCC 6051		40 % reduction in $D_{100\,C}$	
	Glycerol	*B. subtilis* var *niger*-40		63 % reduction in $D_{100\,C}$	
		B. subtilis ATCC 6051		74 % reduction in $D_{100\,C}$	
Manothermosonication (Ultrasound + Heat + Mild Pressure)					
PG Branson sonifier ultrasound	Sterile distilled water	*B. subtilis* var. niger ATCC (9372)	20 kHz, 300 kPa, 70 °C for 12 min, 150 µm	3 log reduction	Raso *et al.* (1998b)

2.1.3. Effect on quality

US on application to different media results in production of free radicals including hydroxyl and hydrogen free radicals. These radicals may react with certain components in food and affect food quality. Also, US tends to disrupt biological tissues creating high surface area for enzyme activity (Ercan *et al.*, 2013). In a study, Chemat *et al.* (2004) showed the effect of high power ultrasound (20 kHz; 150W; 2min) on refined sunflower oil and found that peroxide value increased from 5.38 meq O_2/ kg for untreated oil to 6.38 meq O_2 /kg. They also mentioned that there was an increase in turbidity as well as increase in the formation of volatile flavours. In another study, Tiwari *et al.* (2009) found that orange juice treated with US resulted in 5 % reduction in ascorbic acid.

2.1.4. Future studies

US has great potential for microbial inactivation when applied in combination with pressure and/or heat. However, it is noted that when pressure exceeds (> 600 kPa), lethality of

ultrasound was reduced (Vercet *et al.*, 2001). Therefore, studies are needed to overcome the limitations to pressure when applied in combination with US. Secondly, it is important to consider the amplitudes in US treatment as high amplitude can result in higher inactivation. Moreover, the use of US need to be evaluated with energy consumption in comparison to thermal processes. Also, inactivation of microorganisms need to be evaluated in different matrices as previous literature shows that US may suite specific application.

2.2. Ultra high pressure homogenization (UHPH)

Homogenization is defined as a process of forcing liquid through a disruption valve, which results in the reduction of particle size distribution (Georget *et al.*, 2014b). One of the primary objective of homogenization of beverages is to attain stabilized emulsions (Saunal *et al.*, 1982) of oil and water; preventing phase separation (Perrechil *et al.*, 2010). However, pressure applied during conventional homogenization ranges from 30 MPa to 50 MPa; generally, it has little or no impact on spores (Bevilacqua *et al.*, 2007; Gaulin, 1904; Georget *et al.*, 2014b).

Much research was done to reduce thermal treatment in food processing by using high pressure, as it retains the nutritional quality of food by lowering the required thermal treatment (Matser *et al.*, 2004; Rodriguez *et al.*, 2004; Sale *et al.*, 1970; Scurrah *et al.*, 2006). It was shown that pressures higher than 100 MPa has damaging effects on spores (Rodriguez *et al.*, 2004; Sale *et al.*, 1970; Scurrah *et al.*, 2006). Further, the antimicrobial effects of high pressure increased synergistically with increased temperature (Bull *et al.*, 2009). Therefore, high pressure homogenization (HPH) is considered as more effective as it incorporates temperature and pressure effects into a single and a continuous process.

On the basis of pressure, sometimes high pressure homogenization (HPH) can be categorized into two types. Pressure below 200 MPa is normally termed as high pressure homogenization (HPH) and pressure above 200 MPa, is termed as UHPH (Georget *et al.*, 2014b).

Numerous investigations have been made to replace conventional sterilization by UHPH, as UHPH have damaging effects on spores as UHT treatment (Bevilacqua *et al.*, 2007; Espejo *et al.*, 2014; Georget *et al.*, 2014a). In comparison to conventional sterilization, lower treatment time during UHPH resulted in minimal nutrient damages, less effect on sensory attributes, prevent phase separation and minimize textural changes (Cruz *et al.*, 2007; Ferragut *et al.*, 2011; Pereda *et al.*, 2007). Thereby, UHPH has opened new doors for its use in pasteurization and sterilization.

2.2.1. Mechanism of action

The primary mechanism of spore inactivation by high pressure was discussed by Black *et al.* (2007). In this review, spore germination, germination pathways and effect of different pressures on spores were discussed. It was suggested that moderate pressure (100-300 MPa) can result in germination of spores by inducing nutrient receptor pathway and thereby, could be inactivated by heat. While higher pressure (above 400 MPa) results in Ca-DPA release from spores making them heat sensitive (Black *et al.*, 2007). On the other hand, UHPH applies high pressure and temperature simultaneously. Therefore, UHPH is comparatively more effective because it induces thermal stress as well as shear stress at the same time. Multiple studies were done to evaluate the effect of pressure and thermal treatment on spores using UHPH; it has been found that pressure and thermal treatment provide synergistic effects on inactivation of spores (Espejo *et al.*, 2014; Roig-Sagués *et al.*, 2015).

2.2.2. Microbial spore inactivation

Table 2-2 shows a literature review on UHPH inactivation of spores. Many researchers showed that UHPH can inactivate heat resistant spores successfully in milk and other beverages to achieve sterilization. Espejo *et al.* (2014) studied inactivation of multiple spores in milk using UHPH with applied pressure of 300 MPa. With an inlet temperature (T_{inlet}) of 74 ºC, a valve temperature (T_{valve}) of 129.6 ºC,outlet temperature (T_{outlet}) 16.7 ºC, 4.78 log reduction in *B. subtilis* spores and 4.39 log reduction in *G. stearothermophilus* spores were obtained. Also, Georget *et al.* (2014a) obtained 5 log reduction of *B. subtilis* spores in PBS buffer at T_{inlet} of 85 ºC and T_{valve} of 145 ºC.

Table 2-2: Literature review on microbial spore inactivation using high pressure homogenization

Spores	Media/ Food	Maximal log Reduction	Operating Conditions	Source
Aerobic spores (Natural)	Soy milk	Less than 2.18	Pressure $_{Max}$: 300 MPa $T_{inlet\ Max:}$ 80 ºC $T_{valve\ Max:}$ 144 ºC	Poliseli-Scopel *et al.* (2014)

Aerobic spores (Natural)	Soy milk	Greater than 2	Pressure $_{Max}$: 300 MPa T $_{inlet\ Max:}$ 40 ºC T $_{valve\ Max:}$ 108 ºC	Cruz *et al.* (2007)
Aerobic spores (Natural)	Milk (3.5 % Fat)	Greater than 1	Pressure $_{Max}$: 300 MPa T $_{inlet\ Max:}$ 40 ºC T $_{valve\ Max:}$ 103 ºC	Pereda *et al.* (2007)
Aerobic Spores (Natural)	Almond beverages (with and without lecithin)	4	Pressure $_{Max}$: 300 MPa T $_{inlet\ Max:}$ 75 ºC T $_{valve\ Max:}$ 129 ºC	Valencia-Flores *et al.* (2013)
Aerobic spores (Natural)	Soymilk and Almond milk	Greater than 4	Pressure $_{Max}$: 300 MPa T $_{inlet\ Max:}$ 75 ºC T $_{valve\ Max:}$ 135 ºC	Ferragut *et al.* (2011)
Alicyclobacillus acidoterrestris (DSMZ 2498, r4 and c8)	Malt extract broth	DSMZ 2498: 1-2 r4: 0.5-1 c8: 0.25	Pressure $_{Max}$: 170 MPa T $_{inlet\ Max}$: Not given T $_{valve\ Max:}$ 55 ºC	Bevilacqua *et al.* (2007)
Alicyclobacillus acidoterrestris (DSMZ 2498 & γ4)	Laboratory medium apple juice	γ4: 0.82 DSMZ 2498: 0.67	Pressure $_{Max}$: 140 MPa T $_{inlet\ Max}$: Not given T $_{valve\ Max:}$ Not given Exit temperature: 40ºC	Bevilacqua *et al.* (2012)
Alicyclobacillus acidoterrestris spores (CECT 7094)	Orange Juice	Greater than 5.5	Pressure $_{Max}$: 300 MPa T $_{inlet\ Max:}$ 80 ºC T $_{valve\ Max:}$ 130 ºC	Roig-Sagués *et al.* (2015)
Alicyclobacillus acidoterrestris (N-1100, N-1108, N-1096, SAC, and OS-CAJ)	*Bacillus acidoterrestris* thermophilic broth	Less than 0.2	Pressure $_{Max}$: 300 MPa T $_{inlet\ Max:}$ Not given T $_{valve\ Max:}$ Not given	Chen *et al.* (2013)

Alicyclobacillus hesperidum spores (CECT 5324)	Orange Juice	5.0	Pressure Max: 300 MPa T_{inlet} Max: 80ºC T_{valve} Max: 130ºC	Roig-Sagués *et al.* (2015)
Bacillus cereus SV3 and SV98 spores	Sterilized double distilled water	SV3: 2.2 SV98: 1.9	Pressure Max: 150 MPa T_{inlet} Max:20 ºC T_{valve} Max:45 ºC No. of cycles: 3	Chaves-López *et al.* (2009)
Bacillus cereus CECT 5144	Milk	6.47	Pressure Max: 300 MPa T_{inlet} Max: 85ºC T_{valve} Max: 139ºC	Espejo *et al.* (2014)
Bacillus coagulans DSMZ 2356	Milk	6.57	Pressure Max: 300 MPa T_{inlet} Max: 85ºC T_{valve} Max: 139ºC	Espejo *et al.* (2014)
Bacillus licheniformis DSMZ 13	Milk	6.33	Pressure Max: 300 MPa T_{inlet} Max: 85 ºC T_{valve} Max: 139 ºC	Espejo *et al.* (2014)
Bacillus licheniformis ATCC 14580	Ice cream mix	0.75	Pressure Max: 200 MPa T_{inlet} Max: 50 ºC T_{valve} Max: 88 ºC	Feijoo *et al.* (1997)
Bacillus sporothermodurans DSMZ 10599	Milk	6.91	Pressure Max: 300 MPa T_{inlet} Max: 85 ºC T_{valve} Max: 139 ºC	Espejo *et al.* (2014)
Bacillus stearothermopilus ATCC 7953 spores	Skim Milk	Less than 1	Pressure Max: 300 MPa T_{inlet} Max: 45 ºC T_{valve} Max: 84 ºC No. of Cycles Max: 16	Pinho *et al.* (2011)
Bacilus subtilis CECT 4002	Milk	5.22	Pressure Max: 300 MPa T_{inlet} Max: 85 ºC	Espejo *et al.* (2014)

			Valve Temperature Max: 139 ºC	
Bacillus subtilis PS 832 spores	PBS Buffer 0.01 M	2	Pressure Max: 350 MPa T $_{inlet}$ Max: 80 ºC Valve Temperature Max:>145 ºC	Georget *et al.* (2014a)
Bacillus subtilis SV 50 and SV 108	Sterilized double distilled water	SV 50 : 1.9 SV 108: 1.6	Pressure Max: MPa T $_{inlet}$ Max: ºC T $_{valve}$ Max: ºC No. of cycles: 3	Chaves-López *et al.* (2009)
Clostridium sporogenes PA 3679 spores	Skim Milk	Less than 1	Pressure Max: 300 MPa T $_{inlet}$ Max: 45 ºC T $_{valve}$ Max: 84 ºC No. of Cycles Max: 16	Pinho *et al.* (2011)
Geobacillus stearothermophilus ATCC 7953 spores	PBS Buffer 0.01 M	5	Pressure Max: 350 MPa T $_{inlet}$ Max: 80 ºC T $_{valve}$ Max:>145 ºC	Georget *et al.* (2014a)
Geobacillus stearothermophilus CECT 47	Milk	5.26	Pressure Max: 300 MPa T $_{inlet}$ Max: 85 ºC T $_{valve}$ Max: 139 ºC	Espejo *et al.* (2014)

Operational parameters:

Inlet temperature, outlet temperature and valve temperature:

During operation of UHPH, T $_{inlet}$ is considered to be a main contributor in spore inactivation. It was found that higher the T $_{inlet}$ can result in higher T $_{valve}$ depending on the pressure applied and thereby, can results in higher spore inactivation.

Feijoo *et al.* (1997) studied the effect of T $_{inlet}$ from 33 ºC to 50 ºC on *B. licheniformis* spores at 200 MPa and observed that inactivation of spores were increased from 0.53 log to 0.75 log reduction. In another study, Espejo *et al.* (2014) investigated the effect of different , T $_{inlet}$ on inactivation of *Bacillus* spores. It was shown that increase in , T $_{inlet}$ from 55 to 85 ºC resulted

in increase of 3.34 log reduction of B. *cereus* spores, 4.77 log reduction of *B. licheniformis* spores, 6.21 log reduction of *B. sporothermodurans* spores, 3.78 log reduction of *B. coagulans,* 4.42 log reduction in *G. stearothermophilus* spores and 4.55 log reduction in *B. subtilis* spores. , T_{inlet} (55 ^{o}C to 85 ^{o}C) also resulted in increased T_{valve} from 113 ^{o}C to 139 ^{o}C at the pressure applied (300 MPa). A similar trend was obtained by Georget *et al.* (2014a) on spores of *B. subtilis* and *G. stearothermophilus* in PBS buffer. At pressures of 300 MPa and 350 MPa, it was found that there is no inactivation in spores at temperatures of 37^{o}C (*B. subtilis*) and 55^{o}C (*G. stearothermopilus*). However, increase in T_{inlet} to 80 ^{o}C resulted in up to five log reduction in *B. subtilis* and two log reduction in *G. stearothermophilus* spores. In another study, Roig-Sagués *et al.* (2015) studied the effect of different inlet temperatures on *A. acidoterrestris* and *A. hesperidum*. On increasing T_{inlet} from 20 ^{o}C to 80 ^{o}C, inactivation of spores increased from 0.5 log to 5.5 log approximately for *A. acidoterrestris* spores and 0.2 to 5 log approximately for *A. hesperidum*. On the other hand, Dong *et al.* (2015) reported 3.5 log reduction in spores of *Alicyclobacillus acidoterrestris* at 350 MPa with T_{valve} higher than 150 ^{o}C.

Operational pressure:

Operational pressure of UHPH was found to be an important contributor in spore inactivation. This operational pressure is directly linked to T_{valve} where an increase in operational pressure results in increased T_{valve}. Few researchers have observed that increase in operational pressure at constant T_{inlet} resulted in higher inactivation of spores.

Feijoo *et al.* (1997) studied the effect of different pressures (0, 50, 100, 150, 200 MPa) with T_{inlet} (33, 36, 44, 50 ^{o}C) on reduction of *B. licheniformis* spores. In this study, a slight spore reduction was observed by increase of pressure. For instance, with an T_{inlet} of 50 ^{o}C, an increase in pressure from 50 MPa to 200 MPa resulted in 19 to 68 % spore inactivation respectively. A similar trend was obtained by Pereda *et al.* (2007) where they found 1.1 log reduction with an T_{inlet} of 40 ^{o}C and 300 MPa. Bevilacqua *et al.* (2007) studied the effect of pressure on spore inactivation of *Alicyclobacillus acidoterrestris* on pH 3.5 and 4.5. It was found that increase in pressure from 140 MPa to 170 MPa increased inactivation of 0.16 log reduction at pH 3.5 for one of the strain. In another study, spore log count of soymilk based product was reduced from 2.37 to 0.36 on application of pressure at 200 MPa and reduced further to 0.24 at 300 MPa at T_{inlet} of 40 ^{o}C (Cruz *et al.*, 2007). Further, Valencia-Flores *et al.* (2013) found that increase in pressure from 200 MPa to 300 MPa, resulted in 3.22 log reduction in total spore count in almond beverages.

Number of cycles:

For HPH, number of cycles has an important role in inactivation. In fact, an increase in temperature resulted from first cycle have an impact on second run. Chaves-López *et al.* (2009) studied the effect of number of cycles on *B. cereus* and *B. subtilis* spores. More than five log reduction was obtained on three cycles at 150 MPa in *Bacillus cereus* SV 3 spores. On contrary, Pinho *et al.* (2011) found that 16 cycles at 300 MPa resulted only 0.67 log reduction in *B. stearothermophilus* spores. This could be due to the resistance of *G. stearothermophilus* to high pressure.

Residence time:

One of the primary benefits of using UHPH is its low residence time or holding time at the valve, which is short (estimated residence time based on measured flow rates is less than a sec) in comparison to UHT which is around 3-4 sec under same conditions (Espejo *et al.*, 2014).

2.2.3. Effect on quality

UHPH can reduce particle size and thereby, can increase stability during storage. In a study on soymilk, UHPH treatment resulted in intense reduction in particle size in comparison to base product and UHT treated milk (Cruz *et al.*, 2007). Similar results were obtained by Ferragut *et al.* (2011), in which soymilk and almond milk treated by UHPH resulted in improved emulsion stability. Poliseli-Scopel *et al.* (2014) found that UHPH treated milk (144 ^{o}C for 0.7 s) did not show any microbial growth in six months of storage which was similar to UHT (142 ^{o}C for 6 s). In addition to microbial quality, no noticeable effect was observed in sensory quality of UHPH milk. High colloidal stability was observed which is similar to results obtained by Cruz *et al.* (2007), Ferragut *et al.* (2011) and Valencia-Flores *et al.* (2013) (Table 2-3).

While, Pereda *et al.* (2007) observed reduction in viscosity of UHPH treated milk in comparison to pasteurized milk. Further they also mentioned UHPH treated milk gave a shelf life between 14 and 18 days similar to pasteurized milk.

Moreover, when fruit juices were UHPH treated, it resulted in retention of vitamin C, polyphenols and antioxidants (Jacobo *et al.*, 2014). UHPH treatment of orange juice and clementine samples with pH 3.1, reduced pectin methyl esterase enzyme activity to less than 10 % (Navarro *et al.*, 2014).

Table 2-3: Literature review on effect of UHPH on quality of liquid foods

Product	Parameters	Effect on quality	Reference
Almond milk	Pressure 350 MPa Inlet temp 85 ^{o}C	• Significant reduction in particle size was observed • Almond proteins antigens were not detected in UHPH treated product. • Vitamin B_1 and Vitamin B_2 were similar to raw milk • 50 % reduction in free sulfhydryl groups were observed	Briviba *et al.* (2016)
Soy milk	Pressure 200 MPa, 300 MPa	• Intense particle size reduction was observed, although aggregates were formed at 300 MPa. • UHPH products were more stable (less particle settling) than UHT milk.	Cruz *et al.* (2007)
Soy milk Almond milk	Pressure 200 MPa, 300 MPa Inlet temp 55 ^{o}C, 65 ^{o}C, 75 ^{o}C	• Finer emulsion was observed. Although aggregation was observed. • Increased oxidation was observed in almond milk.	Ferragut *et al.* (2011)
Soy milk	Pressure 300 MPa Inlet temp 80^{o}C Oulet temp 144^{o}C	• UHPH treated milk produce high colloidal stability during storage. • No difference in sensory attributes was detected by panel members between UHT and UHPH treated milk.	Poliseli-Scopel *et al.* (2014)
Almond Beverages with lecithin and without lecithin	Pressure 200 MPa, 300 MPa Oulet temp 130^{o}C	• UHPH treatments of Almond beverages with lecithin resulted in decrease of particle size and thus, higher physical stability. • UHPH treated samples resulted in higher peroxide value at day 1 • Hydrophobicity increased in UHPH treated Almond beverages with lecithin in comparison to Almond beverages without lecithin.	Valencia-Flores *et al.* (2013)
Soy milk	Pressure 200 MPa, 300 MPa Inlet temp 55^{o}C, 65^{o}C, 75^{o}C	• Particle size was reduced • Sedimentation was reduced	Poliseli-Scopel *et al.* (2012)

		• Lower hydro peroxide formation was observed in comparison to UHT milk	

2.2.4. Future studies

UHPH shows an immense potential to inactivate bacterial spores and thereby can extend shelf life of liquid food products. Sheep milk has a good potential in consumer market due to its high nutritional value. It comprised of twice the quantity of protein/fat contents than cow's milk. However, it is difficult to process sheep milk through conventional thermal treatment as high temperature causes protein denaturation and thereby results in sedimentation. Hence, UHPH might result in better product quality for sheep milk while inactivating spores to the required level.

2.3. Ultraviolet

The use of electromagnetic radiations are well known for its antimicrobial effects. UV radiation was first studied for the treatment of drinking water. Later on, US FDA approved the use of UV for surface treatment of foods and juice products (having turbulent flow with minimum Reynolds number of 2200) (21 CFR179.39). UV light lies in the region from 100 nm to 400 nm and classified as UVA (315 to 400 nm), UV-B (280 to 315 nm), UV-C (200 to 280 nm), and vacuum UV (100 to 200 nm) (Figure 2-6). UV-C light has germicidal wavelength, and it alters the genetic material of microbial cells affecting reproduction of microorganisms (Baysal et al., 2013; Caminiti et al., 2010).

In designing new non-thermal methods, energy requirement is considered as an important factor for commercial adaptability. The use of UV requires low energy for microbial inactivation in comparison to thermal processing. The reason for its commercial acceptability lies in its low energy consumption and easy adaptation to current processing facilities as a batch or continuous process.

Light characteristics and its importance in UV treatment

A number of light characteristics are important to consider in design of UV reactor. These includes interference of light, reflection, refraction, dispersion and transmittance properties of materials involved.

Interference

Interference can be described as a phenomenon when two waves of the light add together to form a new pattern (Figure 2-2). It occurs as constructive interference and destructive interference. Constructive interference occurs when the troughs of the waves align together while destructive interference occurs when trough of one wave align with peak of other wave. In result, constructive interference results in brighter spots while destructive interference results in darker spots. Considering the double slit effect, UV reactor may also result in the formation of bright and dark spots based on the particle available in liquid treated and reactor design (De Pree, 2010; Khan *et al.*, 2017).

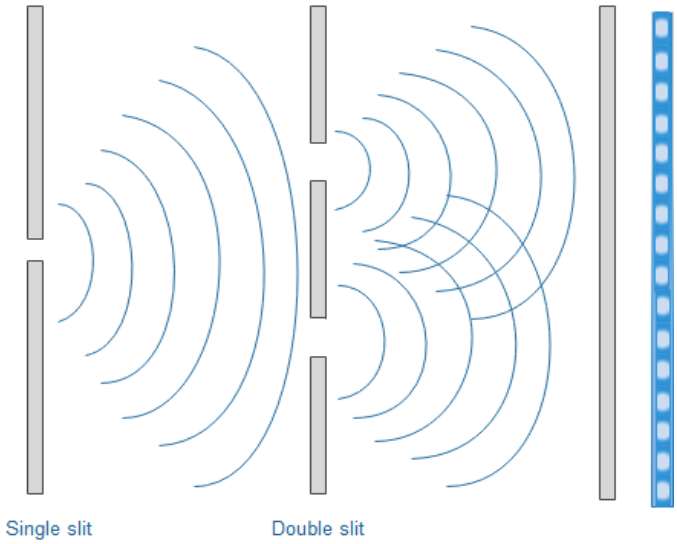


Figure 2-2: Young's Double-Slit Experiment

Reflection

Reflection is defined as change of direction when a light is incident towards an object and reflects back at an angle. The law of reflection states that the angle of incidence is equal to angle of reflection (angle of incidence is the angle at which light approaches towards an object and angle of reflection is the angle at which light return back) (Figure 2-3). In UV reactor design reflective surfaces (e.g., Aluminium) can improve the UV light available for liquids treated. Also, UV light incident towards liquids treated is important to consider to minimize reflected light and maximize refracted light to be available for liquid foods (De Pree, 2010; Khan *et al.*, 2017).

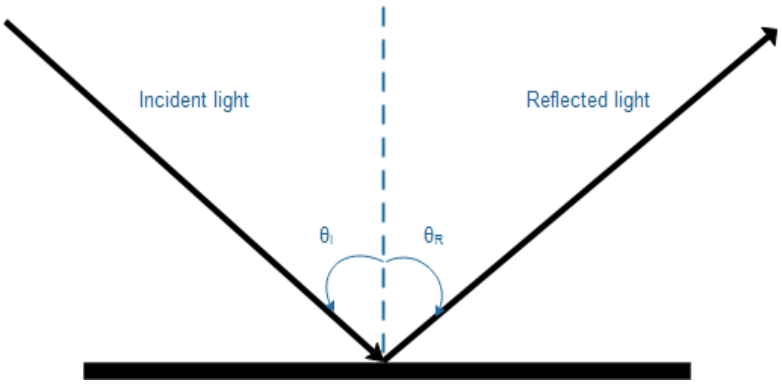


Figure 2-3: Reflection from an object

Refraction

The refraction occurs when light waves change direction as it passes through the medium (Figure 2-4). Refractive index is defined as the ratio of speed to light in vacuum to speed of light in the medium (De Pree, 2010; Khan *et al.*, 2017).

$$n = \frac{c}{v}$$

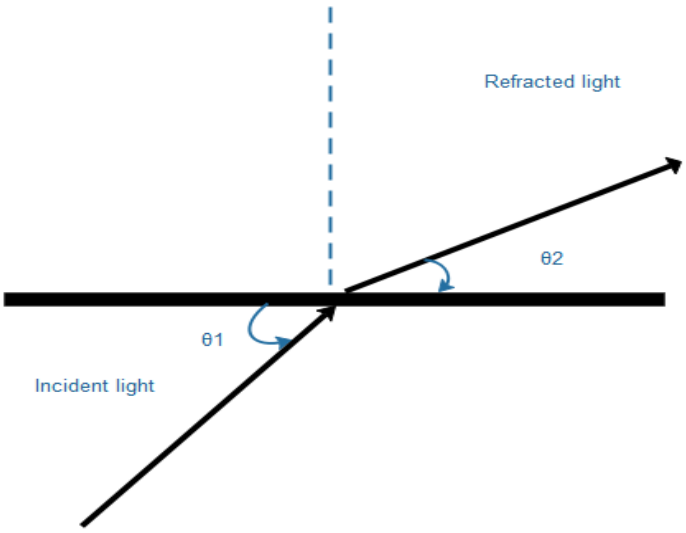


Figure 2-4: Refraction of light from a surface

This can also be mathematically explained as

$$n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$$

Where, θ_1 shows angle of incidence, θ_2 shows angle of reflection/refraction, n_1 is the index of incidence medium and n_2 is the index of refracted/ refracted medium.

Scattering

On exposure of UV light to liquids with suspended solids results in multiple light paths. This occurs as light scatters with the surface from suspended particles (Figure 2-5). Thereby, mixing can expose new layers to high luminous zone with liquids having high suspended solids (De Pree, 2010; Khan *et al.*, 2017).

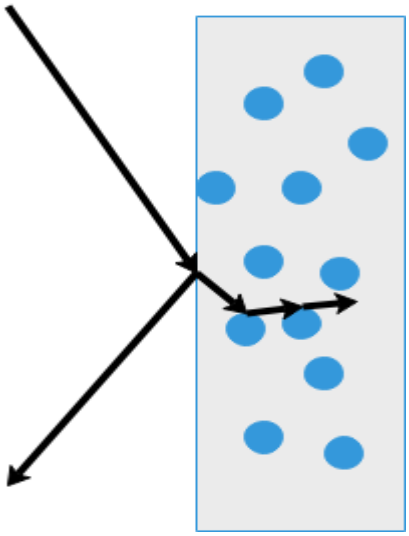


Figure 2-5: Incident light through a medium

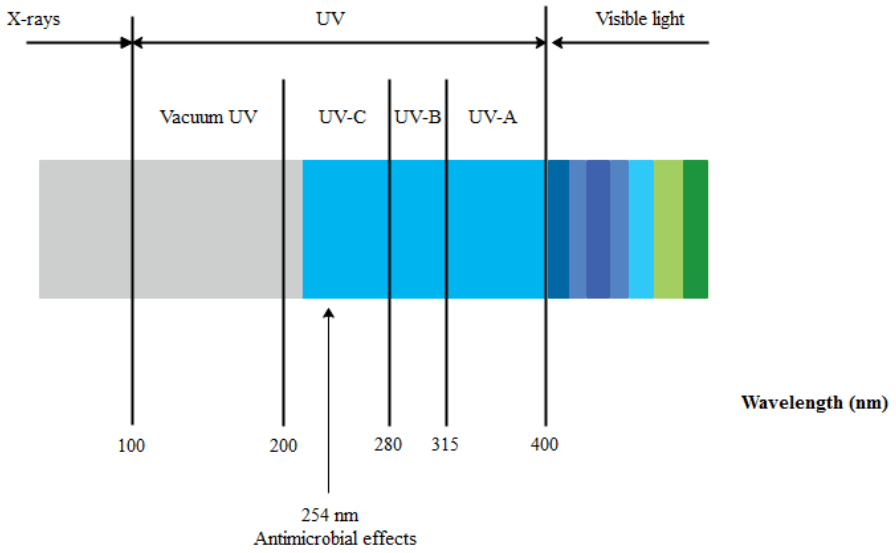


Figure 2-6: Spectrum of UV light (Adapted from Dai *et al.* (2012))

2.3.1. Mechanism of action

UV possesses broad spectrum that ranges from 100 nm to 400 nm. However, UV at 253.7 nm is highly effective against microorganisms. Amongst microorganisms (vegetative cells and spores), spores are highly resistant to UV and require high dosage for their inactivation (Datta *et al.*, 2015). UV-C radiation primarily targets genetic material of spore DNA and results in the formation of photoproducts (Gayán *et al.*, 2014a). Also, UV results in the formation of reactive oxygen species (ROS) that can attack DNA and proteins of microbial cell (Li *et al.*, 2016).

2.3.2. Effect on spore inactivation

The lethality of UV radiation is more pronounced in water and clear liquids in comparison to opaque liquids. Therefore, UV treatment of milk and other liquid dairy products is challenging because of its low transmission. However, many researchers have developed UV reactors that possess thin pathway to increase UV exposure to maximum level (Alberini *et al.*, 2015; Gayán *et al.*, 2014b; Koutchma, 2009; Koutchma *et al.*, 2009). One of the well-known commercially available process has been developed by Sure Pure AG, Zug, Switzerland which has been widely studied for its effect on microbial and physiochemical characteristics of milk. This turbulator imparts swirling motion and high turbulence to facilitate exposure of fresh layers to high UV luminous zone. Thus, it provides microbial inactivation while minimizing the quality damage (Cappozzo *et al.*, 2015). The developed hydrodynamic model suggests that coiled tube reactor is more effective to microbial inactivation in comparison to other UV units (Simmons *et al.*, 2012). With the dosage of up to 2 J/ml, UV-C treatment using thin film turbulent flow can cause 5-log reduction in *Listeria monocytogenes, Salmonella Senftenber, Escherichia coli and Staphylococcus aureus* (Crook *et al.*, 2015). However, even with lower dosage of 1.760 J/ml, sensory defects were observed in cow's milk (Rossitto *et al.*, 2012). Further, in comparison to vegetative cells, spores are quite resistant to UV and as at present, limited research has been done.

Table 2-4 represents the literature review on effect of UV radiation on different spores in liquid food products. Baysal *et al.* (2013) studied spores of *Alicyclobacillus acidoterrestris* DSM 3922 in grape and apple juice and obtained 5.5 and 2 log inactivation respectively. Particularly for dairy products, there are few research studies addressing spores inactivation. Choudhary *et al.* (2011) found 2.72 log reduction (skim milk) and 2.65 log reduction (raw milk) in

endospores of B. *cereus* by using coiled tube UV reactor providing pathway of 1.6 mm to liquid flow. In continuation of this, Bandla *et al.* (2012b) obtained 3.29 log reduction in soy milk by using coiled tube UV reactors.

Table 2-4: Effect of UV treatment on spores

Ultraviolet treatment				
Food	**Spores**	**Operating conditions**	**Log reduction**	**References**
Grape juice	*Alicyclobacillus acidoterrestris* DSM 3922	~400 mJ/cm^2 254 nm	5.5	Baysal *et al.* (2013)
Apple Juice			2	
Skim cow milk	*B. cereus* ATCC Perceptrol strain	11.187 mJ/cm^2 23-26 ^{o}C	2.72	Choudhary *et al.* (2011)
Raw cow milk			2.65	
Soymilk	*Bacillus cereus* ATCC Preceptrol strain	11.1 sec, 11.187 mJ/cm^2 253.7 nm 25-30 ^{o}C	3.29	Bandla *et al.* (2012b)
Peach Nectar	*Aspergillus flavus*	20.3 mJ/cm^2 25 ^{o}C	4	Flores-Cervantes *et al.* (2013)
	Aspergillus niger		3	
Citrate buffer	*Alicyclobacillus acidocaldarius* STCC 5137	23720 mJ/ml	3.24	Gayán *et al.* (2013)
	Bacillus cereus STCC 9818		2.93	
	Bacillus coagulans STCC 4522		2.25	
	Bacillus licheniformis STCC 4523		3.85	
	Geobacillus stearothermophilus STCC 12980		4.05	

Water, Used wash water from juice processing, Grape juice concentrate	*Alicyclobacillus acidoterrestris* K47 Spoiled grape	254 nm 500 mJ/ml	More than 4	Groenewald *et al.* (2013)
Water	*Bacillus subtilis* ATCC 6633	254nm 70 mJ/cm^2 660 mJ/ml.sec	3.5	Wang *et al.* (2010)
Sterile nanopure water	*Bacillus subtilis* ATCC 6633	254nm 90 mJ/cm^2	More than 5	Pennell *et al.* (2008)
Surface exposure	*Bacillus subtilis*	~60 mJ/cm^2	More than 4	McDonald *et al.* (2000)

Operational parameters/ Factors:

Transmittance/ Absorptivity coefficients:

The transmission of liquids to UV is considered as a key aspect in effectivity of Ultraviolet radiation. Increase in absorptivity of liquids (opaque liquids) resulted in a decline in lethality. Baysal *et al.* (2013) found that inactivation is directly related to absorption coefficient. Also, Gayán *et al.* (2013) studied the effect of different molar absorptivity on lethality of Ultraviolet with spores of *B. coagulans*. With UV treatment of 20 J/ml at 25 ºC, approx. 3 log reduction observed with citrate buffer (absorptivity coefficient 8.8 cm^{-1}) and less than 1 log with phosphate buffer (absorptivity coefficient 17.0 cm^{-1}). As the absorption coefficient increases, penetration for UV absorption decreases considerably, and therefore absorption coefficient is an important factor in designing UV reactor (Koutchma, 2009).

Thickness of treatment media:

Path length or thickness of media tends to play an important role in inactivation of microorganisms and spores, and an increase in path length can cause a decrease in inactivation rate (Koutchma, 2009). Bandla *et al.* (2012b) investigated different tube diameters to assess its effect on inactivation rate. In this work, PFA tubes (1.6 mm and 3.2 mm) were wrapped around UV lamp and soymilk was allowed to flow through UV reactor. On application of UV treatment using 1.6 mm diameter tube, a maximum log reduction of 5.6 in *E. coli* and 3.29 in *B. cereus* spores was obtained at a residence time of 11.3 s, (11.187 mJ/cm^2).

Dosage:

Different forms of microorganisms possess variation in sensitivity to Ultraviolet. While comparing dosages required for microbial inactivation, bacteria (vegetative cells) requires 25 J/m^2 in comparison to bacterial spores (220 J/m^2) (Datta *et al.*, 2015). As discussed earlier, spores require ultimately high UV dosage to inactivate them. Many papers in literature reported energy as J/m^2 for UV treatment since, UV radiation was used initially for surface sanitation purposes. For liquid treatment, UV treatment dosage can be reported as J/l as described by (Groenewald *et al.*, 2013).

Equation 2-1

$$\text{Dosage (J/l)} = \text{Total UV-C output power (W)} / \text{Flow rate ls}^{-1}$$

The delivered dosage of Ultra-violet light can be calculated by

Equation 2-2

$$D = I \times t$$

Where D= treatment dose (J/m^2) , I= Intensity or dosage rate (W/m^2) and t= contact time/retention time (Datta *et al.*, 2015).

However, it is important to consider dosage delivery that can be estimated using actinometric method (Müller *et al.*, 2017). This involves the treatment of iodometric solution with UV treatment as expressed by

Equation 2-3

$$8KI + KIO_3 + 3H_2O + hv \rightarrow 3I_3^- + 6OH^- + 9K^+$$

In comparison to thermal treatment, UV treatment uses much lower amount of energy to obtain same level of lethality. In a study, Tran *et al.* (2004) found that UV treatment required 2.0 kW h/m^3 to obtain the same results by thermal treatment with energy 82 kW h/m^3. Also, results from many studies showed lower energy consumption by UV (Bandla *et al.*, 2012b; Baysal *et al.*, 2013; Flores-Cervantes *et al.*, 2013; Gayán *et al.*, 2013; Groenewald *et al.*, 2013; Pennell *et al.*, 2008; Wang *et al.*, 2010).

2.3.3. Effect on quality

UV possesses a tremendous effect on microbial inactivation. However, there are some concerns with the use of UV for milk products as UV can result in photo-oxidative changes to milk and

affect the overall nutrition value. UV treatment can affect quality in the following ways (Koutchma *et al.*, 2009):

- UV light at wavelength of 254 nm has energy of 112.8 kcal/Einstein (Einstein---one mole of photons). Theoretically it can affect O-H, C-C, C-H, H-N, and S-S bonds if absorbed. It can result in dissociation into radicals, decomposition into molecular products, isomerization, dimerization, and ionization.
- Nucleic acids are the strongest 253.7 nm light absorbers. Only the purine and pyrimidine bases on the nucleic acid strands absorb, and the polymeric backbone does not absorb. At 253.7 nm, only compounds containing conjugated bonds, such as aromatic-ring and double-ring molecules might be affected in result of UV treatment.
- It is usually stated that the following nutrients are "light sensitive": vitamin A, carotenes, cyanobalamin (vitamin B12), vitamin D, folic acid, vitamin K, riboflavin (vitamin B2) tocopherols (vitamin E), tryptophan, and unsaturated fatty acid residues in oils, solid fats, and phospholipids. Thereby, UV can reduce these nutrients and might affect the overall nutrition value of the product.
- Aromatic amino acids (phenylalanine, tryptophan, and tyrosine) absorb UV strongly at 254 nm and might be affected.

2.3.4. Future studies

UV treatment possesses immense potential to inactivate vegetative cells and spores and thereby, can extend shelf life. However, its lower transmittance to opaque liquids makes it difficult to treat liquids like whey and skim milk. Thus, it is important to consider to improve design of UV reactor to maximize transmission properties. Also, there is a need to evaluate the use of UV in combination with heat or other emerging technologies to assess any sensitization effects from UV to subsequent treatment.

CHAPTER 3

Investigation of the use of ultrasonication followed by heat for spore inactivation

This chapter is based on the following publication

Ansari, J. A., Ismail, M., & Farid, M. (2017). Investigation of the use of ultrasonication followed by heat for spore inactivation. *Food and Bioproducts Processing, 104,* 32-39.

Preface

Conventional thermal sterilization is the most commonly used technique to inactivate microbial spores in low acidic liquid food products. In recent years, food researchers have investigated ultrasonication in combination with heat as an alternate technology for the reduction of microbial spores. However, the competitive advantage of this process over conventional thermal sterilization has not been properly assessed. In this study, energy delivered by ultrasound was used to provide the heating needed, which fulfils 55 % of the thermal energy requirement in addition to the effect caused by cavitation. The effect of pre-treatment with ultrasonication (20 kHz, 750 W) on decimal reduction time (D values) of *B. subtilis* spores ATCC 6633 was evaluated in three different suspending media (water, whole milk and rice porridge) and were compared with thermal treatment. Among these, pre-treatment with ultrasonication (114 µm, 1.1 W/ml, 5 min) of whole milk resulted in 35 % reduction in D value compared to thermal only treatment at 100^{o}C whereas under the same treatment conditions, water and rice porridge gave only 18 % and 4 % reduction respectively. These reduction in D values through the use of combined technology is minimal unless excessive ultrasonication is used, which is commercially not viable.

3.1. Introduction

In thermal sterilization, low acidic liquid food products are heated to very high temperatures to inactivate microbial spores and thereby preserve food. Prolonged exposure to high temperature (120 ^{o}C-140 ^{o}C) results in the deterioration of nutrition value, texture, colour and flavour of food. Over the last few years, numerous studies have been done to reduce the heat intensity during sterilization by using combination of thermal and emerging technologies (Raso *et al.*, 2003).

US has been found to possess antimicrobial effects in addition to its positive role in extraction (Chavan *et al.*, 2013; Duba *et al.*, 2015; Guerrouj *et al.*, 2016; Haque *et al.*, 2016; Kadam *et al.*, 2015; Piyasena *et al.*, 2003; Vinatoru, 2001), homogenization (Wu *et al.*, 2000), mixing, defoaming and emulsification (Delmas *et al.*, 2015; Feng *et al.*, 2010). Antimicrobial role of US was first discovered in anti-submarine warfare that resulted in harmful effects on sea life. Later on, research focuses on mechanism of ultrasonication for destruction of microorganisms (Earnshaw *et al.*, 1995).

Cavitation is the phenomenon that results in the production of microbubbles on exposure of a liquid to ultrasound waves. These microbubbles release high amount of energy and generates high pressure on collapse. The release of high amount of energy and pressure tends to provide microbial inactivation (Feng *et al.*, 2011). Another theory which explains the lethal effect of ultrasound suggests that sonication of any liquid, results in the formation of free radicals that attack DNA within microbial cells (Earnshaw *et al.*, 1995).

Microbial cells are sensitive to sonication treatment while sonication alone has only little effect on spores. However, some studies reported inactivation of spores when ultrasound was used in combination with heat (Evelyn *et al.*, 2015b; Garcia *et al.*, 1989), pressure (Raso *et al.*, 1998b) and chemicals (Sierra *et al.*, 1971). Table 2-1 shows an overview of literature found on ultrasonic inactivation of spores. It shows that ultrasonication was applied in three different ways, termed as pre-treatment with ultrasonication (controlled temperature) followed by heat, Thermosonication (ultrasonication and heat applied simultaneously) and Manothermosonication (ultrasonication, heat and pressure applied simultaneously). Pre-treatment with ultrasonication resulted in decreasing thermal decimal reduction time (D value) of *B. cereus, B. subtilis, B. licheniformis* and *B. stearothermophilus* spores (Burgos *et al.*, 1972; Ordoñez *et al.*, 1976; Palacios *et al.*, 1991). This heat sensitizing effect could be explained by the release of low molecular weight components from spores. It was observed that the release of calcium-dipicolinic acid (DPA), fatty acid, acyl glycerols and glycolipids from *B. stearothermophilus* spores on ultrasonication treatment resulted in subsequent reduction in heat resistance (Palacios *et al.*, 1991). Release of DPA could be considered as an important factor in reducing spores heat resistance, since DPA provides a protective effect for spore DNA (Black *et al.*, 2007; Douki *et al.*, 2005; Setlow, 2006b). Furthermore, thermosonication resulted in higher inactivation of spores thereby resulted lower D values in comparison to thermal only treatment (Evelyn *et al.*, 2015a, 2015b, 2015c; Garcia *et al.*, 1989; Milani *et al.*, 2016). The reason for higher inactivation could be thermal effects as well as cavitation effects. But, the effect of thermosonication diminished as it approaches boiling temperature (Garcia *et al.*, 1989). Also, manothermosonication possess higher inactivation with spores, utilizing shear and thermal stress with cavitation (Raso *et al.*, 1998b).

In all previous studies, experiments were designed to apply ultrasonication in ice bath or high temperature water bath to maintain constant temperature (Burgos *et al.*, 1972; Evelyn *et al.*, 2015b; Garcia *et al.*, 1989; Khanal *et al.*, 2014; Ordoñez *et al.*, 1976). These studies have not considered capturing the energy that was delivered by the ultrasonication equipment. In most

cases, ultrasonication resulted in a loss of large amount of energy which was not addressed due to its dissipation through the cooling mode. Ultrasonication is an energy efficient process and the energy delivered during ultrasonic treatment must be used to raise the temperature of the product to assist microbial inactivation without significant loss of energy. This could result in a more economical and energy efficient treatment (Hielscher, n.d.).

The objective of the work presented in this paper is to utilize the energy delivered by ultrasound for both raising product temperature and for the effects of cavitation needed to cause spore inactivation. The paper will question the benefit of using ultrasonication in combination with heat for sterilization.

3.2. Materials and methods

3.2.1. Media/Food matrix

In this study, double distilled water, whole milk (labelled composition: protein 3.5 g / 100 ml, fat 3.5 g /100 ml, carbohydrates 4.7 g/100 g) and rice porridge were selected as suspension media. Double distilled water was sterilized and cooled before inoculation with spores. UHT whole milk was purchased from a local supermarket. Rice porridge was prepared as described by Evelyn *et al.* (2015b) with slight changes. Raw Jasmine rice (labelled composition: protein 8.2 g/100 g, fat 1 g/100 g, carbohydrates 77.3 g/100 g, dietary fibre 1.1 g/100 g) was boiled with sterilized water (6.25% w/w). Once, rice was cooked, it was cooled and blended to obtain rice porridge.

3.2.2. Microbiological studies

3.2.2.1.*B. subtilis* spore preparation and sporulation

B. subtilis ATCC 6633 was chosen as it is considered to be a biological indicator for validation of moist heat sterilization (Pharmacopeia., 2016). *B. subtilis* ATCC 6633 was obtained from Fort Richard Laboratories (New Zealand). The original freeze-dried culture was initially transferred to cooked meat medium (Fort Richard Laboratories, New Zealand) and incubated for 7 days at 35 ^{o}C. The culture was then plated on tryptic soy agar (TSA) plates and incubated at 30 ^{o}C for 24 h.

From TSA plates, multiple colonies were transferred to nutrient broth by using sterile water and incubated at 37 ºC for 48 h with shaking. Aliquots of 0.1 ml of nutrient broth were spread plated on sporulation medium and incubated for 13-15 days at 30 ºC. The sporulation medium was prepared by using 23 g nutrient agar powder (DIFCO), 1 mg Manganese sulphate (Merck) and 0.5g Calcium chloride (RDH). Sporulation was monitored by using phase contrast microscopy (Motic Microscope BA 410 Series, Canada) as described by Evelyn *et al.* (2015b). After ~75% sporulation within two weeks, spores were harvested by flooding the sporulated agar plate with 3 ml sterile distilled water and scraping the colonies with sterile loop spreader. Collected suspensions were centrifuged three times at 1600 rpm (298 g) for 15 min at 4 ºC to obtain spore pellets. After each centrifugation cycle, supernatant was discarded and pellets were washed with sterile water. After centrifugation, resultant pellets were suspended in sterile water to obtain a suspension of ~ 1×10^{10} spores / ml as determined by plate count. This suspension was heated to 80 ºC for 10 min in order to inactivate vegetative cells and stored at 4 ºC until use. An intermediate spore suspension (~10^{8-9} / ml) was prepared by dilution of stock suspension.

For experiments, a portion of intermediate spore suspension (1 ml) was inoculated in 99 ml of water, whole milk, rice porridge separately and mixed thoroughly to obtain a final concentration of ~10^{6-7} spores /ml.

3.2.2.2.Enumeration of spores

B. subtilis spore concentrations before and after treatments were determined by spread plating into plate count agar by making appropriate dilutions and incubating at 30 ºC for 24-48 h.

3.2.3. Experimental design and data analysis

In the first part of the experiments, thermal inactivation of *B. subtilis* spores was measured in water, whole milk and rice porridge suspension. In the second part, ultrasonication was applied on spore suspension of *B. subtilis* (water only) at two different amplitudes (45 µm, 114 µm) and the temperature increase over time was recorded (as shown in Figure 3-1). Heat inactivation of spores were determined as described in section 3.2.3.1. In the third part, water, whole milk and rice porridge suspension containing *B. subtilis* spores were pretreated with most appropriate conditions of ultrasonication followed by heat and the decimal reduction times were measured.

3.2.3.1. Studies on thermal inactivation of spores

Heat resistance of spores in water and whole milk (treated or control) were determined by using capillary tube method (Franklin *et al.*, 1958; Stern *et al.*, 1954; Van Zuijlen *et al.*, 2010). Glass capillaries (50 µl) were sealed from one side by using Bunsen burner and were sterilized. A sterile micro syringe was used to fill inoculated samples (water or whole milk) into a capillary tube while the other side of the tube was sealed carefully to avoid any heat effects. Capillary tube method was used due to its negligible come up time during treatment (Block, 1977).

For thermal treatment, the capillary tubes were immersed in an oil bath heated to different time intervals depending on temperature range (80 -120 ^{o}C) studied. After thermal treatment, the capillary tubes were immediately placed in an ice bath to drop the temperature rapidly and then washed with ethanol and sterile water. Afterwards, tubes were clipped off from both ends and flushed with sterile water under aseptic conditions. Microbial count was done as described in section 3.2.2.2.

Thermal inactivation of spores inoculated in rice porridge was determined as described by Evelyn *et al.* (2015b). In this case, capillary method was not used, as it was not possible to insert the rice porridge into the capillary tube due to its high viscosity. One ml of spore suspension was packed in laminated plastic bags and sealed by thermosealer (Multivac C200, Germany). Heat treatment was provided for different time intervals at temperatures between 80-100 ^{o}C.

Survival curves were obtained by plotting logarithmic spore reduction against time at specific temperatures. For each specific temperature, approx. one to two logarithmic reduction was obtained from log (N/N_o) vs time plots to determine D values. Also, z-values were obtained by plotting thermal death time curves i.e. logarithmic D values against temperature (Garcia *et al.*, 1989).

3.2.3.2.Ultrasonication in combination with heat

Immediately after ultrasonication treatment, spore suspension was transferred to a capillary tube for thermal studies. Thermal inactivation was determined as described in section 3.2.3.1.

Ultrasound equipment

A 750 W ultrasonic unit (Sonics & materials, Inc., USA) that operates with a frequency of 20 kHz was used in the experimentation. The unit could be operated up to 100% amplitude (114

μm) by using probe SM-0220. The standard horn (0.5 inch or 13mm) is of Titanium Alloy Ti-6Al-4V and is capable of treating 250 ml of sample.

Ultrasonic treatment of sample

Spore suspension (100 ml) was treated by using ultrasonication unit in a beaker using continuous mode to achieve rapid increase in temperature. Prior to ultrasonication, the probe was rinsed with ethanol followed by sterilized water to sanitize it. During ultrasonication, beaker was placed in a polystyrene container to minimise heat losses and a thermocouple was used to record the temperature profile against time. Initial temperature, final temperature, treatment time, and ultrasound amplitude were recorded.

3.2.3.3. Specific energy requirements during ultrasonication and thermal studies

For thermal studies, sensible heat gained by the sample was calculated as follows:

Equation 3-1

$$Q = mC_p\Delta T$$

Where, Q is the thermal energy required to raise the temperature, m is the mass of the sample, C_p is the specific heat capacity and ΔT is the increase in temperature. The sensible heat was calculated for spore suspension, beaker and Ultrasonication probe. The sum of sensible heat (suspension, beaker, ultrasonication probe) was considered for calculation of efficiency.

For ultrasonication, the thermal energy conversion efficiency (η) can be calculated as follows.

Equation 3-2

$$\eta = \frac{Q}{P \cdot t} \times 100$$

Where, P is the power input (W/ml), Q is the thermal energy output (J/ml) and t is the time (sec).

3.2.4. Statistical analysis

Microsoft Excel 2013 (Microsoft Inc., USA) was used for statistical treatment of data. Tukey's test with a confidence level of 95% was performed to compare results of thermal treatment and ultrasonication pretreatment with thermal. Survival curves for spores were plotted and

regression coefficient (R^2) values were used to examine linearity. In addition, mean and standard deviation for replicates were calculated using Excel 2013.

3.3. Results and discussion

3.3.1. Thermal inactivation of spores

Table 3-1 shows the D value of *B. subtilis* spores in water, whole milk and rice porridge after thermal only treatment. As expected, the D values decreased with increasing temperature.

Table 3-1: Thermal resistance of *B. subtilis* ATCC 6633 spores (Data points are mean ± standard deviation of replicates ($n \geq 3$). First order kinetic models have been applied to fit experimental data. The Regression coefficient (R^2) obtained is given in brackets

Media	Temperature (°C)	D value (min) Thermal only
Water	80	140.20 ± 22.13 (0.97)
	90	16.16 ± 3.87 (0.85)
	100	3.01 ± 0.36 (0.97)
	110	0.34 ± 0.09 (0.97)
	120	0.10 ± 0.01 (0.97)
Whole milk	80	321.39 ± 16.50 (0.99)
	90	38.95 ± 2.16 (0.99)
	100	4.51 ± 0.63 (0.91)
	110	0.59 ± 0.03 (0.99)
	120	0.09 ± 0.01 (0.9)
Rice Porridge	80	145.28 ± 2.24 (0.97)
	90	48.04 ± 0.82 (0.99)
	100	4.97 ± 0.74 (0.96)

The resulting D values for water ranged from 140 min (80 °C) to 0.10 min (120 °C), for whole milk, it ranged from 321 min (80 °C) to 0.09 min (120 °C) and for rice porridge, it ranged from 145 min (80° C) to 4.97 min (100 °C). D values published in literature varies with the strain studied (Garcia *et al.*, 1989; Georget *et al.*, 2014a; Siemer *et al.*, 2014). In this study, D values obtained for water were similar to the values reported by Georget *et al.* (2014a) for PBS buffer containing *B. subtilis* PS832 but was lower than the values reported by Garcia *et al.* (1989) for *B. subtilis* var *niger*-40. Furthermore, higher D values were observed for whole milk in

comparison to water but the difference diminished as treatment temperature increased. This trend was similar to that found by Garcia *et al.* (1989), where they obtained higher D values for whole milk than water. This could be explained by the protective effect of fat on microorganisms (Senhaji, 1977).

3.3.2. Thermal inactivation of spores after pre-treatment with Ultrasonication

3.3.2.1. Effect of temperature and amplitude

The ultrasonication pre-treatment of water inoculated with *B. subtilis* spores was carried out at different amplitudes that resulted in different final temperatures. Table 3-2 gives the conditions obtained during treatment at two different amplitudes of 45 µm and 114 µm.

Table 3-2: Operational parameters used during ultrasonication of water

Operating Parameters	**Ultrasonication Amplitude**	
	45 µm	**114 µm**
Initial Temperature (°C)	21.8	20.4
Final Temperature (°C)	70.3	75.1
Temperature increase (°C)	48.5	54.7
Thermal Energy (J/ml)	203	229
Power input (W/mL)	0.3	1.1
Time (min)	17.6	5
Efficiency (%)	87.22	94.53

Ultrasonication energy has increased the temperature by approx. 50-55 °C. Figure 3-1 shows the temperature–time profile at the two different amplitude settings. At lower amplitude of 45 µm (40 %), a time of 18 min was taken to increase temperature to 70 °C from 20°C. While increasing the amplitude to 114 µm (100 %) resulted in a rapid temperature increase, reaching 75 °C within 5 min only. This shows that an increase in amplitude increases the applied power during sonication and the power values at 45 µm and at 114 µm amplitude were 0.3 W/ml and

1.1 W/ml respectively. Similar observations were shown by Khanal *et al.* (2014), who obtained higher power at increased amplitudes. On the other hand, many studies applied sonication by maintaining low or high temperatures using water bath (Burgos *et al.*, 1972; Evelyn *et al.*, 2015b, 2015c; Garcia *et al.*, 1989; Khanal *et al.*, 2014; Ordoñez *et al.*, 1976) and have not utilized the energy delivered by ultrasound for temperature increase. Higher amplitude also resulted in increasing the thermal conversion efficiency as well.

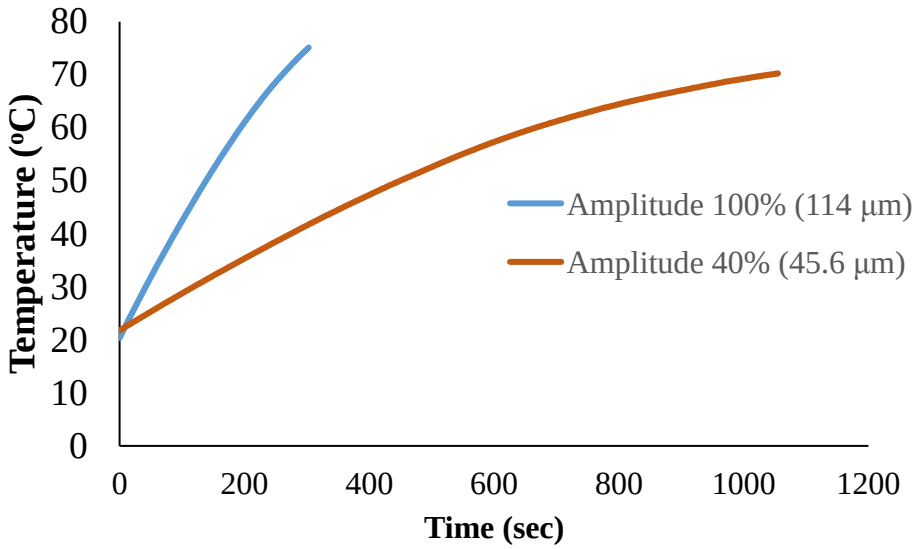


Figure 3-1: Temperature profile during Ultrasonication of water at different amplitudes (40 % and 100 %)

Therefore, in this study, energy delivered by ultrasonication was not only used to provide cavitation effects but also used to capture the heat generated, which is needed to assist microbial inactivation. It was done with the intention of obtaining a synergistic effect on the sterilization process where ultrasonication was followed by heat shock. This effect was investigated for distilled water that was pre-treated with ultrasonication at different temperatures and amplitudes by measuring the D values of *B. subtilis* spores and then compared with thermal only as given in Table 3-3.

Pre-treatment with ultrasonication has resulted in slightly lower D values compared to thermal only (values are significantly different $p < 0.05$). However, ultrasonication at a higher amplitude of 114 µm resulted in similar D values in comparison to lower amplitude while consuming less time (values are not significantly different $p > 0.05$). However, a marked increase in inactivation was observed by Raso *et al.* (1998b) on their study with *B. Subtilis*

spores using manosonication (20 kHz, 300 kPa, 70 ºC, 12min). They reported that inactivation of spores increased from 75 to 99.9 % when the amplitude was increased from 90 to 150 µm. This indicates that effect of amplitude is higher when applied with pressure.

Table 3-3: Comparison of D values of water with *B. subtilis* spores (ATCC 6633) at different thermal treatment temperatures (Data points are mean ± standard deviation ($n \geq 3$). First order kinetic models have been applied to fit experimental data. The Regression coefficient (R^2) obtained is given in brackets

D values (min)			
Temperature (ºC)	**Thermal only**	**Pre-treatment with ultrasonication followed by heat**	
		45 µm	**114 µm**
100	3.01 ± 0.36 (0.97)	2.51 ± 0.08 (0.99)	2.48 ± 0.19 (0.99)
105	1.17 ± 0.09 (0.90)	0.87 ± 0.06 (0.85)	0.76 ± 0.18 (0.99)
110	0.34 ± 0.09 (0.97)	0.18 ± 0.03 (0.90)	0.22 ± 0.06 (0.85)
115	0.16 ± 0.02 (0.84)	0.11 ± 0.02 (0.87)	0.11 ± 0.01 (0.85)

3.3.2.2. Effect of media

The study on the effect of ultrasonication pre-treatment (114 µm, 5 min, 1.1 W/ml) was extended to whole milk and rice porridge and their log reduction values together with that of water at 100 ºC are given in Figure 3-2. The log reduction of spores in milk was 1.1 log for thermal treatment and 1.8 for thermal treatment combined with ultrasonication treatment. Also, a similar set of readings for water and rice porridge show minor advantage of this combined technology. Therefore, this slight difference in log reduction is not sufficient to justify the pre-treatment with ultrasonication. On the other hand, Evelyn *et al.* (2015b) studied inactivation of psychotropic *B. cereus* spores after thermosonication (0.33 W/ml, 210 µm, 1.5 min, 70 ºC) in different media including skim milk, cheese slurry, beef slurry and rice porridge to investigate product quality during refrigerated storage. They obtained distinctly different log reductions; lowest in skim milk (0.3-0.4), but had obtained greater than 3 log reductions in beef slurry, rice porridge and cheese slurry. Evelyn *et al.* (2015b) explained the difference in inactivation of spores is due to the solid content in different media. Moreover, higher inactivation in beef slurry, rice porridge and cheese slurry could be because they tested on inactivation of heat sensitive microorganisms, which was psychrotrophic *Bacillus cereus*.

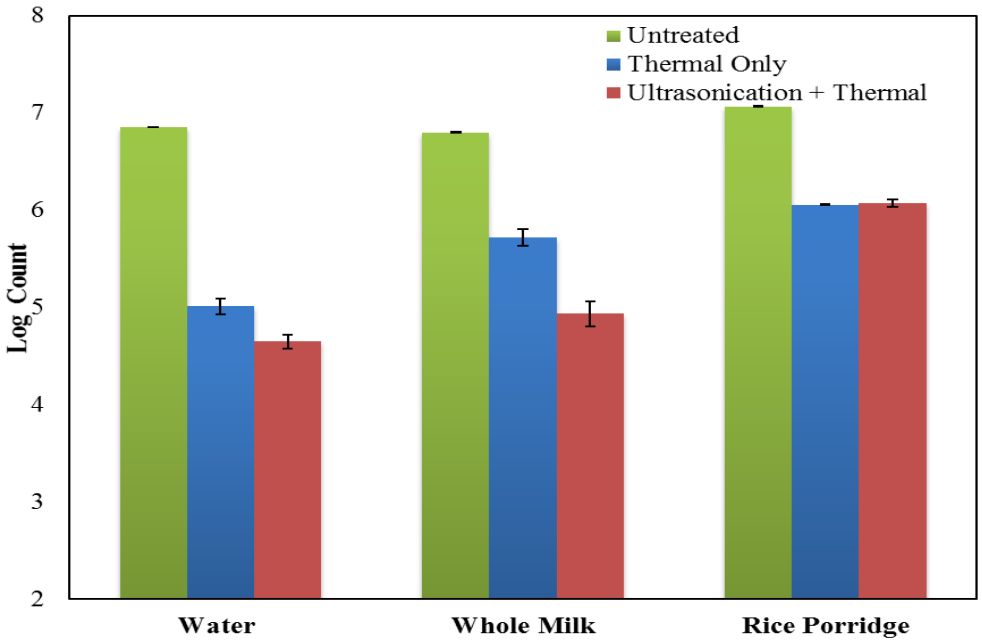


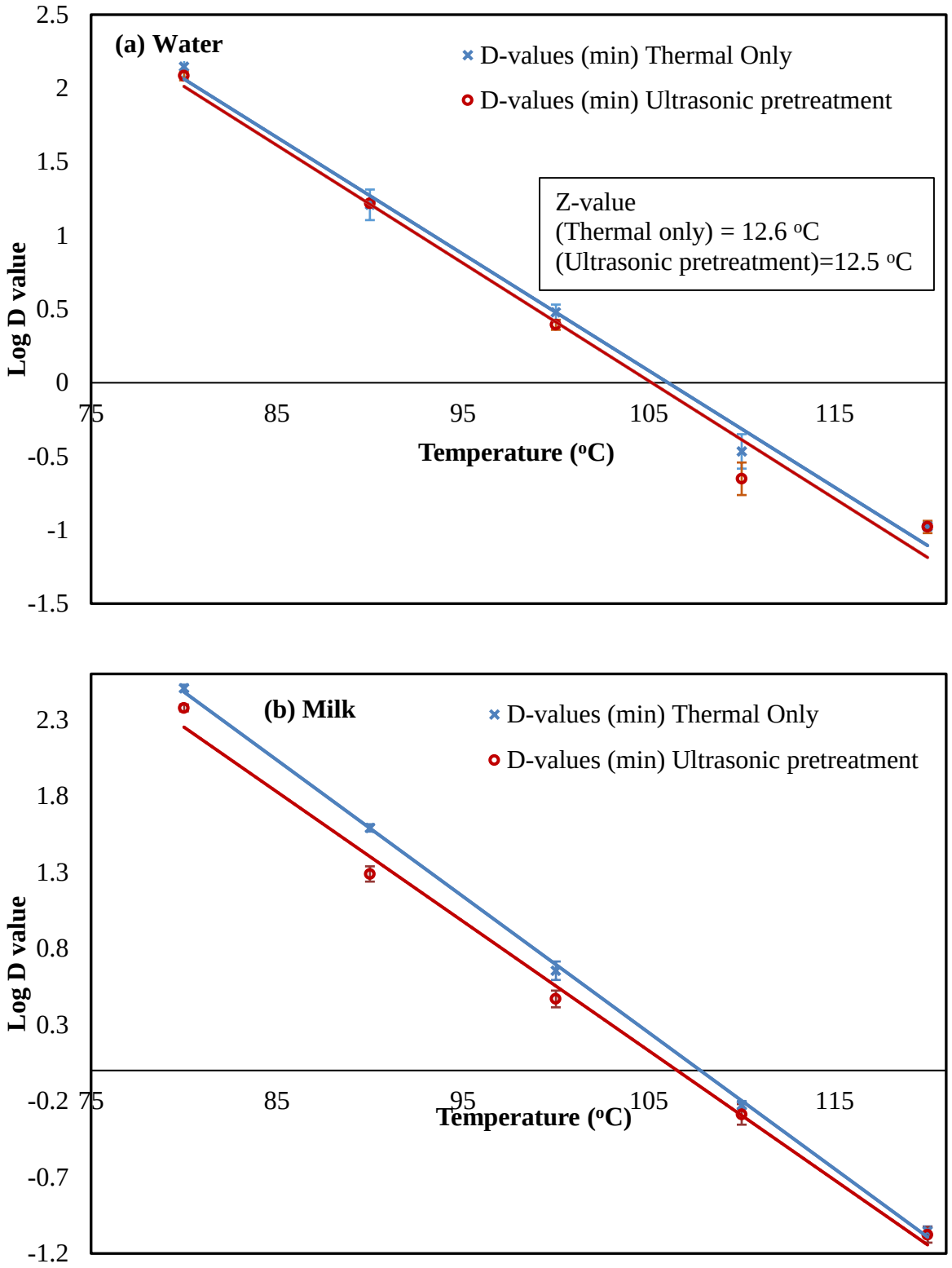
Figure 3-2: *B. subtilis* spores (ATCC 6633) log reduction in different media after thermal treatment at 100 ^{o}C for 5 min and Ultrasonication pre-treatment (1.1 W/ml, 114 µm, 5 min, 100 ml) followed by thermal treatment at 100 ^{o}C for 5 min. (error bars are standard deviation ($n \geq 3$))

Furthermore, the log reductions on different media were carried out at varied temperatures between 80 ^{o}C and 120 ^{o}C and the calculated D values after ultrasonication pre-treatment with higher amplitude of 114 µm are given in Table 3-4. D values for whole milk at 100 ^{o}C was reduced from 4.51 min for thermal only treatment to 2.94 min on ultrasonication pre-treatment which is a 35 % reduction. This result is consistent with the study by Garcia *et al.* (1989), who obtained approx. 43 % reduction in D_{100C} (from 11.36 to 6.84 min) with *B. subtilis* var. *niger*-40 after ultrasonication pre-treatment for whole milk.

Table 3-4: Thermal resistance of *B. subtilis* ATCC 6633 spores after Ultrasonication pre-treatment (1.1 W/ml, 114 µm, 5 min). (Data points are mean ± standard deviation ($n \geq 3$). First order kinetic models have been applied to fit experimental data. The Regression coefficient (R^2) obtained is given in brackets

Media	**Temperature (ºC)**	**D values (min) of pretreatment by ultrasonication to 75ºC followed by heat**
Water	80	121.57 ± 8.58 (0.99)
	90	16.50 ± 0.06 (0.98)
	100	2.48 ± 0.19 (0.99)
	110	0.22 ± 0.06 (0.85)
	120	0.11 ± 0.01 (0.94)
Whole milk	80	238.30 ± 12.40 (0.96)
	90	19.42 ± 2.24 (0.97)
	100	2.94 ± 0.37 (0.96)
	110	0.52 ± 0.08 (0.99)
	120	0.08 ± 0.01 (0.97)
Rice Porridge	80	128.24 ± 2.99 (0.98)
	90	47.43 ± 1.27 (0.98)
	100	4.78 ± 0.19 (0.81)

The log D vs temperature curves as shown in Figure 3-3, demonstrates further the temperature dependency of *B. subtilis* spore inactivation in different media. The z values calculated from log D vs. temperature plots before and after ultrasonication pretreatment for water were not significantly different ($p > 0.05$). However, a slight increase was observed in z-value after ultrasonication pretreatment (values are significantly different $p < 0.05$) for milk. These results show that ultrasonication pretreatment does have little or no effect on z-values. Also, the study of Ordoñez *et al.* (1976) with US (20 kHz, 10 min, volume 5 ml) observed no change in z-values of *B. subtilis* 189 spores, when ultrasonication was applied. This further shows that pretreatment with ultrasonication followed by heat as carried out at a range of temperatures had no significant effect.


(a) Water
D-values (min) Thermal Only
D-values (min) Ultrasonic pretreatment
Z-value
(Thermal only) = 12.6 ºC
(Ultrasonic pretreatment)=12.5 ºC
Log D value
Temperature (ºC)
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
75
85
95
105
115
(b) Milk
D-values (min) Thermal Only
D-values (min) Ultrasonic pretreatment
Log D value
Temperature (ºC)
2.3
1.8
1.3
0.8
0.3
-0.2
-0.7
-1.2
75
85
95
105
115

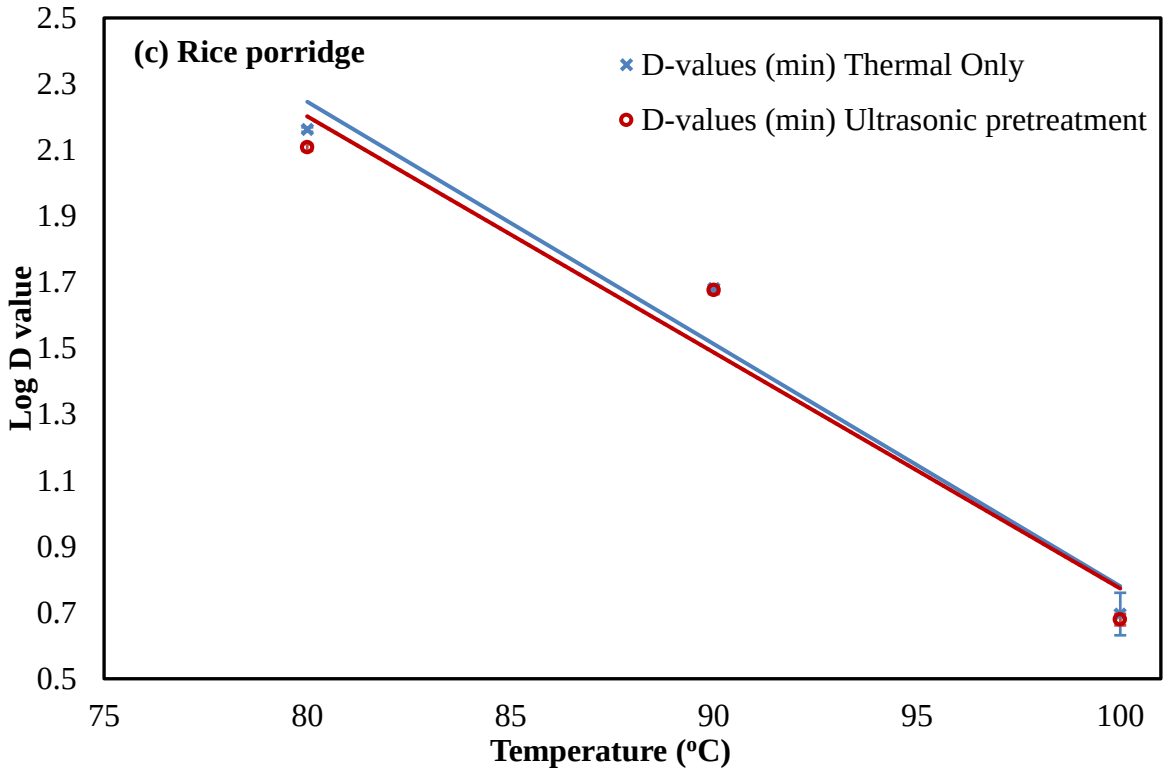


Figure 3-3: Thermal vs. ultrasonication pre-treatment (114 µm, 1.1 W/ml) followed by thermal (a) water (b) milk (c) rice porridge. Results are expressed as mean ± standard deviation (n ≥ 3)

3.3.2.3. Energy requirement in Ultrasonication process

Energy consumption is a significant factor in designing any commercial sterilization process. In this study, ultrasonication was utilized to pre-heat the sample to 75 °C followed by additional heat treatment. For example, the experimental run of microbial inactivation at 120 °C, ultrasonication has provided 229 J/ml in 5 minutes (1.1 W/ml) to increase temperature to 75 °C from 20 °C while thermal treatment has consumed a further 188 J/ml to achieve 120 °C. Total energy required was 417 J/ml. This shows that 55 % of it has been obtained from ultrasonication itself, a process which is energy efficient. On the other hand, many studies applied high amount of energy during ultrasonication and ignored the energy consideration (Burgos *et al.*, 1972; Khanal *et al.*, 2014; Ordoñez *et al.*, 1976). Khanal *et al.* (2014) applied high energy (approx. 2207 J/ml) during ultrasonication pretreatment (amplitude 91.2 µm for 10min). This is very excessive energy compared to thermal treatment but the use of ice bath cooling has hidden this fact (energy consumption). In the study of Khanal *et al.* (2014), approx. 2459 J/ml was applied to achieve 0.559 log reduction in spores of *B. coagulans* ATCC 12245, 0.42 log reduction in *B. licheniformis* ATCC 6634 and 0.73 log reduction in *G. stearothermophilus*. Similarly, Burgos *et al.* (1972) and Ordoñez *et al.* (1976) applied

ultrasonication using ice bath into a small volume of sample. Furthermore, Milani *et al.* (2016) confirmed that ultrasonication is an energy intensive method to inactivate *Saccharomyces cerevisiae* ascospores in beer. To achieve 2.5 log reduction of ascospores 2612 J/ml was required by thermosonication in comparison to 188.8 J/ml by thermal energy.

3.4. Conclusion

This study investigated the potential of using ultrasonication in combination with heat as a sterilization technology. The results presented in this work offer a new perspective to apply ultrasonication not only to create the well-known cavitation effect but also to utilise the ultrasound energy in increasing the temperature of the treated sample. However, microbiological studies with *Bacillus subtilis* spores showed only a slight advantage in spore inactivation (D values) in water, whole milk and rice porridge. Therefore, the slight reduction in D values found through the application of sonication in combination with heat may not be justifiable to apply this combination of technology in industry. This study has shown that, it is necessary to consider energy requirements carefully in any new process development such as the one considered here for microbial spore inactivation.

CHAPTER 4

Investigate the efficacy of UV pretreatment on thermal inactivation of *B. subtilis* spores in different types of milk

This chapter is based on the following publication

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Preface

Shortwave ultraviolet (UVC) radiation is commonly used for sterilization of drinking water. However, its low transmittance within opaque liquids limits its use for milk and other liquid food products. The objective of this study is to assess the efficacy of UV pretreatment on thermal inactivation of *B. subtilis* spores in different types of milk. In this work, UV treatment was applied by using a coiled tube reactor with a perfluoroalkoxy tube coiled around a quartz sleeve containing 254 nm UV lamp. It was observed that UV pretreatment (D_{Act} 2.37 ± 0.126 J/ml) combined with thermal treatment at 110 °C for 30 sec resulted to approximately 6 log reduction in skimmed cow milk, 2.89 log reduction in whole cow milk and 1.10 log reduction in sheep milk. The results show that UV can be a good alternative to sterilization of skim milk at lower temperatures compared to ultra high temperature (UHT) treatment (135 °C, 3 sec).

4.1. Introduction

In thermal sterilization (e.g., UHT), low acid liquid food products are heated at high temperatures to inactivate thermophilic microbial spores in order to produce a shelf stable product. Commonly, *B. subtilis*, *Clostridium sporogenes*, *Bacillus coagulans* and *G. stearothermophilus* spores are used for validation of sterilization processes (Pharmacopeia., 2016). A 6-log reduction is required to assure sterilization and referred as "Sterilization assurance level (SAL)" (Mosley, 2008). However, exposure to high temperature (130 -140 °C) even for shorter time, results in the deterioration of nutritional value, texture, colour and flavour of food (Cappozzo *et al.*, 2015; Popov-Raljić *et al.*, 2008; Renner, 1988; Van Boekel, 1998). Numerous studies have been conducted to reduce the heat intensity during sterilization by using a combination of thermal and non-thermal preservation methods (Li *et al.*, 2016). The use of UV (Ultraviolet) as non-thermal technology has been explored for its antimicrobial effects (Bandla *et al.*, 2012b; Baysal *et al.*, 2013; Gayán *et al.*, 2013; Keyser *et al.*, 2008). In a recent decision from EU commission, UV treated (1045 J/l) milk was approved to obtain extended shelf life milk (Poulsen, 2016). This shows that with improved design of reactors, UV could also have the potential to achieve sterilization of milk. Moreover, UV is a technology that could be operated in a continuous manner and consumes low energy; making it suitable for commercial processing.

UV possesses broad electromagnetic spectrum between 100 nm to 400 nm, which is mainly classified into UV-A (320-400 nm), UV-B (280-320 nm), UV-C (200-280 nm) and vacuum

UV (100-200 nm). UV-C, specifically at 253.7 nm was found to be most effective in terms of microbial inactivation (Coohill *et al.*, 2008) and is attributed for its high germicidal action against bacteria, virus and fungal organisms (Guerrero-Beltrán *et al.*, 2004; Roig-Sagués *et al.*, 2018; Tran *et al.*, 2004). In addition to vegetative cells, UV possesses a great potential to inactivate bacterial spores (Bandla *et al.*, 2012b; Choudhary *et al.*, 2011; Gayán *et al.*, 2013; Pennell *et al.*, 2008; Wang *et al.*, 2010). UV mainly targets the DNA of spores to produce pyrimidine dimers that are known as spore photoproducts (SP) (Moeller *et al.*, 2007). These dimers inhibit the transcription and replication process of DNA, resulting in cell death (Friedberg *et al.*, 2005). However, Setlow (2006b) speculates the presence of a spore repair mechanism which arises due to the presence of an enzyme within the spore photoproduct called 'spore photoproduct lyase'(SPL) that could reverse pyrimidine dimers, after UV treatment. Thereby, this repair mechanism could create an increased resistance of spores to UV.

UV has a major limitation to penetrate within opaque liquids which could result in no or very low microbial inactivation (Datta *et al.*, 2015). In order to increase penetration, researchers have developed and used different designs of UV reactors. These UV reactors involve thin film flow reactors, turbulent flow reactors and coiled tube reactors (Dean, 1927; Gayán *et al.*, 2014b; Koutchma *et al.*, 2009; Tran *et al.*, 2004; Ye *et al.*, 2008). These reactors were designed primarily to reduce UV path length, thereby improve its treatment efficacy. The increase of turbulence is another way of improving exposure to UV radiation. The key processing factors in designing a UV-C treatment system are exposure time, UV dosage, Reynold's number, i.e., turbulence and UV transmittance of the liquid food. The limited transmission of UV through opaque liquids and the tendency of spores to get repaired after UV treatment, suggest the need for a combination of UV with other technology.

Studies on UV inactivation of spores using continuous flow reactors are scarce. Bandla *et al.* (2012b) reported 3.29 log reduction in *B. cereus* spores (ATCC Perceptrol® strain) inoculated in soymilk using a coiled tube reactor. Similarly, Choudhary *et al.* (2011) reported 2.72 log reduction of *B. cereus* spores in skimmed cow milk and 2.65 log reduction in raw cow milk using a coiled tube reactor. Gayán *et al.* (2013) reported that *Bacillus coagulans* (STCC 4522), *B. cereus* (STCC 9818), *Alicyclobacillus acidocaldarius* (STCC 5137), *Bacillus licheniformis* (STCC 4523) and *Geobacillus steraothermophilus* (STCC 12980) spores were reduced by 2.25, 2.93, 3.24, 3.85 and 4.05 log respectively in citrate buffer using an annular thin film flow reactor. They showed that UV pretreatment sensitized *B. coagulans* (STCC 4522) spores making its inactivation in subsequent thermal treatment more effective.

B. subtilis is an obligate aerobe that possesses the capability to produce enzymes that affect the quality of milk (Gopal *et al.*, 2015). It is also resistant to both UV and thermal treatments (Datta *et al.*, 2015) and used for validation of sterilization processes (Pharmacopeia., 2016). On the other hand, milk is a medium that is difficult to treat with UV because it possesses high absorbance and hence UV alone cannot achieve sterilization. However, Gayán *et al.* (2013) reports that a combination of UV with heat may improve spore inactivation in citrate phosphate buffer. Nevertheless, to the best of our knowledge, the use of UV followed by heat has not been studied for inactivation of spores in milk. Therefore, it is the objective of the work presented in this paper to evaluate the effect of combined UV and heat to inactivate *B. subtilis* spores in different types of milk.

4.2. Materials and methods

4.2.1. Media/Food matrix

Double distilled water, skimmed cow's milk (SCM), whole cow's milk (WCM) and sheep milk (SM) were selected as suspension media. SCM and WCM were obtained in liquid form in 1 L aseptic packs purchased from local supermarket. Sheep milk was provided by Spring Sheep Company in a powder form and reconstituted to 1: 6 ratio with sterilized distilled water as per manufacturer's instructions. The fat contents labelled on packaging was 0.1 % in SCM, 3.5 % in WCM and 5.24 % in SM after reconstitution. These different types of milk products (SCM, WCM and SM) that has different total solids and fat contents were chosen to analyse antimicrobial effect of UV treatment.

Physical properties of milk:

Physical properties (total solids, pH, viscosity, Density and absorption coefficient at 254 nm) of SCM, WCM and SM were measured.

Total solids of SCM, WCM and SM was obtained accordingly by AOAC method 990.19. Aluminium dishes were pre-dried in oven at 100 ^{o}C for 2 h and stored in desiccator. Milk sample (3 g) was weighed in the dried aluminium dishes and placed in water bath at 38 ± 1 ^{o}C for 25 min and oven dried at 100 ± 1 ^{o}C for 3 h. Total solids (%) was calculated as

Equation 4-1

$$Total\ solids\ (\%) = \frac{(W_2 - W_1) - B}{Sample\ weight} \times 100$$

Where, W_2 is mass in gram of dish + dried milk, W_1 is the mass of empty dish and B is blank (difference of empty aluminium plate before and after drying)

The pH of milk products was measured by immersing pH electrode in samples under constant reading. Prior to measurements, pH was calibrated against standard solution (pH 4, pH 7, and pH 10). The viscosity of milk samples was measured by using Rheometer (AR-G2, TA instrument, Texas, USA). Milk sample was transferred to a suitable sample container and a shear rate of 100 s^{-1} was applied. The viscosity was determined by plotting the shear stress against shear rate. The density of milk products was measured by using relative density bottle.

Absorption coefficients of different milk products were measured through UV-Vis spectrophotometer (Perkin Elmer Lambda 35) at 254 nm by diluting 1 ml of milk with 99 ml of distilled water (Bandla *et al.*, 2012b). The absorption coefficient was calculated by taking the ratio of obtained absorption values at 254 nm to the path length of quartz cuvette (1 cm).

4.2.2. Microbiological studies

4.2.2.1. Bacterial strains and sporulation

B. subtilis (ATCC 6633) was obtained from Fort Richard laboratories, New Zealand. *B. subtilis* (ATCC 6633), freeze-dried culture was added to cooked meat medium (Fort Richard Laboratories) and incubated for seven days at 35 ^{o}C. After incubation, a wire loop filled with microbial suspension was plated on Nutrient agar (NA) (Difco) plates and incubated at 30 ^{o}C for 24 h. From NA plates, multiple colonies were transferred to nutrient broth and incubated at 37 ^{o}C for 24 h with continuous shaking. Aliquots of 0.1 ml of prepared inoculum were plated on solidified sporulating medium. Sporulation medium was prepared by using 23 g of NA, 1 mg manganese sulphate (Merck) and 0.5 g calcium chloride (RDH) in one litre of water. Sporulation was monitored by using phase contrast microscopy (Motic microscope BA 410 Series, Canada) as described by Evelyn *et al.* (2015b). After 75 % sporulation, spores were harvested by flooding the plates with 3 ml of sterilized water using a spreader. Collected spores were centrifuged three times at 1600 rpm (298 g), 4 ^{o}C and for 15 min. After each centrifugation run, the supernatant was discarded and sterilized water was added to the same volume. Finally, obtained suspension was thermally treated at 80 ^{o}C for 10 min to inactivate vegetative cells and

then was stored at 4 °C. For experiments with UV, approx. 2 ml of spore suspension was inoculated to achieve spore count of (~10^7 – 10^8 CFU/ ml) in milk samples (for 200 ml).

4.2.2.2. Enumeration of spores

Spore concentrations before and after treatment were determined by spread plating onto plate count agar (PCA) and incubation at 30 °C for 24-48 h by making appropriate dilutions.

4.2.3. UV equipment and experimental plan

4.2.3.1.Thermal inactivation of spores

Thermal resistance of spores in different media were determined by using capillary tube method (Franklin *et al.*, 1958; Stern *et al.*, 1954; Van Zuijlen *et al.*, 2010). Glass capillaries were filled with 50 µl of the inoculated sample and sealed from both edges using Bunsen burner. Immense care was taken to avoid any heating during the process of sealing. Capillary tube method was used due to its negligible come up time during treatment (Block, 1977).

For thermal treatment, the capillary tubes were immersed in an oil bath maintained at 110 °C for different time intervals (up to 25 sec for water with every 5 sec, up to 80 sec for SCM with every 20 sec, up to 120 sec for WCM with every 40 sec, up to 240 sec for SM with every 60 sec) to achieve at least one to two log reductions. The temperature (110 °C) was selected as it may provide the possibility of HTST process after UV treatment. For initial count, untreated capillaries were used and clipped off in the same way as treated ones and considered as control. After thermal treatment, the capillary tubes were immediately transferred to an ice bath to lower its temperature rapidly and then washed with ethanol and sterile water. Afterwards, tubes were clipped off from both ends and flushed with sterile water under aseptic conditions. Then the microbial count was measured as described in Section 4.2.2.2.

Survival curves were obtained by plotting logarithmic spore reduction against thermal treatment period time at a constant temperature. The decimal reduction time (D value) was calculated by taking reciprocal of the slope of Equation 4-2 as given as follows:

Equation 4-2

$$Log\ \frac{N}{N_o} = -\frac{t}{D_T}$$

Where N_o is the initial microbial population, N is the survival population after treatment, t is the time in sec and D_T is the D value at specific temperature.

4.2.3.2. UV experiments

UV Equipment:

A UV reactor was designed and constructed at the Department of Chemical and Materials Engineering, University of Auckland, and was similar to the one described by Choudhary *et al.* (2011). The UV lamp (EGPH369N/S, 0.369 m length) and quartz sleeve (EQS 450, ID 21.7 mm, OD 24.4 mm) were obtained from Davey water products, New Zealand. UV-C source power was given as 5.6 W (Total lamp power of 19 W, low pressure mercury lamp, Davey water products) at a wavelength of 254 nm. Perfluoroalkoxy (PFA) tube with total length of 7.62 m (ID 1.6 mm, OD, 3.2 mm), was obtained from Thermofischer Scientific, New Zealand. PFA tube was used because it possesses high transmittance (80 %) to UV radiation, stability to high temperature and chemicals (Bandla *et al.*, 2012a; Cambié *et al.*, 2016).

Figure 4-1 shows a schematic diagram of the coiled tube reactor. UV-C lamp was placed inside a quartz sleeve, while the PFA tube was coiled over the quartz sleeve tightly without any gap spaces. The coiled reactor was placed inside a stainless steel barrel to avoid any UV exposure to the surrounding environment.

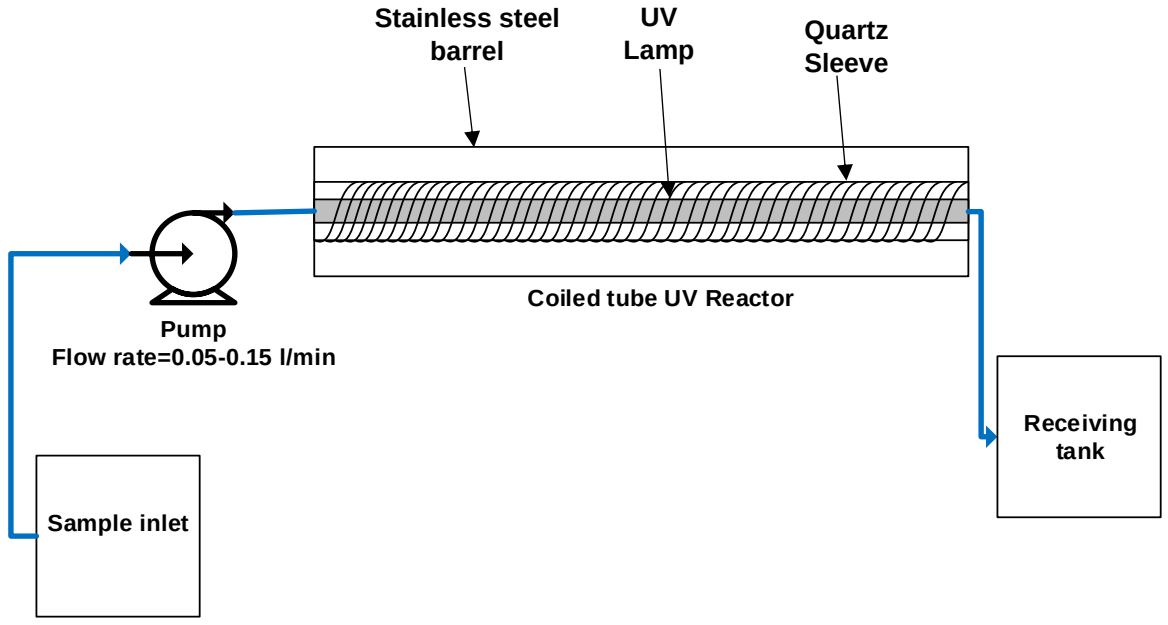


Figure 4-1: Schematic diagram of UV treatment using a coiled tube reactor (Not to scale).

UV Equipment Operation and cleaning:

A peristaltic pump (Model No. 7553-75, Cole-Parmer Instrument Company) was used for pumping the milk or water through the reactor. Flow rate and inlet temperature (20 ^{o}C) were set and the same settings were used for all experiments. After each operation, the reactor was rinsed with sterilant 'Oxonia' solution for 5 min followed by 70 % ethanol for 2-3 min. After cleaning, the system was rinsed with sterilized water for 5 min to remove all traces of Oxonia and ethanol. The microbial count of rinsing water after cleaning was taken as described in section 4.2.2.2 to ensure proper cleaning. UV lamp was switched on 5 min prior to any experiments as a warm-up time as per instructions of manufacturer. UV-C energy consumption for treated sample was calculated as dosage per unit volume since it is the most appropriate method for a liquid product. The obtained value was multiplied with transmission of Quartz sleeve (90 %) and PFA tube (80 %) (Choudhary *et al.*, 2011).

Equation 4-3

$$\text{UV-C}\left(\frac{\text{J}}{\text{ml}}\right) = \frac{\text{UV-C Power of lamp (W)}}{\text{Flow rate}\left(\frac{\text{ml}}{\text{s}}\right)} \times T_{Quartz} \times \text{T}_{Tube}$$

Delivered UV dose (D Act):

UV treatment results in triiodide formation and could be expressed using the following equation as given by (Müller *et al.*, 2014).

Equation 4-4

$$8KI + KIO_3 + 3H_2O + hv \rightarrow 3I_3^- + 6OH^- + 9K^+$$

It was carried out by treating aqueous solution of 0.6 M potassium iodide and 0.1 M potassium iodate in 0.01 M borate buffer (pH 9.25) at the specified flow rate (9 L/h) as described by Rahn *et al.* (2003) and Müller *et al.* (2014). The UV dose (D_v in J/ml) was calculated by using the following relation:

Equation 4-5

$$D_{ACT} = \frac{A_{352\,nm} \times P_{253nm}}{pl \times \emptyset \times \varepsilon_{352\,nm}}$$

Where $A_{352\,nm}$ represents measured absorbance at 352 nm, $P_{253.7\,nm}$ is the number of Joules per Einstein of 253.7 nm photons (4.716×10^5 J einst^{-1}), pl is the path length of the quartz

cuvette (1 cm), $\emptyset$ is the quantum yield (mol einst^{-1}) and $\varepsilon_{352\,nm}$ is the molar absorption coefficient of triiodide at 352 nm (27600 dm^{3}mol^{-1})

A correction to quantum yield was applied using the initial temperature (^{o}C) and concentration of the iodide ($A_{300\,nm} \times 1.061^{-1}$)

Reynolds number (Re) of the flowing liquid during treatment was calculated as follows:

Equation 4-6

$$Re = \frac{D \times \rho \times v}{\mu}$$

Where D is diameter of the coiled tube, ρ is density of the treated liquid, v is velocity of the liquid and μ is its dynamic viscosity. Dynamic viscosity of water was taken as 0.001 Pa. s.

Experimental design:

In the first part of experiment, thermal treatment of *B. subtilis* spores (ATCC 6633) was done at 110 ^{o}C for different types of milk and water using capillary technique as described in section 4.2.3.1.

In the second part of experiments, three flow rates (50 ml/min, 100 ml/min, and 150 ml/min) were selected for UV inactivation of *B. subtilis* spores in both water and SCM. Subsequently, the highest flow rate (150 ml/min) was applied in all other experiments and subsequent inactivation were measured. For a single pass at 50 ml/min,100 ml/min and 150 ml/min, an increase of 3.0 ^{o}C, 2.36 ^{o}C and 1.3 ^{o}C in temperature were observed respectively.

In the third set of experiments, thermal treatment of the UV pre-treated milk was conducted at 110 ^{o}C (10 to 50 sec) for the different milk types using capillary method as described in section 4.2.3.1. At least two independent experiments were performed for each thermal treatment, UV treatment and combined UV followed by thermal treatment.

4.2.3.3. Statistical analysis

Microsoft Excel 2013 (Microsoft Inc., USA) was used for statistical analysis of data. Mean and standard deviation for replicates were calculated using Excel 2013. Also, Single factor ANOVA was performed to analyse the effect of Reynolds number and dosage (J/ml) on inactivation using confidence level of 95 %.

4.3. Results and discussion

4.3.1. Thermal inactivation of spores

Table 4-2 shows the D values of *B. subtilis* spores in water, SCM, WCM and SM at 110 ^{o}C after thermal treatment. The D value obtained for water was 0.31 ± 0.01 min, similar to the one obtained by Conesa *et al.* (2003) which was 0.32 min at 110 ^{o}C for *B. subtilis* AdHCl spores. Similarly, Cregenzán-Alberti *et al.* (2017) obtained 0.32 min for *B. subtilis* DSM 618 spores in water. However, slightly lower value was observed (0.26 min) for *B. subtilis* PS 832 at pH 7 in ringer solution by Siemer *et al.* (2014).

The D values obtained for *B. subtilis* (ATCC 6633) in different types of milk are also shown in Table 4-2 where the value was lowest for SCM (0.44 ± 0.01 min) and highest for SM (0.94 ± 0.06 min). A higher value of 0.63 min was reported by Cregenzán-Alberti *et al.* (2017) in reconstituted SCM for *B. subtilis* endospores DSM 618. This difference might be due to difference in strains and sporulation media. Mazas *et al.* (1995) analysed the effect of sporulation media on D values and found that the composition of sporulation media can affect D values of microbial spores. The total solids (%) of the treated milks were in the following order SCM (8.81 ± 0.05) < WCM (12.00 ± 0.02) < SM (16.19 ± 0.03) (Table 4-1) These results show that increased solid content leads to increase spore resistance and hence less effective thermal treatment. Setlow (2006b) explained this increased spore resistance is due to the low water content in the core of spore. In addition, fat possesses a protective effect to microorganisms (Senhaji, 1977). The results of this study are also supported by Jagannath *et al.* (2003), who found higher D values for UHT WCM in comparison to UHT SCM.

Table 4-1: Physical properties of milk products

Sample	SCM	WCM	SM
Total solids	8.81 ± 0.05	12.00 ± 0.02	16.19 ± 0.03
Absorption Coefficient (cm^{-1})	170.60 ± 0.05	326.13 ± 1.50	337.30 ± 6.95
Moisture content	91.19 ± 0.05	88 ± 0.05	83.81 ± 0.05
Viscosity (Pa s)	0.0017	0.0018	0.0023
pH	6.57	6.55	6.51
Density (Kg/m^3)	1031	1027	1023
Re at 150 ml/min	1206	1135	885

Table 4-2: Thermal inactivation D values (min) of *B. subtilis* spores in Water, SCM, WCM and SM at 110 ºC

Media	D values (min)
Water	0.31 ± 0.01
SCM	0.44 ± 0.01
WCM	0.69 ± 0.04
SM	0.94 ± 0.06

4.3.2. Ultraviolet (UV) treatment of spores

In this study, UV treatment was carried out at different flow rates to highlight the importance of Re. In addition, the effect of UV dosage on spore inactivation was analysed by circulating the liquid through the reactor multiple times. A single pass through the reactor at 150 ml/min corresponds to D_{Act} 0.790 ± 0.126 J/ml of UV-C treatment. Table 4-3 shows the inactivation of *B. subtilis* spores ATCC 6633 in water using UV treatment at different UV dosage and

residence time. However, the effect of increasing Re is not significant since UV transmittance is high in water. High log reduction of 5.17 ± 0.46 was achieved even at low UV dosage of D_{Act} 0.790 ± 0.126 J/ml at Re equals 1989 in a single pass. Therefore, during multiple passes, the log reduction was higher than the detection limit as shown in Table 4-3.

Table 4-3: Inactivation of *B. subtilis* spores ATCC 6633 in water with UV treatment at different operating conditions (Log reduction showed as mean ± SD of replicates)

WATER					
Flow rate (ml/min)	**No. of passes**	**Reynold number**	**D_{Act} (J/ml)**	**Residence time (s)**	**Log inactivation**
50	One	663	1.823 ± .0025	18.4	Greater than 6.90 (Initial count 7.9)
100	One	1326	1.139 ± 0.006	9.2	5.09 ± 0.19
	Two		2.278 ± 0.012	18.4	Greater than 6.90 (Initial count 7.9)
150	One	1989	0.790 ± 0.042	6.1	5.17 ± 0.46
	Two		1.58 ± 0.084	12.3	Greater than 6.90 (Initial count 7.9)
	Three		2.37 ± 0.126	18.4	Greater than 6.90 (Initial count 7.9)

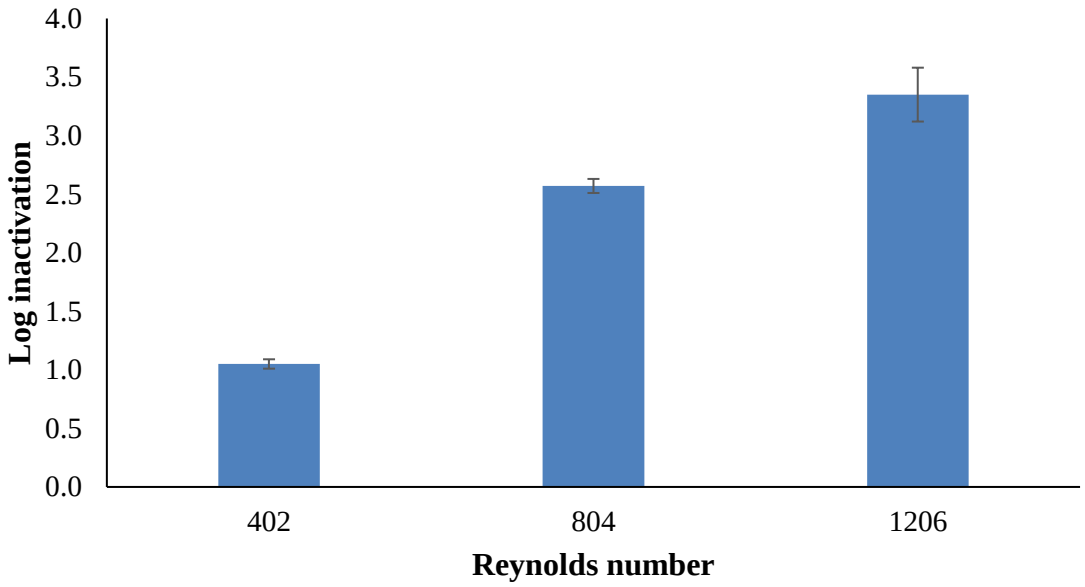


Figure 4-2: Effect of Reynolds number (Re) on inactivation of *B. subtilis* ATCC 6633 spores with UV treatment (18.4 sec) in SCM

Figure 4-2 shows the inactivation of *B. subtilis* spores with different values of Re for SCM using the continuous flow UV reactor. For the same residence time ~18.4 sec, the log inactivation was increased from 1.05 ± 0.04 at Re = 402 to 3.35 ± 0.23 at Re = 1206. This effect of increase in Re could be explained due to more effective mixing during flow as well as mixing in between multiple passes through the UV reactor, which improved UV exposure and thereby resulted in enhanced inactivation. A similar trend was also disclosed by Bandla *et al.* (2012a) who studied *B. cereus* spores in soymilk using coiled tube reactor with PFA tube (ID 1.6mm). For the same dosage of 11.187 mJ/cm^2 (11.3 s), the log inactivation was increased from 2.09 at Re = 349 to 3.22 at Re = 1376. Bandla *et al.* (2012a) explained that smaller diameter tubing induces circular flow causing efficient mixing allowing better exposure to UV. Choudhary *et al.* (2011) also studied coiled tube reactor with *B. cereus* spores in SCM and raw cow milk (RCM). For the same dosage of 11.187 mJ/cm^2 (11.3 s) in RCM, the log inactivation was increased from 1.28 at Re =181 to 2.65 at Re = 713. In SCM, it was increased from 1.59 at Re = 265 to 2.72 at Re = 1064.

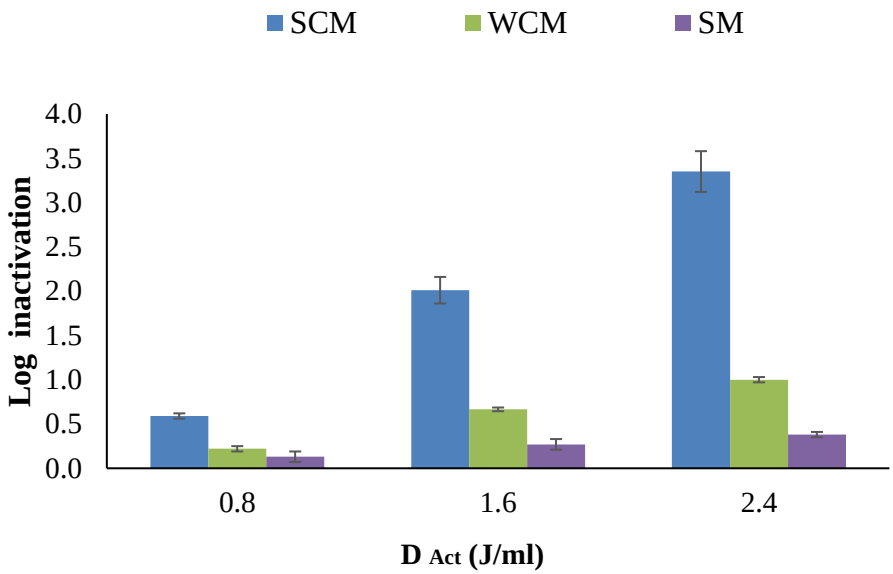


Figure 4-3: Inactivation of *B. subtilis* ATCC 6633 spores in SCM, WCM and SM (Flow rate 150 ml/min)

Figure 4-3 shows the inactivation of *B. subtilis* spores in different media keeping the flow rate same at 150 ml/min using different dosages. Further, mixing effect that arose in between multiple passes, maximize microbial exposure to UV. In addition, pulsatile flow from pump also responsible for better mixing and hence, better UV exposure (McDonough *et al.*, 2019).

The results show that spore reduction in SCM was higher than WCM and SM. This could be due to different fat contents of milk products (SM > WCM > SCM). Secondly, the solid content of milk products were also in similar order (SM > WCM > SCM). High solid content not only affects thermal treatment as mentioned previously but also hinders effective UV treatment as they are directly related to absorption coefficient. The absorption coefficient (cm^{-1}) of 170.60 ± 0.05 for SCM, 326.13 ± 1.50 for WCM and 337.30 ± 6.95 for SM further confirms that UV penetration was highest for SCM among different types of milk. Thereby SCM needed lower dosage for the same log reduction when compared to WCM or SM resulting in UV treatment effectivity in the following order SCM > WCM > SM. As mentioned previously, there are only few studies that are carried out for spore inactivation with UV (Bandla *et al.*, 2012b; Choudhary *et al.*, 2011; Gayán *et al.*, 2013). Out of these studies only Choudhary et al. (2011) investigated *B. cereus* spores in SCM and raw cow milk and reported 2.72 and 2.65 log reduction whereas Bandla *et al.*, 2012b and Gayán *et al.*, 2013 did studies on soya milk and citrate buffer with UV. Therefore, this shows that inactivation results are media and microorganism dependent.

4.3.3. Effect of UV pretreatment on thermal inactivation of spores

Table 4-4 shows the inactivation of spores using UV (150 ml/min, D_{Act} 2.37 ± 0.126 J/ml) in combination with thermal treatment at 110 °C for specified time intervals. It was observed that UV (150 ml/min, D_{Act} 2.37 ± 0.126 J/ml) in combination with heat provides 5.95 ± 0.34 log reduction in SCM in 30 sec and 5.18 ± 0.07 log reduction in 50 sec in whole milk. These results also show that longer thermal treatment time was required to achieve the same level of inactivation of spores in WCM than SCM. Further, this combination was not effective for SM which gave only 1.45 ± 0.14 log reduction with the same UV and thermal treatment (110 °C for 50 sec).

Table 4-4: Inactivation of *B. subtilis* ATCC 6633 spores in different types of milk after UV treatment (D_{Act} 2.37 ± 0.126 J/ml, 150 ml/min, 18 sec) and thermal treatment at 110 °C.

Treatment time at 110 °C (sec)	Log inactivation (UV + Thermal)		
	SCM	WCM	SM
10	4.77 ± 0.33	1.40 ± 0.11	0.59 ± 0.03
20	5.47 ± 0.36	1.96 ± 0.20	0.73 ± 0.04
30	5.95 ± 0.34	2.89 ± 0.09	1.10 ± 0.06
40	Below detection limit based on initial log count of 7	4.02 ± 0.08	1.24 ± 0.07
50	---	5.18 ± 0.07	1.45 ± 0.14

In summary, Figure 4-4 provides a comparison of spore inactivation when thermal, UV and UV combined with thermal treatments were applied. As expected, the spore reduction obtained with thermal alone at 110 °C for 30 sec is minimal for all types of milk. It also shows that UV alone is not sufficient to get considerable spore inactivation. For e.g., UV and thermal treatment alone resulted in 3.35 and approximately 1 log reduction in SCM respectively. On the other hand, UV in combination with heat gave pronounced sensitization effect for subsequent thermal treatment resulting in a synergistic effect in all types of milk. This combined treatment was most effective in SCM with 5.95 ± 0.34 log reductions while for WCM and SM resulted in 2.89 ± 0.09 and 1.10 ± 0.06 log reduction respectively.

Unlike vegetative cells, UV treatment of spores results in the formation of thymidyl-thymidine adduct which is known as spore photoproduct (SP) (Setlow, 2006b). As mentioned previously, spore photoproducts possess the capability to repair rapidly by a number of mechanisms including enzymatic repair since some spores possess enzymes even in their dormant form (Warth, 1980). For instance, enzyme spore photoproduct lyase (Spl) could reverse spore photoproduct (Setlow, 2006b). The improvement in UV inactivation when followed by heat might be explained by the heat sensitivity of spore photoproduct lyase. Warth (1980) studied the heat sensitivity of enzymes extracted from *B. cereus* spores and reported that the enzymes were inactivated between temperatures of 47 to 70 °C.

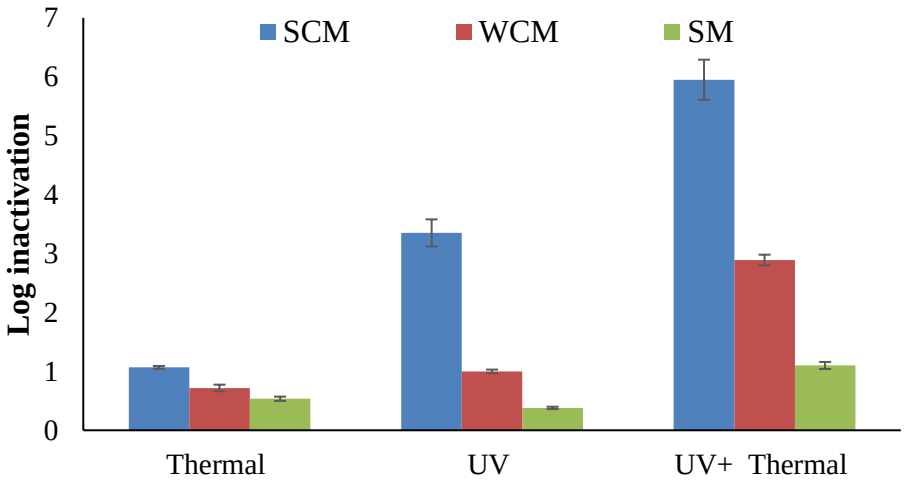


Figure 4-4: Inactivation of *B. subtilis* ATCC 6633 spores in SCM, WCM and SM. Thermal: 110 ^{o}C for 30 sec, UV: D_{Act} 2.37 ± 0.126 J/ml at flow rate of 150 ml/min, UV + Thermal: UV (D_{Act} 2.37 ± 0.126 J/ml at flow rate of 150 ml/min) + Thermal (110 ^{o}C for 30 sec, ~354 J/ml).

4.3.4. Energy consumption

Further, Figure 4-4 that shows significant increase in the inactivation of *B. subtilis* spores through the use of combination of UV and thermal has also have an effect on energy consumption. Thermal treatment from 20 ^{o}C to 110 ^{o}C would require ~354 J/ml while the estimated energy needed for the use of UV is only 4.84 J/ml (UV-C only) or 22.8 J/ml (total power consumption) for the combined treatment. Since combination of UV and thermal treatment can achieve sterilization at lower temperature than conventional treatment, it has the additional benefit of energy saving that could make this technology more attractive to the industry.

4.4. Conclusion

The potential of using UV in combination with heat as an alternative method for thermal sterilization has been investigated in this work. The results show that UV treatment sensitized spores, making thermal treatment more efficient, especially for SCM. This benefit was less with WCM and SM as transmittance of UV is lower than SCM due to high total solids in them. Therefore, this combined technology could be an economical alternative to conventional sterilization of SCM and probably for other types of milk if UV transmission could be improved further.

CHAPTER 5

Inactivation of *G. stearothermophilus* spores using Ultraviolet treatment in combination with heat

This chapter based is based on the draft of the paper ready for submission to an international journal.

Preface

UHT treatment is commonly used for sterilization of low acidic pumpable foods that involves a thermal treatment at high temperatures for short duration. Presently, emerging technologies are studied to lower the temperature required for sterilization. Ultraviolet light in the germicidal range (UV-C) could provide an alternative to conventional sterilization when applied with mild heat. In this study, UV treatment using a coiled tube reactor that gives enhanced exposure was used. The effect on *G. stearothermophilus* spores, which is one of the highly heat-resistant spores that can spoil milk was analysed. It was observed that UV pretreatment (4.84 J/ml) combined with thermal treatment resulted in 5.38 ± 0.01 log reduction in skim milk (120 ^{o}C for 10 sec), 2.70 ± 0.24 log reduction in whole milk (120 ^{o}C for 3 min) and 1.08 ± 0.11 log reduction in sheep milk (120 ^{o}C for 3 min). The results show that UV-C pre-treatment could lower the adverse effects during thermal sterilization.

5.1. Introduction:

Bacterial spores are identified as round shaped dormant form of bacterial cell, produced in adverse environmental conditions and possess the ability to survive for millions of years (Cano *et al.*, 1995). They are more resistive to heat in comparison to vegetative cells and therefore, need a high treatment temperature for their inactivation. *G. stearothermophilus* spores are one of the most highly heat resistant spores and are commonly used for validation of moist heat sterilization (Pharmacopeia., 2016). Its resistance to heat could be attributed to its high content of hexose and dipicolinic acid in comparison to *B. cereus, B. subtilis* and *B. coagulans* spores (Setlow *et al.*, 2006; Warth *et al.*, 1963).

Ultra-high temperature (UHT) treatment has been used to reduce *G. stearothermophilus* spores in liquid milk to achieve sterilization (Casillas-Buenrostro *et al.*, 2012). However, UHT results in adverse changes in organoleptic properties and nutritional value due to high temperature exposure. These changes are attributed to Maillard and oxidative reactions that mainly affect proteins, fats and vitamins (Cappozzo *et al.*, 2015; Renner, 1988; Van Boekel, 1998).

Presently, emerging technologies in combination with heat are considered as a good approach to reduce sterilization temperature of foods with the objective to retain their quality (Ansari *et al.*, 2017; Georget *et al.*, 2014a; Poliseli-Scopel *et al.*, 2014; Spilimbergo *et al.*, 2003; Tran *et al.*, 2004). Emerging technologies that are advanced in research include Pulsed electric field (PEF), Ultraviolet (UV), High pressure processing (HPP), Ultrasound and Ultra high pressure

homogenization (UHPH). However, only few technologies possess the capability to inactivate these spores with low energy requirements. UV is one of the technologies that possesses these characteristics with its tremendous effect on spores. Further, in a recent decision, EU commission authorized the placement of UV treated milk as a novel food to the market (Poulsen, 2016). This current progress, made in regulatory compliance, will drive commercialization of UV treatment at a faster pace.

UV treatment possesses the capability to sterilize clear liquids because of its high sporicidal efficacy (Gayán *et al.*, 2013). However, UV has low transmittance within opaque liquids, which makes its application difficult for low acidic products such as liquid milk (Datta *et al.*, 2015). Some of the efficiently designed UV processing units could provide high inactivation of spores even in opaque liquids. Coiled tube reactor is one of these designs that induces efficient turbulence and a secondary flow due to circular movement referred to as "Dean flow" , providing better UV exposure (Bandla *et al.*, 2012a; Choudhary *et al.*, 2011). The intensity of Dean flow can be measured by Dean number (De) defined by the following equation (Dean, 1927; Gayán *et al.*, 2014b)

Equation 5-1

$$D_e = Re\sqrt{\frac{D_h}{D_c}}$$

Where Re is Reynold number, D_h represents hydraulic diameter, and D_c represents coil diameter (Figure 5-1). D_e value higher than 150 results in a secondary flow and is responsible for improved UV inactivation of microorganisms (Choudhary *et al.*, 2011; Lu *et al.*, 2010).

Spores are more resistant to both thermal and UV treatment than vegetative cells. This high resistance is owing to the repair mechanism which is associated with spore inactivation. Exposure of spores to UV results in the formation of spore photoproducts, which can be reversed by enzyme spore photoproduct lyase (Spl) present in spores (Setlow, 2006b). Thermal treatment immediately after UV treatment may hinder such repair by reducing the activity of this enzyme and hence, a combination of UV with heat could result in higher inactivation of spores.

G. stearothermophilus can cause a flat sour defect in milk by producing acid and is responsible for reduced shelf of food (Kakagianni *et al.*, 2016; Ledenbach *et al.*, 2009). Only a few studies have considered the inactivation of these spores using UV-C treatment. Gayán *et al.* (2013)

reported 4.25 log reduction of *G. stearothermophilus* spores using citrate phosphate buffer as a suspended medium, having high UV transmittance. To best of our knowledge, UV inactivation of *G. stearothermophilus* spores has not been studied in opaque liquid such as milk. Therefore, the objective of this work is to study the effect of UV treatment on inactivation of *G. stearothermophilus* spores in milk and to investigate if UV treatment could sensitize spores for the subsequent thermal treatment.

5.2. Materials and methods

5.2.1. Media/Food matrix

Distilled water, SCM, WCM and SM were used as inoculation media for spores. UHT treated SCM and WCM were purchased from a local market in 1 L aseptic packs. SM was provided in powder form by Spring Sheep Company, New Zealand and was reconstituted by dissolving it in water at a ratio of 1:6. The fat contents labelled on packaging was 0.1 % in SCM, 3.5 % in WCM and 5.24 % in SM after reconstitution.

Total solids

Total solids of SCM, WCM and SM was determined as described by AOAC 990.19. Milk sample (3 g) was weighed in aluminium dishes (oven pre-dried at 100 ^{o}C for 2 h) and placed in water bath for 38 ± 1 ^{o}C for 25 min and oven dried at 100 ± 1 ^{o}C for 3 h. Total solids (%) was calculated as

Equation 5-2

$$Total\ solids\ (\%) = \frac{(W_2 - W_1) - B}{Sample\ weight} \times 100$$

Where, W_2 is mass in gram of dish + dried milk, W_1 is the mass of empty dish and B is blank

5.2.2. Microbiological studies

5.2.2.1. Bacterial strain and sporulation

The bacterial strain used in this study was *G. stearothermophilus* (ATCC 7953). The spores was obtained from New Zealand Reference culture collection, Medical section (NZRM).

The freeze-dried culture of *G. stearothermophilus* (ATCC 7953) spores was revived by transferring it into cooked meat medium and incubated it at 55 ^{o}C for 24 h. After incubation, a wire loop filled with spore suspension was plated on nutrient agar (NA) and incubated at 55 ^{o}C for 24 h. From NA plates, multiple colonies were transferred to TYE broth (Tryptone 1%, Yeast extract 0.5%, Dipotassium hydrogen phosphate 0.2%) and incubated in a shaking incubator at 250 rpm and 55 ^{o}C for 24 h. Aliquots of 0.2 ml of TYE broth was transferred to sporulation medium and incubated at 55 ^{o}C for 3 days covered in polyethylene bags to avoid dehydration. Sporulation media was composed of 23 g of NA (Difco), 1 mg Manganese sulphate (Merck) and 0.5 g Calcium chloride (RDH) in 1 L of water. After three days, these bags were removed from the incubator and placed at room temperature for another three days. Sporulation was monitored by using phase contrast microscopy (Motic microscope BA410 series, Canada) as described by Evelyn *et al.* (2015b). After 75-90 % sporulation, spores were harvested by flooding the plates with sterilized water and then centrifuged at 4800 g, 4 ^{o}C for 15 min until supernatant becomes clear (minimum three times). The obtained suspension was heat pasteurized at 80 ^{o}C for 10 min and refrigerated at 4 ^{o}C until further use.

5.2.2.2. Enumeration of spores

Spore concentrations before and after treatments were determined by spread plating on NA plates and incubation at 55 ^{o}C for 24-48 h after making appropriate serial dilutions.

5.2.3. UV equipment and experimental plan

5.2.3.1. Studies on thermal inactivation of spores

The thermal resistance of spores was determined by the capillary method. Glass capillary method is commonly used for the determination of thermal inactivation kinetics as it provides rapid heating and cooling within negligible come-up time (Block, 1977). Capillaries were filled with inoculated samples and sealed afterward from both ends using Bunsen burner with proper care to avoid localized heating. Sealed capillaries were transferred to a heated oil bath maintained at 120^{o} C for different time intervals. The temperature (120 ^{o}C) was selected as it may provide the possibility of HTST process after UV treatment. After heat treatment, capillaries were transferred to a cooling bath at 0-4 ^{o}C and then washed with ethanol followed by rinsing with sterile water. Contents of capillaries were removed by clipping off from both ends, and microbial counts were measured as described in Section 5.2.2.2. D value was

determined by plotting logarithmic reduction against time. Untreated capillaries were clipped off in the same way as treated ones and initial count was taken from them.

5.2.3.2. UV experiments

UV Equipment

In this study, a coiled tube reactor was used for treating liquid milk with UV radiation at 254 nm. It was designed and fabricated as described by Choudhary *et al.* (2011) with some changes carried out at the Department of Chemical and Materials Engineering, University of Auckland. The UV lamp (EGPH369N/S, UVC 5.6 W with a lamp power of 19W) and quartz sleeve (EQS 450, ID 21.7 mm, OD 24.4 mm) were obtained from Davey water products, New Zealand. Perfluoroalkoxy (PFA) tube with dimensions ID 1.6 mm, OD 3.2 mm, and a length 7.62 m was obtained from Thermofischer Scientific, New Zealand. The UV lamp was enclosed in a quartz sleeve and PFA tube was coiled tightly on the quartz sleeve providing maximum exposure to UV (Figure 5-1). The whole unit was placed in a stainless steel chamber to avoid exposure to UV during experiments. A peristaltic pump was used for pumping liquids through the reactor at a flow rate of 150 ml/min.

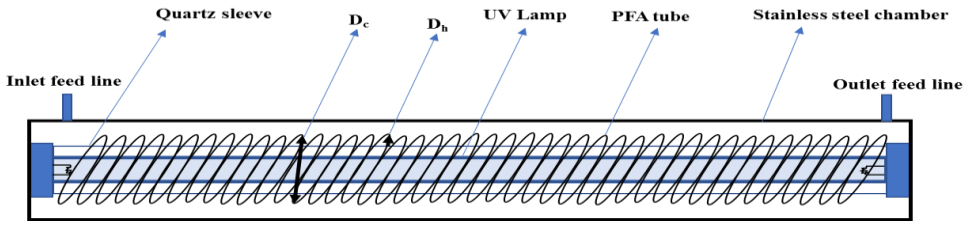


Figure 5-1: Schematic diagram of coiled tube reactor (Not to scale).

UV Equipment operation and cleaning

UV lamp was switched on 5 min before any experiment as warm-up period. Inoculated samples were passed multiple times through the reactor with an average flowrate of 150 ml/min to provide different UV dosages. After each treatment, Oxonia cleaning solution was circulated through the reactor for 5 min, followed by pumping ethanol (70 % v/v) for another 5 min to sanitize the UV unit. Finally, sterilized water was circulated for 5 min to remove all traces of Oxonia and ethanol. The UV-C dosage was calculated as the energy used per unit volume while considering the transmittance factors through quartz tube and PFA tube as described by (Choudhary *et al.*, 2011; Müller *et al.*, 2014). Accordingly, T_{Quartz} and $T_{PFA\ Tube}$ were taken 90 % and 80 % respectively (Choudhary *et al.*, 2011; Geveke, 2005).

$$UVC\ \left(\frac{J}{ml}\right) = \frac{UVC\ Power\ of\ lamp\ (W)}{Flow\ rate\ \left(\frac{ml}{s}\right)} \times T_{Quartz} \times T_{PFA\ Tube}$$

Reynold number during treatment was calculated as follows:

$$Re = \frac{D \times \rho \times V}{\mu}$$

Where *D* is the inner diameter of coiled tube, ρ is the density, V is the velocity and μ is the dynamic viscosity of the liquid treated. Dynamic viscosity and density were calculated using the correlation developed by Bakshi *et al.* (1984). Dynamic viscosity for water was taken as 0.001 Pa.s (Swindells *et al.*, 1952).

Absorption coefficient

Absorption coefficients of the different media were measured through UV-Vis spectrophotometer (Perkin Elmer, Lambda 35) at 254 nm by diluting 1 ml of milk to 100 ml using distilled water (maintained at different temperatures) as it was not possible to measure absorption coefficient with the original milk sample due to its low transmittance (Bandla *et al.*, 2012b). The absorption coefficient was calculated by the ratio of absorption values at 254 nm to the path length of quartz cuvette (1 cm).

Experimental design

In the first part of the experiments, thermal inactivation kinetics of *G. stearothermophilus* spores (ATCC 7953) was studied at 120 ^{o}C for all liquid media using the capillary technique as described in section 5.2.3.1.

In the second part of experiments, liquid media with inoculated spores were pumped through the UV reactor at a flow rate of 150 ml/min for a multiple passes so as to expose the liquid to different UV doses (1.61 J/ml, 3.23 J/ml, and 4.84 J/ml).

In the third part of experiments, the UV treatment of the different types of milk (150 ml/min, 4.84 J/ml, 20 ^{o}C) was followed by thermal treatment at 120 ^{o}C for up to 3 min.

5.2.3.3.Statistical analysis

Microsoft Excel 2013 (Microsoft Inc., USA) was used for statistical analysis of data. Mean and standard deviation of replicates were calculated using Excel 2013. Also, Tukey's test with a confidence level of 95 % was performed to compare inactivation at different temperatures.

5.3. Results and discussion

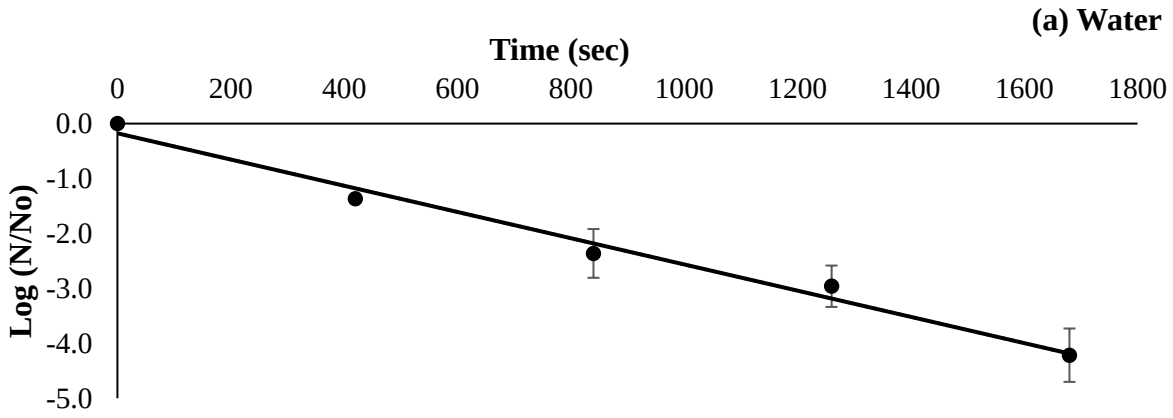
5.3.1. Thermal inactivation of spores

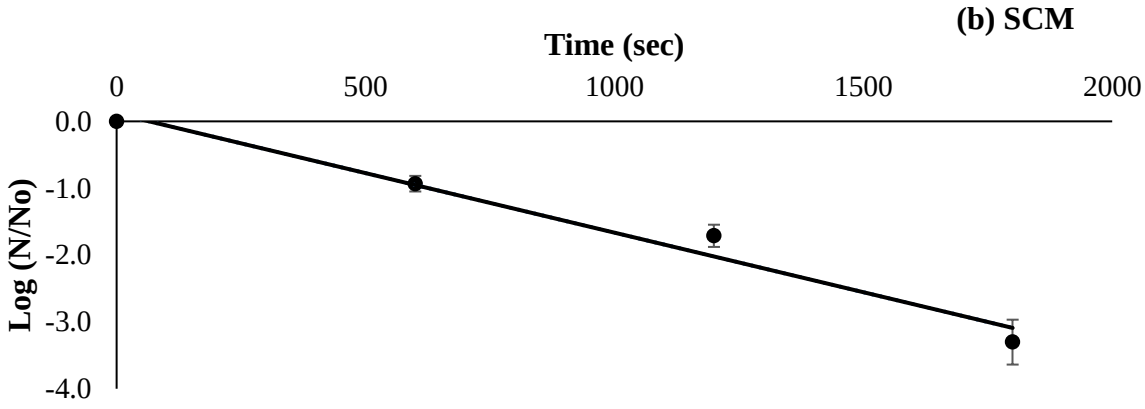
Figure 5-2 gives the logarithmic reductions of *G. stearothermophilus* spores in water, SCM, WCM and SM when thermally treated at 120 ^{o}C for different times. The linearity of the graphs confirms first order kinetics enabling D value calculation from its gradient. Table 5-1 shows the corresponding D values of *G. stearothermophilus* spores in water, SCM, WCM and SM at 120 ^{o}C. D value of SM was the highest followed by WCM, SCM and lowest for water.

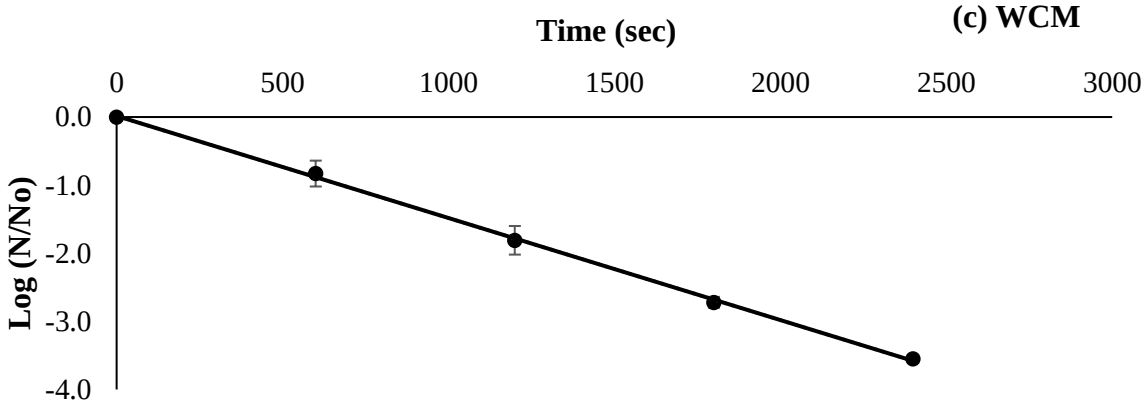
The D values observed for water was 7.00 ± 0.45 min at 120 ^{o}C and is quite similar to the value observed by Marquis *et al.* (1985) which was 6.5 min. However, slightly higher values (8.31 ± 0.77 min) were obtained by Lopez *et al.* (1996). Also, Wimalaratne *et al.* (2008) have reported D value of 8.4 min in milli Q water for the same strain used as in this study. This slight difference could be explained by the different procedures used for sporulation. For SCM, as expected the D value was 9.40 ± 0.84 min, which was lower than that of WCM and SM. Among different milk products, D value for SCM was lowest and highest for SM. It could be explained by their total solids content which was in the following order SCM (8.81 ± 0.05) < WCM (12.00 ± 0.02) < SM (16.19 ± 0.03). Jagannath *et al.* (2003) found that D values were higher for UHT whole milk in comparison to UHT skim milk.

Table 5-1: Thermal inactivation D values (min) of *G. stearothermophilus* spores in Water, SCM, WCM and SM at 120 ^{o}C

Media	D values (min) at 120 ^{o}C
Water	7.00 ± 0.45
SCM	9.40 ± 0.84
WCM	11.12 ± 0.03
SM	11.44 ± 0.57


(a) Water
Time (sec)
0
200
400
600
800
1000
1200
1400
1600
1800
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
Log (N/No)


(b) SCM
Time (sec)
0
500
1000
1500
2000
0.0
-1.0
-2.0
-3.0
-4.0
Log (N/No)


(c) WCM
Time (sec)
0
500
1000
1500
2000
2500
3000
0.0
-1.0
-2.0
-3.0
-4.0
Log (N/No)

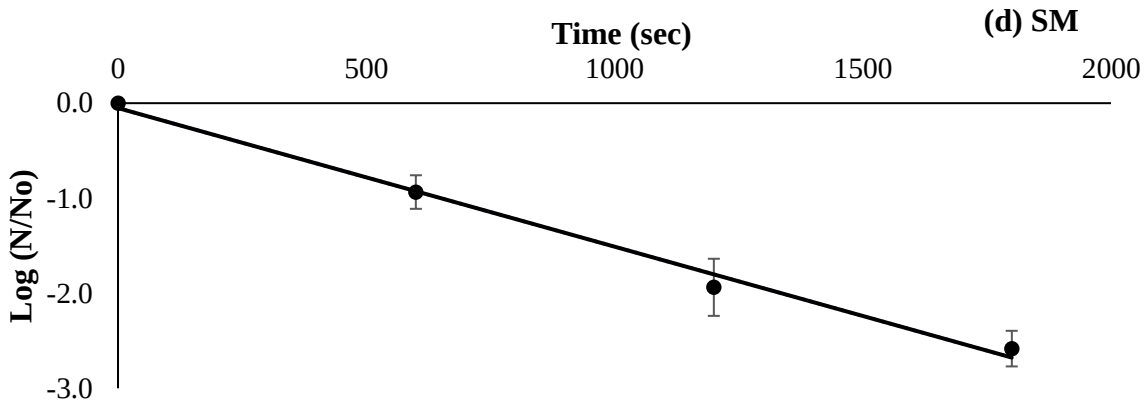


Figure 5-2: Thermal inactivation curves for *G. stearothermophilus* ATCC 7953 spores at 120 ^{o}C (a) Water, (b) SCM (c) WCM (d) SM

5.3.2. Ultraviolet (UV) treatment

Table 5-2 shows UV inactivation of *G. stearothermophilus* spores in water. With one pass at 150 ml/min, approx. 4 log reduction was obtained which increased to greater than the detection limit of 5.3-log reduction following the second pass. In water, very high level of inactivation of spores was observed even at low dosage as UV transmission in water is very high.

In comparison to water, the high UV absorption in different milk types (Table 5-3) resulted in a low inactivation of spores. Absorption coefficient (cm^{-1}) of UV in liquid media was measured using UV/Vis Spectrophotometer at three different temperatures of 4 ^{o}C, 20 ^{o}C and 50 ^{o}C. These measurements were done to understand if temperature possesses any effect on transmission that is mainly attributed to macromolecules present in the liquid. In SCM, an increase in absorption coefficient was observed with the increase in temperature from 4 ^{o}C to 50 ^{o}C (values are significantly different $p < 0.05$) and hence resulted in slightly decrease in inactivation values. In WCM and SM, absorbance was lower at 50 ^{o}C than at 4 ^{o}C (values are significantly different $p < 0.05$). Matak *et al.* (2007) explained that solubilization of fat crystals at higher temperature in these two milk resulted in a decrease in absorbance. Further, as expected, absorptivity was highest in sheep milk (337.30 ± 6.95 cm^{-1}) and lowest in skim milk (170.60 ± 0.10 cm^{-1}) at 20 ^{o}C showing higher transmittance in skim milk and lower transmittance in sheep milk. This lower transmittance in sheep milk results in lower inactivation of spores due to limited exposure. Also, an increase in the inactivation was observed on the increase in inlet temperature from 4 ^{o}C and 20 ^{o}C ($p < 0.05$), which can be

explained by combined effect of temperature and improved transmittance. Therefore, subsequent experiments were done at an inlet temperature of 20 ^{o}C to consider ambient conditions during milk collection.

Figure 5-3 shows spore inactivation in SCM, WCM and SM using UV-C treatment under ambient conditions with increase in dosage. There are multiple reasons for the difference in inactivation trends observed in SCM, WCM and SM. First, transmittance was highest for SCM followed by WCM and SM. Secondly, increase in total solids resulted in a decrease in Reynold number (Re = 1382 for skim milk, Re = 977 for whole milk and Re = 687 for sheep milk at 20 ^{o}C) at the same flow rate. Therefore, for the same dosage, the UV transmittance and its effectiveness in inactivation of spores is higher in skim milk than for whole milk and sheep milk. In addition, a coiled tube reactor induces secondary flow referred as Dean flow in addition to turbulence assisting high inactivation with UV. In all cases, De number was higher than 150 signifying the occurrence of secondary flow during UV treatment. Further, multiple passes provided the possibility of intimate mixing of spore suspension in between treatment and thereby, provided the opportunity for better exposure to UV during treatment (Bandla *et al.*, 2012a). In addition, pulsatile flow from pump also responsible for better mixing and hence, better UV exposure (McDonough *et al.*, 2019). Also, viscosity of these milk were different making Reynold number higher for SCM and lower for SM. In result, exposure to Ultraviolet was higher for SCM and lowest for SM.

Table 5-2: Ultraviolet inactivation of *G. stearothermophilus* spores in water with coiled tube reactor at a flow rate of 150 ml/min

WATER				
No. of passes	**Reynold number**	**Dosage (J/ml)**	**Residence time (sec)**	**Log inactivation**
One	1989	1.61	6.1	3.88 ± 0.11
Two		3.23	12.3	> 5.3

Table 5-3: Absorption coefficient (cm^{-1}) and subsequent inactivation of *G. stearothermophilus* spores ATCC 7953 at different inlet temperatures at flow rate of 150 ml/min

Media	Inlet Temperatures	Absorption coefficient at inlet temperatures (cm^{-1})	Inactivation at 4.84 J/ml
SCM	4 ºC	164.47 ± 0.76	4.11 ± 0.22
	20 ºC	170.60 ± 0.10	3.09 ± 0.15
	50 ºC	184.50 ± 0.66	3.84 ± 0.30
WCM	4 ºC	324.43 ± 0.88	1.47 ± 0.27
	20 ºC	326.13 ± 1.50	1.81 ± 0.16
	50 ºC	310.00 ± 3.73	1.70 ± 0.05
SM	4 ºC	391.97 ± 14.26	0.24 ± 0.08
	20 ºC	337.30 ± 6.95	0.45 ± 0.04
	50 ºC	308.73 ± 13.23	0.29 ± 0.03

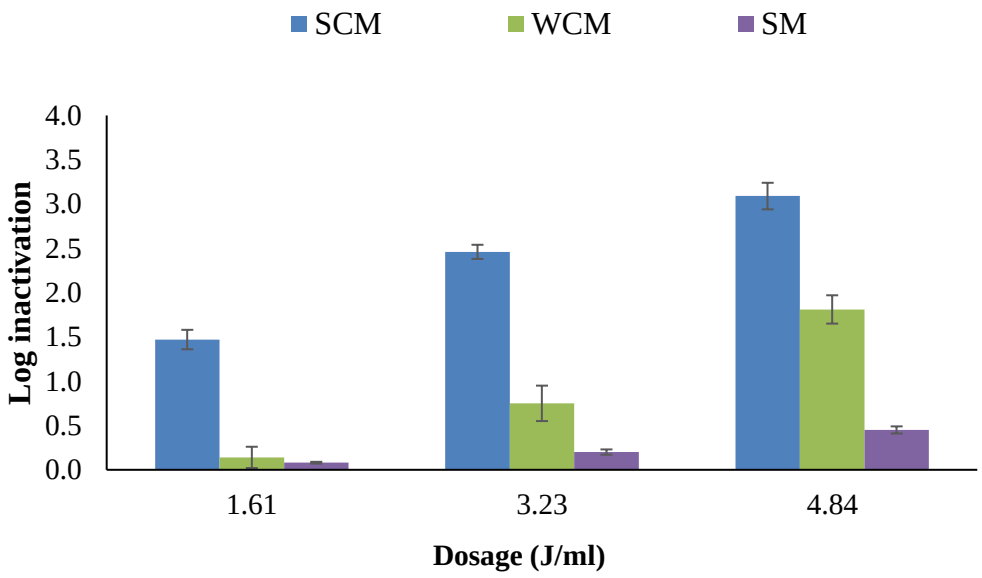


Figure 5-3: Inactivation of *G. stearothermophilus* ATCC 7953 spores in SCM, WCM and SM at a flow rate of 150 ml/min.

5.3.3. UV pre-treatment followed by thermal treatment

UV pre-treatment followed by heat was applied to obtain a hurdle effect and thereby to enhance spore inactivation as relevant to sterilization. The combination of UV pre-treatment (4.84 J/ml at 150 ml/min) followed by heat (120 ºC for 20 sec) gave more than 5 log reduction in *G.*

stearothermophilus spores in SCM as shown in Figure 5-4. This synergistic inactivation could be explained as heat possibly damaged the repair mechanism after UV treatment which was associated with enzyme Spore photoproduct lyase (Spl) as discussed in Section 5.1. However, this cannot be confirmed unless extended shelf life measurements are done. Table 5-4 shows the inactivation of *G. stearothermophilus* spores when UV treatment (4.84 J/ml, 150 ml/min) was followed by thermal treatment at 120 ºC, for different times. As expected, SCM gave the highest log reduction of 5.38 at the lowest heating time of 10 sec. while, WCM and SM required much more treatment time to inactivate due to their low UV transmission. However, an incremental increase in inactivation of spores with heat was observed with increase of time in all types of milk showing that UV treatment sensitized spores.

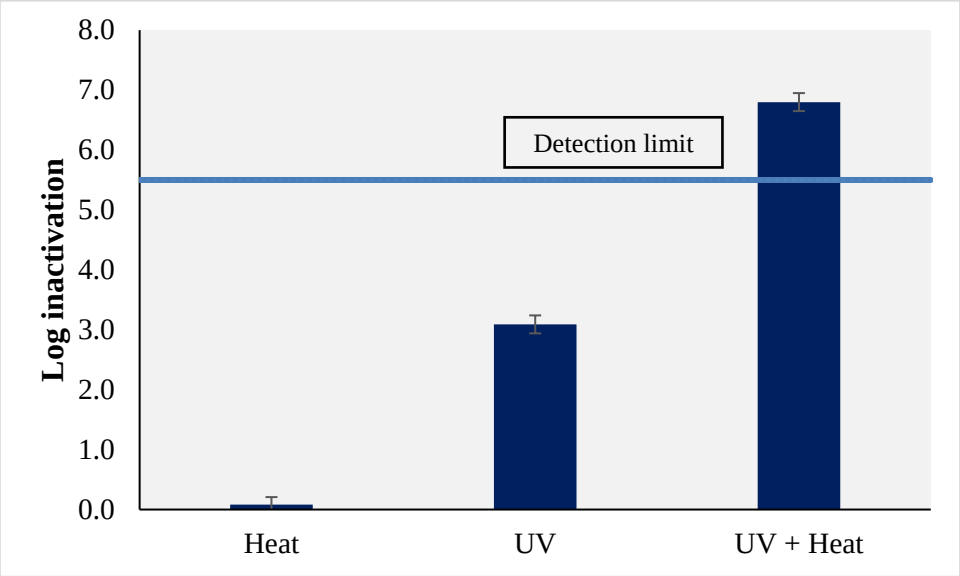


Figure 5-4: Inactivation of *G. stearothermophilus* ATCC 7953 spores in SCM. Heat: 120 ºC for 20 sec, UV: 4.84 J/ml at flow rate of 150ml/min, UV + Heat: UV (4.84 J/ml at flow rate of 150 ml/min) + Heat (120 ºC for 20 sec).

Table 5-4: Inactivation of *G. stearothermophilus* ATCC 7953 spores in different types of milk after Ultraviolet treatment (4.84 J/ml—with three passes, 150 ml/min) followed by thermal treatment at 120 ºC for different times.

Treatment time at 120 ºC	Log inactivation (UV + Thermal)		
	SCM	WCM	SM
10 s	5.38 ± 0.01	1.91 ± 0.17	0.59 ± 0.17
1 min		2.05 ± 0.26	0.80 ± 0.11
2 min	---	2.51 ± 0.22	0.94 ± 0.15
3 min		2.70 ± 0.24	1.08 ± 0.11

5.4. Conclusion

The potential of using UV in combination with heat as an alternative method of sterilization was investigated in this work. It was found that UV absorptivity depends on temperature and has a major effect on the inactivation of *G. stearothermophilus* in SCM, WCM and SM. The results found in this work shows that UV treatment sensitized spores, making thermal treatment more efficient, particularly in SCM. The effect was not so pronounced in WCM and SM. Therefore, this combination of technology can provide an alternative to conventional thermal sterilization, mostly for SCM while other high solid content milk would require more work to improve the design of the UV reactor.

CHAPTER 6

Extension of shelf life of pasteurized trim milk using ultraviolet treatment

This chapter is based on the draft of the paper ready for submission to an international journal.

Preface

The demand for minimally processed foods has increased in the last few years and gains high acceptability among consumers as it has better nutritional value than processed foods. Pasteurized milk is minimally processed and consumed largely for its fresh taste and higher nutritional value compared to Ultra High Temperature (UHT) and powdered milk. However, one of the constraints is its limited shelf life under refrigeration as it cannot retain quality and safety for more than 14 days. Emerging technologies can extend the shelf life of milk while using low energy. Ultraviolet (UV) is well known to inactivate spores as well as vegetative cells. In this study, it was shown that 2.64 J/ml of UV-C treatment applied on pasteurized trim milk (PTM) can extend shelf life up to 53 days under refrigeration. This finding was also supported by the inactivation of 3.40 ± 0.14 log of thermo-resistant *G. stearothermophilus* spores (ATCC 7953) in milk with similar UV operating conditions. Therefore, microbial study together with physicochemical properties demonstrated that pasteurization followed by UV can enhance the shelf life of trim milk considerably.

Industrial relevance: UV treatment consumes very low energy and can extend the shelf life of milk as per the market requirement. In addition, UV treatment can reduce carbon emission making it environmentally friendly compared to conventional thermal technologies. Also, it requires low installation cost which makes it suitable for both small and large scale production, particularly for developing countries.

6.1. Introduction

UHT sterilization and HTST pasteurization treatments are used to preserve milk thereby, extending its shelf life. UHT sterilization is carried out by exposing milk to 135 ^{o}C for a few seconds to achieve sterilization while HTST pasteurization is achieved by treating milk at 72 ^{o}C for 15 sec to inactivate enzymes and pathogenic microorganisms. UHT sterilization results in the production of many volatile sulfur compounds and imparts cooked flavour in milk although it increases its shelf life up to 180 days (Al-Attabi *et al.*, 2009). On the other side, HTST pasteurized milk tastes similar to fresh milk and considered as minimally processed but has a shelf life of only 14 days under refrigeration. This limited shelf life of HTST pasteurized milk makes it difficult to transport from sites of milk production to consumers. For e.g. there is an increase in demand for minimally processed milk from Australia and New Zealand to

Asian countries. However, logistics and transportation of pasteurized milk between different continents of the world is challenging with a shelf life of only 14 days.

This demand for minimally processed milk in export markets has resulted in an interest in Extended Shelf Life (ESL) milk. ESL offers an option between HTST pasteurized milk and UHT sterilized milk, which is shelf stable. ESL processing requires a 'total quality approach' i.e. the ability to manage the entire chain. Some of the key differences between ESL milk and HTST pasteurized milk are in the quality of raw milk, degree of microbial inactivation and packaging conditions. It is essential that control measures are in place at farm level to obtain good quality milk (Doll *et al.*, 2017). Further during ESL milk production, it is required to inactivate psychrotrophic bacteria and its spores together with pathogenic microbes with minimal chemical changes (Deeth, 2017). Psychrotrophic bacteria are mainly responsible for spoilage under refrigerated conditions. The spores of some of these psychrotropic bacteria in milk (*Bacillus* spp , *G. stearothermophilus, B. licheniformis, B. subtilis, B. coagulans* and *B. cereus*) are considered thermoresistant and could survive HTST pasteurizaion treatment (Samaržija *et al.*, 2012). Therefore, it is vital to inactivate them, to achieve shelf life of milk up to 60 days. In addition to that, aseptic packaging plays an important role in avoiding recontamination after processing.

Current ESL milk production methods employ bactofugation or microfiltration with HTST pasteurisation, which provide shelf life of 21-30 days (Table 6-1). This shelf life is not sufficient to access export markets other than through air freight at high cost. On the other hand, use of emerging technologies together with HTST pasteurization possess the capability of producing ESL milk with a longer shelf life, as the combined technologies create synergy to inactivate spores together with pathogens at a higher degree in comparison to present technologies. Namely, Pulse electric field, Cold plasma, Ultra high pressure homogenization and Ultraviolet are well known to inactivate spores when combined with thermal treatment (Alkhafaji *et al.*, 2012; Bandla *et al.*, 2012b; Choudhary *et al.*, 2011; Dobrynin *et al.*, 2010; Georget *et al.*, 2014a). Only a few studies assess the use of these technologies for producing ESL milk. Sepulveda *et al.* (2005) investigated pulsed electric field processing on HTST pasteurized milk in order to achieve shelf life of up to 60 d. In a different study using argon gas plasma, no significant change was reported in bacterial count after 6 weeks in comparison to pasteurized milk (Ponraj *et al.*, 2017).

Ultraviolet (UV) is well known for its antimicrobial properties and possesses the capability to inactivate spores. However, UV has low transmission through opaque liquids making it

difficult to treat liquid milk (Datta *et al.*, 2015). In the last few years, different thin film reactors were designed and studied to improve spore inactivation. Coiled tube UV reactor is one of the reactors which induces circular flow referred as Dean Flow and improves mixing which makes it more efficient to inactivate spores (Bandla *et al.*, 2012a; Choudhary *et al.*, 2011; Dean, 1927). Koutchma *et al.* (2013) suggested that UV-C treatment followed by pasteurization can result in an improved shelf life by inactivating spores. Further, chemical characterization study of UV treatment before or after HTST treatment showed no change in raw milk in relation to proximate analysis, FA profile, oxidation or protein profile while considering 14 days of storage (Cappozzo *et al.*, 2015).

Even though HTST pasteurization treatment can inactivate some enzymes as well as pathogenic microorganisms in milk, the survival of psychtrophic spores limits its shelf life as explained previously. To the best of our knowledge, no study has been done using UV treatment on HTST pasteurized milk to enhance the shelf life of milk. Therefore, the objective of this study is to investigate the application of UV treatment on HTST pasteurized milk to extend its shelf life under refrigerated condition.

Table 6-1: Comparison of different processes to enhance shelf life of milk (Rysstad *et al*., 2006)

Process	Treatment conditions applied	Log reduction in aerobic psychrotrophic spores	Expected shelf life	Drawbacks
Pasteurization	72 ^{o}C for 15 sec	None	Up to 14 days	No spore reduction
Bactofugation	Pasteurization in combination with bactofugation	1-2	Up to 21 days	Limited spore removal
Microfiltration	Filtration through membrane followed by pasteurization	2-3	Up to 30 days	Fat need to be pasteurized separately as it cannot pass through

				membranes and limited spore removal
UHT treatment	135 ºC for a few sec	Greater than 8	180 days	High temperature treatment required that results in undesirable changes in quality.

6.2. Materials & methods

6.2.1. Media/Food matrix

HTST pasteurized trim milk (PTM) was obtained from a local supermarket in 3 L bottles. According to labelled composition, it had 3.7 g protein, 0.4 g fats, 4.9 g carbohydrates, 45 mg Sodium and 130mg Calcium in 100 ml of milk. UHT-trim milk (UHT-TM) was obtained in 1 litre tetra packs from a local super market for study on spore inactivation with labelled composition as 3.4 g protein, 0.1 g fat, 4.9 g carbohydrates, 45 mg Sodium and 125 mg Calcium.

6.2.2. Bacterial strain and sporulation

The bacterial strain (*G. stearothermophilus*, ATCC 7953) was obtained from New Zealand reference culture collection (NZRM, New Zealand). *G. stearothermophilus* was selected in this study since it is known as a thermoresistant psychrotrophic bacteria (Samaržija *et al.*, 2012). The freeze-dried culture of *G. stearothermophilus* (ATCC 7953) spores was transferred to cooked meat medium and incubated at 55 ºC for 24 h. A wire loop from incubated suspension was plated on nutrient agar (NA) plates and further incubated at 55 ºC for 24 h. Multiple colonies were transferred to TYE broth (Tryptone 1%, Yeast extract 0.5%, Dipotassium hydrogen phosphate 0.2%) and incubated in shaking incubator at 55 ºC (250 rpm) for 24 h. Aliquots of 0.2 ml of inoculated broth after incubation was spread plated to sporulation medium (23 g of NA (Difco), 1 mg Manganese sulphate (Merck) and 0.5 g Calcium chloride (RDH) for

a litre) and incubated at 55 ^{o}C for 3 days sealed in polyethylene bags to avoid dehydration. These bags were then taken out from the incubator and were placed under ambient conditions for further three days. Then sporulation was monitored by phase contrast microscopy (Motic microscope BA410 series, Canada) as described by Evelyn *et al.* (2015b). After 75 % of sporulation, spores were harvested by washing the plates with sterilized water and the resulting spore suspension was centrifuged at (4800 g, 4 ^{o}C , 15 min) for three times. The obtained suspension was pasteurized at 80 ^{o}C for 10 min to inactivate vegetative cells and subsequently refrigerated at 4 ^{o}C until further use.

6.2.3. UV equipment and experimental plan

6.2.3.1. UV experiments

UV equipment

UV treatment of milk was carried out using a coiled tube reactor designed at University of Auckland as described by Choudhary *et al.* (2011). A Perfluoroalkoxy (PFA) tube (ID 1.6 mm, 3.2 mm, length 7.62 m) obtained from Thermofischer scientific, New Zealand was coiled over the quartz sleeve (EQS 450, ID 21.7 mm, OD 24.4 mm, Davey water products, New Zealand) enclosing the UV-C lamp (EGPH369N/S, UV-C power 5.6 W, Lamp power 19 W) positioned in the centre. The whole setup was placed inside a stainless steel barrel to avoid any exposure to environment during UV treatment. PFA tube was used as it possesses high transmittance (80 % transmission), stability to high temperature and chemicals as mentioned by Choudhary *et al.* (2011)

UV Equipment Operation and cleaning

A peristatic pump was used for pumping milk through the UV reactor at a flow rate of 9 L/h for microbial and physico-chemical studies. Prior to any experiment, UV lamp was switched on for 5 min as warm up time. The unit was sanitized by passing ethanol (70 % v/v) for 2-3 min and then sterilized water was passed for another 5 min to remove any traces of ethanol. This rinsed water was collected into a sterilized container and spread plated to ensure proper cleaning as described in section 6.2.4.1. After UV treatment of milk, Oxonia solution was passed through the reactor for 5 min, followed by ethanol for 2-3 min and rinsed with sterilized water for 5 min.

UV dosage using actinometry

Actinometry can be used for the determination of UV energy delivered to liquid. UV treatment results in triiodide formation and should be expressed by the following equation.

Equation 6-1

$$8KI + KIO_3 + 3H_2O + hv \rightarrow 3I_3^- + 6OH^- + 9K^+$$

It was done by treating aqueous solution of 0.6 M potassium iodide and 0.1 M potassium iodate in 0.01 M borate buffer (pH 9.25) at the specified flow rate (9 L/h) as described by Rahn *et al.* (2003) and Müller *et al.* (2014). The UV dose (D_v in J/L) was calculated by using the following relation:

Equation 6-2

$$D_v = \frac{A_{352\,nm} \times P_{253nm}}{pl \times \emptyset \times \varepsilon_{352\,nm}}$$

Where $A_{352\,nm}$ represents measured absorbance at 352 nm, $P_{253.7\,nm}$ are the number of Joules per Einstein of 253.7 nm photons (4.716×10^5 J einst^{-1}), pl is the path length of the quartz cuvette (1 cm), $\emptyset$ is the quantum yield (mol einst^{-1}) and $\varepsilon_{352\,nm}$ is the molar absorption coefficient of triiodide at 352 nm (27600 dm^{3}mol^{-1})

A correction to quantum yield was applied using the initial temperature (^{o}C) and concentration of the iodide ($A_{300\,nm} \times 1.061^{-1}$)

Equation 6-3

$$\emptyset = 0.73 \times (1 + 0.23 \times [c_i - 0.577]) \times (1 + 0.02 \times [T_i - 20.7^oC])\ Eq\ (3)$$

6.2.3.2. Experimental design

In the first part of the study, Pasteurized trim milk (PTM) was passed multiple times through the UV reactor and was labelled as PTM- UV_1, PTM-UV_2 and PTM-UV_3 for one, two and three passes respectively as shown in Figure 6-1. Then the treated samples were stored under refrigeration at 4 ^{o}C for the shelf life study. Samples were taken out at different time intervals up to 63 days and analysed for total microbial count (TMC) and physico chemical characteristics (pH, conductivity, titratable acidity, and colour).

In the later part of the study, the effect of UV treatment (different dosages) on inactivation of *G. stearothermophilus* spores were analysed with UHT-TM. UHT milk was considered for spore inactivation study as it provides the closest possible sterile source of milk. This second

part of the study was helpful to understand the extension of shelf life resulted on PTM milk from UV treatment.

All the handling and collection of milk was done in a biosafety cabinet to avoid any contamination during/after UV treatment. Further, transfer and filling of UV-treated milk into sterilized containers was also carried out in biosafety cabinet. To ensure sterile environment, microbial count of air was taken by placing an opened agar plate for 2 h in the biosafety cabinet and was later incubated at 32 ^{o}C.

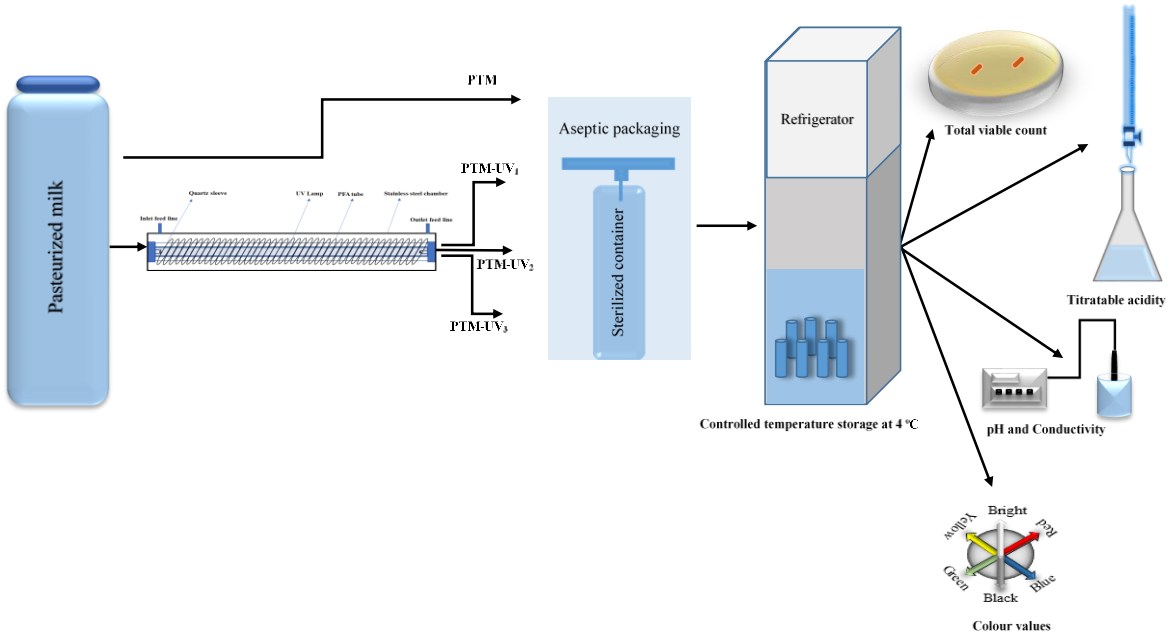


Figure 6-1: Schematic representation of the experimental setup for ESL milk treatment

6.2.4. Microbiological and physico chemical studies

6.2.4.1. Microbial counts

For the ESL study, TMC of the samples was done by spread plating with appropriate dilutions on plate count agar (DIFCO) and incubated at 32 ^{o}C (24-48 h). Meanwhile *G. stearothermophilus* spores, counting was done by spread plating appropriate dilutions of treated and untreated samples on NA agar plates and incubated at 55 ^{o}C for 24-48 h.

6.2.4.2. Physico-chemical analysis

Chemical and physical parameters of PTM, PTM-UV_1, PTM-UV_2 and PTM-UV_3 were analysed as described below.

pH and titratable acidity

For determination of pH, milk samples were transferred to a 100 ml beaker and pH electrode was immersed until constant reading was obtained. Prior to pH measurements, pH meter was calibrated using standard solutions (pH 4.0, pH 7.0, pH 10.0). The titratable acidity was measured by using AOAC method (AOAC 947.05) to analyse the effect of acidity due to UV treatment. Milk sample (10-15 g) was weighed in conical flask and few drops of phenolphthalein (1 % w/v in 50 % ethanol) were added. Finally, it was titrated with 0.1 N NaOH until faint pink colour persists for 30 sec. Sodium hydroxide was standardized by using Potassium hydrogen phthalate (AOAC 936.16). Results were expressed as % lactic acid using the following relationship

1 ml of 0.1 N NaOH = 0.009 g lactic acid

Conductivity

Conductivity is considered as an important tool which is related to microbial growth. Fermentation of lactose and conversion to lactic acid in a media sharply increases its conductivity (Mucchetti *et al.*, 1994). The conductivity (mS/cm) of milk samples was measured using conductivity meter (Seven Compact TM Conductivity S230) by immersing the probe in milk samples until a constant reading was obtained.

Colour

The colour of food is an integral part and is a significant factor in determining its acceptance. Therefore, it is important to find out the effect of UV treatment on the change of colour in milk during storage. It was measured by using CR-400 Chroma meter as described by Makwana (2016). The colour values were obtained using CIE (*Commission Internationale d'Eclairage*) colour scale. Calibration was performed by using calibration plate (CR A43) with a white background. The numerical values of L^* , a^* and b^* are used to calculate the total colour difference (TCD)) as given by the equation.

Equation 6-4

$$\Delta E^* = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$$

Where ΔL^* , Δa^* *and* Δb^* shows the difference between values obtained from pasteurized milk and UV treated milk samples.

Also, L, a, b were measured using ANLAB lab coordinates and TCD was calculated as follows

Equation 6-5

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \quad Eq\ (5)$$

Where ΔL, Δa and Δb shows difference between values obtained from pasteurized milk and UV treated milk samples.

6.2.5. Statistical analysis

Statistical treatment of data was performed using Microsoft excel 2016. Mean and standard deviation were calculated from triplicate reading. A Tukey test was done to find out statistically different values using confidence level of 95 %.

6.3. Results and discussion

6.3.1. Microbial analysis

The success of a potential ESL technology lies in the extent of reduction in psychotropic spores in addition to pathogenic organisms. Therefore, it is required to assess both aspects i.e. TMC during storage and extent of spore inactivation. Thereby, in the first part of this study, TMC of HTST pasteurized milk after UV treatment was measured during refrigerated storage for up to 63 days. In the second part, similar UV operating conditions were assessed in inoculated UHT milk for *G. stearothermophilus* spore inactivation.

Total microbial count during storage:

In this study, PTM was treated with UV to target surviving spores and vegetative cells. Figure 6-2 shows the microbial population of post pasteurization treatment using UV at different dosages (PTM- UV_1, PTM -UV_2 and PTM-UV_3). The dosages were measured using actinometry and was calculated using equation (2) to be 0.88 J/ml, 1.76 J/ml and 2.64 J/ml for PTM- UV_1, PTM -UV_2 and PTM-UV_3 respectively. A count of less than 20,000 CFU/ml (4-log) of milk is considered as A grade quality pasteurized milk (USFDA, 2009). It was found that PTM and PTM-UV_1 (0.88 J/ml of UV) resulted in 4-log count less than 26 days. However, with further UV treatment, it increased to 41 days (PTM-UV_2 with 1.76 J/ml). With three passes through UV reactor (2.64 J/ml of UV), it took 54 days to reach 4-log count. It was

observed that different extent of shelf life can be obtained by varying the dosage of UV treatment. In general, the end of shelf life is attained when the count reaches $10^6 – 10^7$ CFU/ml and results in unacceptable sensory changes in milk (Silcock *et al.*, 2014). Commercially pasteurized trim milk is usually labelled for two weeks of shelf life. In comparison, this study showed that UV treated pasteurized trim milk could be extended up to 54 days before it reaches the limits of Grade A quality.

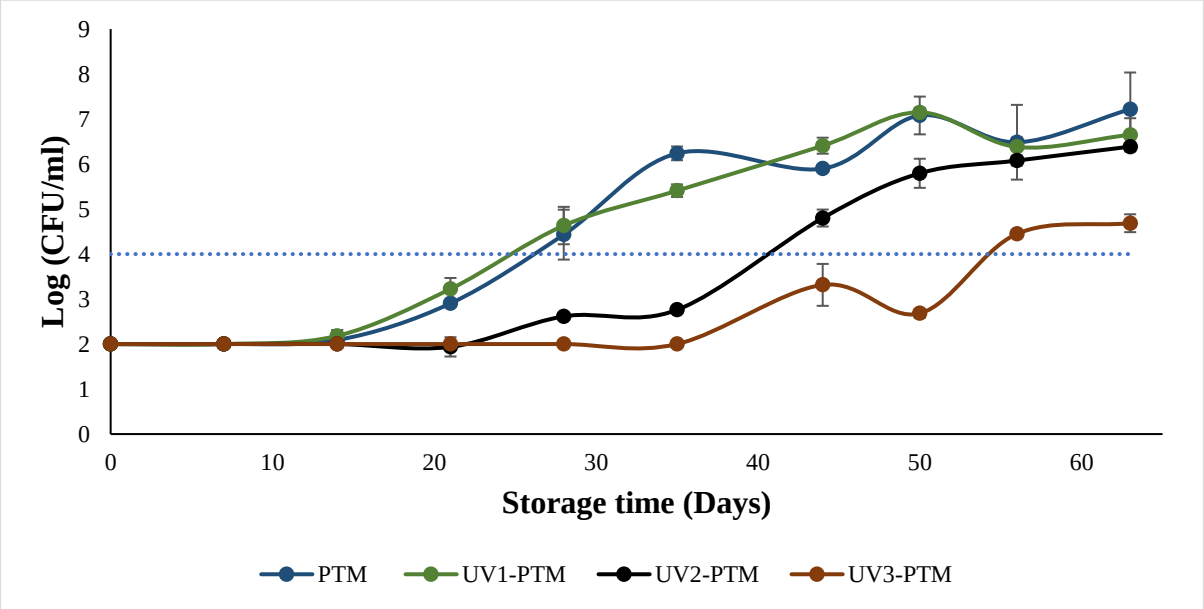


Figure 6-2: Total microbial count (TMC) of UV-treated milk stored at 4 ^{o}C for up to 63 days

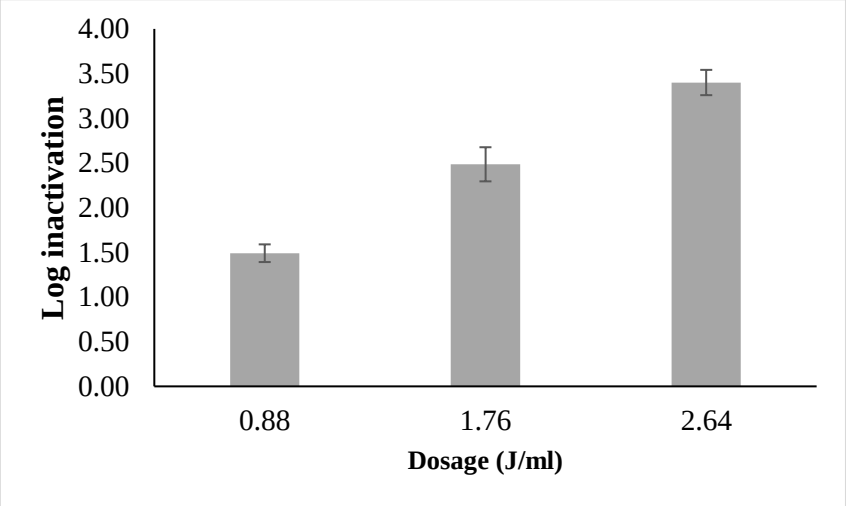


Figure 6-3: Inactivation of *G. stearothermophilus* ATCC 7953 spores in UHT-TM at a flow rate of 9 L/h with UV treatment.

***G. stearothermophilus* spores inactivation:**

Figure 6-3 shows the inactivation of *G. stearothermophilus* spores in inoculated UHT-TM. Inactivation of spores increased with the applied UV dosage when circulated at the same flow rate of 9 L/h. *G. stearothermophilus* spores is considered as one of the most heat resistant spores and 3.40 ± 0.14 log inactivation was observed after three passes through the UV coiled tube reactor. Similarly, Choudhary *et al.* (2011) obtained 2.72 log reduction in *B. cereus* spores in skimmed cow milk with 11.187 mJ/cm^2 (11.3 sec). Therefore, UV treatment can be explained on the basis of its ability to inactivate spores that has survived pasteurization conditions. Thus, combination of pasteurization followed by UV treatment increased microbial inactivation and thereby contributed towards extension of the shelf life of milk considerably.

6.3.2. Physicochemical analysis

Physicochemical changes are attributed due to multiple reasons including oxidative reactions, enzymatic hydrolysis (proteinases and lipases) and production of microbial metabolites. Microbial metabolites are produce by bacteria that can metabolize milk components to form unwanted products. Lactic acid bacteria can metabolize lactose to lactic acid and results in pH drop that can results in subsequent precipitation of proteins (Lu *et al.*, 2017). Also, by products from lactose degradation can result in "sour" and "gassy" defects. Table 6-2 shows pH measurements over time for PTM, PTM-UV$_1$, PTM-UV$_2$ and PTM-UV$_3$ under refrigerated storage at 4 ^{o}C. It was observed that pH dropped from 6.68 ± 0.01 to 6.24 ± 0.01 after 63 days of storage for PTM. ($P < 0.05$) However, PTM-UV$_3$ did not result in statistically significant difference in pH even after 63 days storage ($P > 0.05$). This can be explained on the basis that UV treatment with three passes inactivated microorganisms considerably which also resulted in less changes in pH. It is important to consider natural buffering system present in milk which resists change in pH up to 45 days (Salaün *et al.*, 2005). Therefore, titratable acidity was measured for the samples as shown in Figure 6-4. For all the samples, titratable acidity remains below 0.2 % for up to 45 days of storage. Sepulveda *et al.* (2005) mentioned that, sensory changes can be observed when the % of lactic acid is above 0.2. Accordingly, lactic acid increased above 0.2 % for PTM and PTM –UV$_1$ after 45 days while PTM-UV$_2$ reached this threshold value after 55 days. However, this value in PTM-UV$_3$ was maintained below 0.2 % of lactic acid even after 63 days of storage. This is also supported by microbial count results as shown in Figure 6-2 as PTM-UV$_3$ has the lowest TMC among the four samples after 63 days.

Table 6-2: pH measurements obtained for PTM, PTM-UV_1, PTM-UV_2 and PTM-UV_3 stored at 4 ^oC

Day	pH			
	PTM	PTM-UV_1	PTM-UV_2	PTM-UV_3
0	6.68 ± 0.01	6.69 ± 0.01	6.70 ± 0.01	6.68 ± 0.01
7	6.68 ± 0.01	6.68 ± 0.00	6.70 ± 0.01	6.69 ± 0.01
14	6.68 ± 0.01	6.68 ± 0.00	6.68 ± 0.01	6.68 ± 0.00
21	6.72 ± 0.01	6.70 ± 0.00	6.71 ± 0.00	6.70 ± 0.01
28	6.65 ± 0.00	6.61 ± 0.00	6.65 ± 0.01	6.65 ± 0.01
35	6.69 ± 0.01	6.70 ± 0.01	6.72 ± 0.01	6.71 ± 0.01
44	6.66 ± 0.01	6.56 ± 0.01	6.67 ± 0.01	6.71 ± 0.00
50	6.38 ± 0.01	6.21 ± 0.02	6.68 ± 0.01	6.80 ± 0.02
56	6.35 ± 0.00	6.52 ± 0.01	6.39 ± 0.01	6.74 ± 0.01
63	6.24 ± 0.01	6.20 ± 0.02	6.22 ± 0.01	6.67 ± 0.02

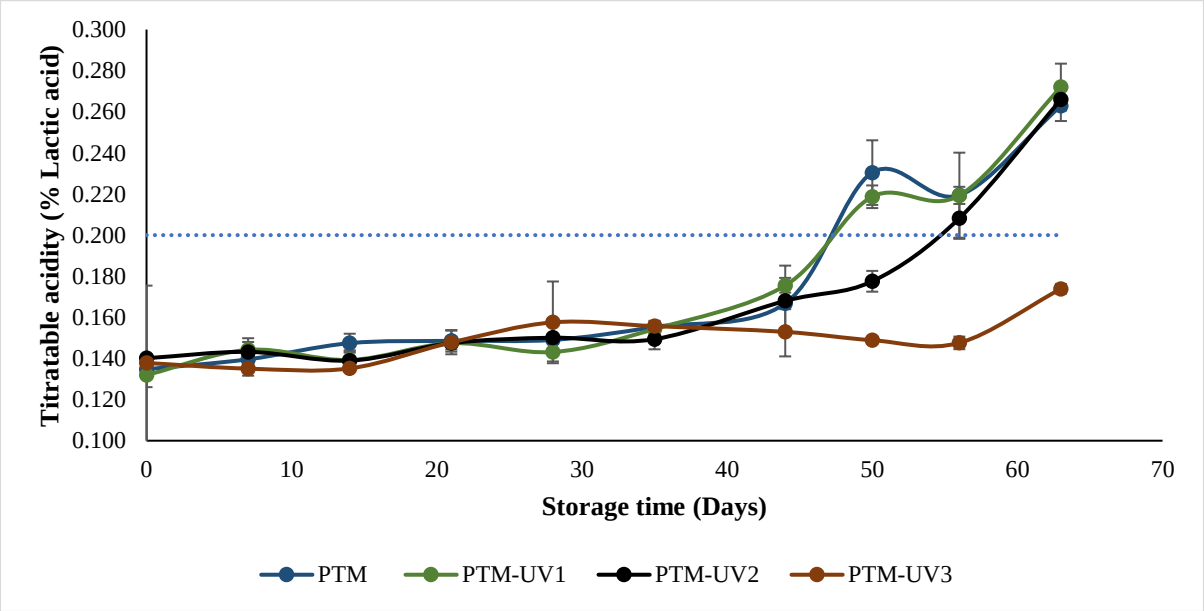


Figure 6-4: Titratable acidity of milk stored at 4 ^oC for upto 63 days

Electrical conductivity of milk is an important parameter and relates to the bacterial contamination. Conductivity is also used to study the changes caused by lactic acid bacteria (Lanzanova *et al.*, 1993). Correlation between acidification and conductivity is considered positive and increase in conductivity results in an increase in acidity (Rug-Lenartowicz, 1954). Table 6-3 shows the conductivity measurements for the milk (PTM, PTM-UV_1, PTM-UV_2 and

PTM-UV_3) with time during controlled temperature storage of 4 ^oC. It was found that conductivity for PTM was increased from 5.23 ± 0.01 to 5.63 ± 0.02 ($P < 0.05$). Similar trends was obtained for PTM-UV_1 (5.19 ± 0.01 to 5.48 ± 0.01), PTM-UV_2 (5.15 ± 0.01 to 5.62 ± 0.02) and PTM-UV_3 (5.14 ± 0.01 to 5.31 ± 0.01) ($P < 0.05$). However, the change in conductivity for PTM-UV_3.was very much less and is coherent with the earlier findings of pH and acidity measurements.

Colour change of milk during storage is considered important and have influence on human perception. Therefore, changes in colour after UV treatment were noted and compared with PTM during storage. Table 6-4 and Table 6-5 shows colour values during storage of samples using CIE and ANLAB colour system. With UV-C treatment, L* and L value were decreased slightly for all of the samples, which shows decrease in lightness of milk during storage. The negative a* value represents green and positive represents red, which shows all of the samples having green shade. The negative b* value denotes blue and positive value represents yellow, which shows that observed values for all samples have blue colour. Total colour difference (ΔE*) value of around 2.3 corresponds to just noticeable difference (Sharma *et al.*, 2002). However, a value lower than 2.3 of colour difference were observed while comparing PTM samples with UV treated samples. This shows that UV treatment did not affect colour value of milk samples.

Table 6-3: Conductivity measurements obtained for PTM, PTM-UV_1, PTM-UV_2 and PTM-UV_3 stored at 4 ^oC

Day	Conductivity (mS/cm)			
	PTM	**PTM-UV_1**	**PTM-UV_2**	**PTM-UV_3**
0	5.23 ± 0.01	5.19 ± 0.01	5.15 ± 0.01	5.14 ± 0.01
7	5.24 ± 0.01	5.23 ± 0.02	5.22 ± 0.02	5.23 ± 0.03
14	5.47 ± 0.02	5.40 ± 0.02	5.40 ± 0.02	5.40 ± 0.03
21	5.45 ± 0.01	5.46 ± 0.02	5.47 ± 0.02	5.42 ± 0.02
28	5.40 ± 0.03	5.46 ± 0.02	5.41 ± 0.01	5.42 ± 0.03
35	5.44 ± 0.01	5.41 ± 0.01	5.35 ± 0.02	5.37 ± 0.01
44	5.49 ± 0.02	5.58 ± 0.02	5.44 ± 0.01	5.39 ± 0.01
50	5.58 ± 0.08	5.77 ± 0.05	5.26 ± 0.04	5.17 ± 0.03
56	5.61 ± 0.04	5.49 ± 0.04	5.64 ± 0.03	5.29 ± 0.02
63	5.63 ± 0.02	5.48 ± 0.01	5.62 ± 0.02	5.31 ± 0.01

Table 6-4: Colour measurements obtained for PTM, PTM-UV_1, PTM-UV_2 and PTM-UV_3 stored at 4 ^oC using CIE system

Day	Colour															
	PTM				PTM-UV_1				PTM-UV_2				PTM-UV_3			
	L*	A*	B*	ΔE*	L*	A*	B*	ΔE*	L*	A*	B*	ΔE*	L*	A*	B*	ΔE*
0	80.39 ± 1.28	-5.61 ± 0.53	0.85 ± 1.35	--	81.18 ± 0.53	-5.63 ± 0.25	1.85 ± 0.63	1.28	81.20 ± 0.15	-5.58 ± 0.06	1.95 ± 0.13	1.37	80.64 ± 0.32	-5.33 ± 0.32	1.32 ± 0.35	0.60
7	81.29 ± 0.17	-6.22 ± 0.04	2.67 ± 0.26	--	81.25 ± 0.04	-6.11 ± 0.05	2.90 ± 0.05	0.25	81.38 ± 0.03	-5.97 ± 0.02	3.17 ± 0.03	0.57	80.97 ± 0.43	-5.80 ± 0.04	2.53 ± 0.40	0.55
14	80.62 ± 1.71	-5.85 ± 0.29	2.96 ± 0.07	--	80.77 ± 0.11	-5.72 ± 0.06	2.41 ± 0.06	0.58	81.17 ± 0.23	-5.76 ± 0.09	2.68 ± 0.32	0.62	80.46 ± 0.29	-5.55 ± 0.08	2.02 ± 0.31	0.99
21	79.77 ± 0.74	-5.65 ± 0.46	1.56 ± 0.81	--	80.62 ± 0.27	-5.89 ± 0.07	2.60 ± 0.20	1.37	80.30 ± 0.40	-5.66 ± 0.09	2.01 ± 0.05	0.70	79.54 ± 0.72	-5.36 ± 0.33	1.38 ± 0.95	0.41
28	79.84 ± 0.21	-5.58 ± 0.10	1.53 ± 0.40	--	80.50 ± 0.51	-5.60 ± 0.33	2.68 ± 0.06	1.32	79.95 ± 0.45	-5.47 ± 0.16	2.06 ± 0.23	0.55	80.49 ± 0.29	-5.51 ± 0.05	2.44 ± 0.07	1.12
35	78.58 ± 0.66	-5.76 ± 0.30	1.70 ± 0.64	--	78.84 ± 0.30	-5.63 ± 0.16	1.86 ± 0.24	0.33	79.47 ± 0.07	-5.75 ± 0.10	2.44 ± 0.08	1.16	79.39 ± 0.13	-5.59 ± 0.12	2.28 ± 0.13	1.01
44	81.63 ± 5.08	-6.20 ± 0.09	2.32 ± 0.26	--	79.37 ± 0.25	-6.26 ± 0.09	2.73 ± 0.20	2.30	79.15 ± 0.06	-6.11 ± 0.04	2.51 ± 0.06	2.48	78.71 ± 0.47	-5.88 ± 0.12	2.46 ± 0.32	2.94
50	79.48 ± 0.30	-5.67 ± 0.36	1.97 ± 0.22	--	79.90 ± 0.43	-5.96 ± 0.13	2.21 ± 0.27	0.56	79.22 ± 0.01	-6.06 ± 0.10	2.32 ± 0.12	0.58	78.91 ± 0.26	-5.85 ± 0.06	2.43 ± 0.13	0.76
56	78.80 ± 0.31	-6.12 ± 0.04	2.30 ± 0.15	--	78.62 ± 0.25	-6.17 ± 0.08	2.65 ± 0.08	0.38	79.43 ± 0.39	-5.80 ± 0.39	2.73 ± 0.11	0.83	78.28 ± 0.04	-5.91 ± 0.11	2.70 ± 0.05	0.69
63	78.75 ± 0.37	-6.23 ± 0.09	2.55 ± 0.17	--	78.45 ± 0.10	-6.25 ± 0.10	2.72 ± 0.13	0.35	79.20 ± 0.28	-5.81 ± 0.38	2.74 ± 0.08	0.64	78.58 ± 0.12	-5.64 ± 0.17	2.55 ± 0.10	0.61

Table 6-5: Colour measurements obtained for PTM, PTM-UV_1, PTM-UV_2 and PTM-UV_3 stored at 4 ^oC using ANLAB colour system

Day	Colour															
	PTM				PTM-UV_1				PTM-UV_2				PTM-UV_3			
	L	a	B	ΔE	L	a	b	ΔE	L	A	B	ΔE	L	a	b	ΔE
0	75.75 ± 1.51	-5.30 ± 0.53	0.80 ± 1.29	--	76.68 ± 0.63	-5.34 ± 0.25	1.77 ± 0.60	1.34	76.70 ± 0.18	-5.30 ± 0.06	1.85 ± 0.12	1.41	76.05 ± 0.38	-5.05 ± 0.15	1.25 ± 0.34	0.59
7	76.81 ± 0.20	-5.87 ± 0.08	2.53 ± 0.24	--	76.68 ± 0.15	-5.64 ± 0.25	2.74 ± 0.05	0.34	76.88 ± 0.03	-5.65 ± 0.01	2.95 ± 0.07	0.48	76.20 ± 0.33	-5.41 ± 0.15	2.25 ± 0.36	0.80
14	75.05 ± 1.62	-5.48 ± 0.29	2.81 ± 0.01	--	76.25 ± 0.04	-5.44 ± 0.01	2.26 ± 0.04	1.32	76.67 ± 0.29	-5.45 ± 0.09	2.69 ± 0.33	1.62	75.86 ± 0.32	-5.24 ± 0.08	1.91 ± 0.29	1.24
21	75.21 ± 0.91	-5.13 ± 0.43	1.54 ± 0.76	--	75.89 ± 0.16	-5.64 ± 0.14	2.42 ± 0.19	1.22	75.42 ± 0.11	-5.31 ± 0.04	1.90 ± 0.06	0.46	75.04 ± 1.15	-5.08 ± 0.35	1.31 ± 0.89	0.28
28	75.29 ± 0.41	-5.27 ± 0.09	1.30 ± 0.36	--	75.77 ± 0.65	-5.42 ± 0.17	2.55 ± 0.06	1.35	75.40 ± 0.50	-5.04 ± 0.23	2.04 ± 0.31	0.79	76.09 ± 0.06	-5.21 ± 0.05	2.25 ± 0.10	1.25
35	73.98 ± 0.62	-5.41 ± 0.29	1.63 ± 0.59	--	74.18 ± 0.08	-5.33 ± 0.07	1.87 ± 0.04	0.31	74.57 ± 0.20	-5.50 ± 0.08	2.28 ± 0.07	0.88	74.52 ± 0.23	-5.26 ± 0.10	2.22 ± 0.08	0.81
44	73.65 ± 0.22	-5.80 ± 0.08	1.86 ± 0.38	--	74.74 ± 0.23	-5.67 ± 0.37	2.41 ± 0.27	1.22	74.31 ± 0.10	-5.54 ± 0.28	2.32 ± 0.10	0.85	74.05 ± 0.37	-5.55 ± 0.13	2.27 ± 0.42	0.63
50	74.80 ± 0.16	-5.51 ± 0.06	1.78 ± 0.15	--	75.44 ± 0.21	-5.49 ± 0.31	2.03 ± 0.25	0.69	74.36 ± 0.03	-5.60 ± 0.07	2.24 ± 0.16	0.64	74.20 ± 0.16	-5.47 ± 0.06	2.38 ± 0.26	0.85
56	73.87 ± 0.37	-5.63 ± 0.16	2.33 ± 0.23	--	73.73 ± 0.25	-5.72 ± 0.05	2.57 ± 0.05	0.29	74.34 ± 0.23	-5.64 ± 0.08	2.49 ± 0.13	0.50	73.30 ± 0.02	-5.54 ± 0.06	2.52 ± 0.05	0.61
63	73.91 ± 0.40	-5.36 ± 0.18	2.25 ± 0.17	--	73.71 ± 0.23	-5.63 ± 0.16	2.68 ± 0.21	0.54	74.35 ± 0.10	-5.52 ± 0.12	2.46 ± 0.12	0.51	73.63 ± 0.46	-5.39 ± 0.12	2.38 ± 0.06	0.31

6.4. Conclusion

The present study evaluated the use of UV treatment to extend the shelf life of commercially pasteurized trim milk. Microbiological and physicochemical studies revealed that the shelf life of milk increased with the increase of UV dosage. It was also shown that the sporicidal effect of UV treatment assisted in obtaining the extension of shelf life of milk approx. up to 50 days. The outcome of this study could help to meet the logistics and transportation challenges in the global trade of ESL milk.

CHAPTER 7

Effect of nitrogen purging on skim milk quality during UV treatment

This chapter is based on the draft of the paper ready for submission to an international journal.

Preface

UV treatment is well known for its antimicrobial effects and current research shows that it has the potential to inactivate microorganisms in milk at much lower temperatures than conventional thermal treatment. However, Ultraviolet irradiation can result in adverse effects on milk quality, which arises due to photo oxidation in the presence of oxygen. Limiting the dissolved oxygen content in milk can minimize oxidative damage and thus, result in better product quality. Nitrogen purging could be an effective method for removing dissolved oxygen from liquids. The present study evaluates the effects of nitrogen purging (prior to UV treatment) on milk quality. It was found that nitrogen purged UV treated milk causes minimal changes to physicochemical properties of milk.

7.1. Introduction

There is an increasing trend in consumption of minimally heat processed foods (Zink, 1997). Milk is considered as nutritionally rich food that contains carbohydrates (lactose), proteins (mainly casein and whey), fats (long chain saturated and unsaturated fatty acid, short chain fatty acids), vitamins and minerals (Jenness, 1988; Månsson, 2008). One of the primary step for Ultra high temperature (UHT) packaged milk includes heat treatment at high temperature (135-142 ^{o}C) for short duration. However, such treatment results in adverse effect on vitamins (Cappozzo *et al.*, 2015; Kilshaw *et al.*, 1982), proteins (Efigênia *et al.*, 1997; Kessler *et al.*, 1991), colour and flavour (Patton, 1955; Shimamura *et al.*, 2012; Van Boekel, 1998).

In the last few years, researchers have considered using emerging technologies in an attempt to reduce temperature during milk processing (Li *et al.*, 2016). One of the well-known disinfection technologies is Ultraviolet (UV) treatment. UV can provide low cost installation and production cost which also makes it suitable for small scale processing with low carbon emissions in comparison to thermal technologies making it environmental friendly (Cilliers *et al.*, 2014). Specifically, treatment with UV-C (254 nm) is well known for its antimicrobial effects in water. However, its low transmittance through opaque liquids limits its penetration and hence, results in little or no inactivation. In the past few years, different designs of UV reactors were developed to improve inactivation of microorganisms in milk. Choudhary *et al.* (2011) found that UV treatment using a coiled tube reactor can enhance inactivation of spores as this design provides mixing and improved exposure due to circular flow in small diameter tubing.

On the other hand, some studies highlighted negative effects of UV treatment on quality of milk. These negative effects are caused by photo oxidation in the presence of oxygen. It is well known that oxygen contribute to oxidative reactions which affect pigments, vitamins, proteins and fats (Davies *et al.*, 2001; Sattar *et al.*, 1975). Guneser *et al.* (2012) and Cappozzo *et al.* (2015) noted that UV treatment decreases light sensitive vitamins. This reduction in vitamins depends on the design of reactor, dosage and UV intensity applied. In addition to the negative impact on vitamins, oxidation of unsaturated lipids results in the formation of different compounds that impart undesirable flavour (Cappozzo *et al.*, 2015; Cilliers *et al.*, 2014; Matak *et al.*, 2004; Poulsen, 2016).The intensity of oxidative reactions depends on temperature, type of fatty acids, distribution of double bonds and amount of dissolved oxygen (Sattar *et al.*, 1975). Considering proteins, UV treatment mainly affect aromatic amino acids and results in undesirable flavour products (Cilliers *et al.*, 2014; Poulsen, 2016). It has been found that amino acids phenylalanine, tryptophan and histidine are degraded by UV treatment (Koutchma *et al.*, 2009). Samuelsson *et al.* (1961) reported methionine degradation in the presence of oxygen and Singleton *et al.* (1963) reported that oxygen is responsible for off flavour development when milk was exposed to 450 nm light (Sattar *et al.*, 1975). Even for UHT milk, oxygen is considered as a major source for stale and oxidized flavours (Deeth *et al.*, 2017). The presence of dissolved gasses in raw milk accounts to 6 % by volume and it was reported that deaeration before UHT treatment improves organoleptic properties in comparison to non-deaerated product (Deeth *et al.*, 2017). Currently, vacuum deaerators are used to remove dissolved gases from liquid milk (Burton, 2012). Similarly, it is hypothesized that, removal of these dissolved gases before UV treatment may result in improved quality by reducing oxidative reactions. Nitrogen purging is one of the best method to remove dissolved oxygen in liquids in comparison to boiling under reduced pressure, sonication, and boiling under atmospheric pressure (Butler *et al.*, 1994).

To the best of our knowledge, the effect of nitrogen purging was not evaluated on quality of UV treated milk. The primary objective of this study was to analyse the effect of nitrogen purging prior to UV treatment on milk quality using a continuous flow coiled tube UV reactor.

7.2. Methodology

7.2.1. Materials

Fresh raw milk was obtained from a local dairy farm and centrifuged at 2500 g at 4 ^{o}C for 30 min. Fresh skim milk (FSM) was prepared from it as described by Mazri *et al.* (2012). Then it was filtered to remove fat particles by using glass wool and the supernatant was used for all UV treatments.

7.2.2. UV experimental design

7.2.2.1.UV Equipment

In this study, a coiled tube UV reactor was designed at the University of Auckland workshop as similar to the one described by Choudhary *et al.* (2011). The UV lamp (EGPH369N/S) was placed in the centre of quartz sleeve (EQS 450, 15 mm in diameter, and 292 mm of arc length) and was connected to a power source. As per specification from manufacturer (Davey water product, New Zealand), UV-C power was 5.6 W with total lamp power of 19 W. Perfluoroalkoxy (PFA, Thermofischer scientific. New Zealand) tube (ID 1.6 mm, OD 3.2 mm) was coiled over quartz sleeve. The reason for PFA tube selection was its high transmittance to UV (~ 80 %) (Bandla *et al.*, 2012a; Cambié *et al.*, 2016).

7.2.2.2. UV Experimental plan

In the first part of experiments, FSM was nitrogen purged at 25 ml/s for 5 min by using a purger that results in bubbles. This glass nitrogen purger distributed the Nitrogen flow into small bubbles. However, there is a drawback of using Nitrogen purging as this result in foaming. The nitrogen purged skim milk (NPSM) and FSM were passed through coiled tube reactor at different flow rates (10, 20, 30, 40 and 50 ml/min) to obtain different residence times (90, 46, 31, 23 and 18 sec). Lower flow rates were used to analyse the effect of different residence times on milk quality with UV treatment. Both NPSM and FSM were analysed for absorbance at 280 nm ($A_{280\ nm}$) and protein carbonyls (PC) to assess photo oxidative damage after UV treatment.

UV-C dosage was calculated as given by Müller *et al.* (2014). The obtained value was corrected for transmission of Quartz sleeve (90 %) and PFA tube (80 %) (Choudhary *et al.*, 2011).

Equation 7-1

$$\text{UV-C}\left(\frac{\text{J}}{\text{ml}}\right) = \frac{\text{UV-C Power of lamp (W)}}{\text{Flow rate}\left(\frac{\text{ml}}{\text{s}}\right)} \times T_{Quartz} \times \text{T}_{Tube}$$

In the second part of experiments, high flow rates were used which resembles with preliminary studies on spore inactivation. NPSM was treated with UV at three different dosages through multiple passes (1.61, 3.23, 4.84 J/ml) at a flowrate of 9 L/h and denoted by UV_1-NPSM, UV_2-NPSM, and UV_3-NPSM. To avoid contact with air in between passes, the empty containers were also purged with nitrogen, keeping only a single opening to collect sample. FSM was also treated using UV (4.84 J/ml, three passes) without nitrogen and denoted as UV_3-SM. Subsequently, all samples (FSM, UV_3-SM, UV_1- NPSM, UV_2-NPSM, and UV_3-NPSM) were analysed for different physical and chemical tests as described in section 7.2.3. Prior to any experiment, UV lamp was switched on for 5 min as warm up time as described by the manufacturer.

7.2.3. Milk physical and chemical analysis

7.2.3.1. Protein Carbonyls

Protein carbonyls (PC) are measured to assess the extent of degradation that would have occurred to protein present in milk as a result of photo oxidative changes during UV treatment (Suzuki *et al.*, 2010). In the first step, it involves the derivatization of carbonyl groups in protein with dinitrophenyl hydrazine (DNPH) to form dinitrophenyl hydrazone (DNP). This DNP can be detected by spectroscopy at 370 nm (Shacter, 2000) and was determined by using the method adapted by Levine *et al.* (1990) and Fenaille *et al.* (2006). A small portion of milk sample (contains equivalent to 2 mg of protein) was added to 1 ml of 10 mM DNPH in 2 M HCl and incubated at room temperature for 30 min. Incubated solution was precipitated by using 10 % (w/v) TCA (final concentration) and the supernatant was separated by micro centrifuge (Heraeus Pico 21 Centrifuge, Thermoscientific) at 7500 × g for 5 min. The obtained protein pellets were washed three times using 1 ml of ethanol: ethyl acetate (50:50) and centrifugation each time. Washed pellets were added to 1 ml of 6 M guanidine hydrochloride (pH 2.3) and absorbance at 370 nm was measured using plate reader (Multimode plate reader, EnSpire manufactured by Perkin Elmer). The PCs were calculated using a molar absorptivity of 2.2×10^4 M^{-1} cm^{-1}(Augustyniak *et al.*, 2015).

7.2.3.2. Absorbance at 280 nm (A280 nm)

$A_{280\ nm}$ is used for the determination of protein content that arises due to the presence of amino acids with aromatic rings (Stoscheck, 1990). Therefore, higher values of absorbance corresponds to high protein contents or higher aromatic amino acids content. This was analysed by precipitating 10 ml of skim milk by adding 5 ml of 30 % Trichloroacetic (TCA) acid followed by incubation at room temperature for 5 min. Finally, precipitated solution was filtered through Whatman no. 2 filter paper and absorbance of obtained supernatant (diluting 1:5) was measured at 280 nm using UV/Vis spectrophotometer (Cilliers *et al.*, 2014; Kwan *et al.*, 1983).

7.2.3.3. Colour

Colour is one of the important parameters for evaluation of milk quality as it possesses strong influence on consumer perception (Imran, 1999). It was measured by using CR-400 Chroma meter as described by Makwana (2016). Calibration was performed by using calibration plate (CR A43) with white background. The colour values were obtained using Hunter colour system (L, a, b) where 'L' indicates lightness between scale of 0 to 100 (black to white), 'a' indicates green and red (negative values represents green and positive values represents red) and 'b' indicates yellow and blue (negative values for blue and positive values for yellow). For comparison between UV treated and untreated milk, total colour difference (ΔE) was calculated by using the following equation as reported by Minolta (1994).

Equation 7-2

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$$

Where ΔL , Δa *and* Δb are the difference between values obtained from raw skimmed milk and treated milk.

7.2.3.4. Rheology parameters

Rheology parameters were analysed to evaluate any effect on flow behaviour of milk samples after UV treatment. It was measured by using rheometer (AR-G2, TA instrument, Texas, USA) as described by Makwana (2016). A volume of milk (25 ^{o}C) was transferred to the sample container in sufficient quantity to immerse the concentric cylinder (28 mm in diameter, DIN rotor) completely and then a shear rate of 200 s^{-1} was applied. The shear stress "σ" (mPa) was plotted against shear rate "γ" (s^{-1}) to evaluate the rheological differences between samples.

7.2.3.5. Particle size distribution (PSD)

The PSD of milk samples gives important information about structural changes in the different components of milk such as formation of complexes, precipitation and denaturation of proteins. The PSD of samples were determined using Master Size 2000 (Malvern Instruments, Malvern., U.K.). Milk samples were analysed with an obscuration value between ~ 4-5 % using refractive index for dispersant (water) as 1.33 and skim milk as 1.5. The measurements were performed in triplicate under ambient conditions immediately after the UV treatment. Values d_{10} , d_{50} and d_{90} were noted which shows the particle diameter at 10%, 50 % and 90 % of the cumulative distribution curve respectively. In addition to that, the surface weighted mean (D [3,2]) and volume weighted mean (D [4,3]) were determined using following equations (Costa *et al.*, 2018)

Equation 7-3

$$D[3,2] = \frac{\sum n_i {d_i}^3}{\sum n_i {d_i}^2}$$

Equation 7-4

$$D[4,3] = \frac{\sum n_i {d_i}^4}{\sum n_i {d_i}^3}$$

Where d_i represents mean diameter of particle, n_i is the number of particles.

7.2.3.6. pH and titratable acidity

For pH determination, milk samples were placed in 100 ml beaker and pH was determined by immersing pH probe into the beaker until constant reading was obtained. The titratable acidity was measured by using AOAC method (AOAC 947.05). For titratable acidity 10-20 g of milk sample was taken in 250 ml of flask, few drops of phenolphthalein (1 % w/v in 50 % ethanol) was added and titrated with 0.1 N Sodium hydroxide (NaOH) till faint pink colour. NaOH was standardized by using Potassium hydrogen phthalate and corrected normality was used for calculations (AOAC 936.16). Results were expressed as % lactic acid using following relationship

1 ml of 0.1 N NaOH = 0.009 g lactic acid

7.2.3.7. Electrical conductivity

Electrical conductivity arises due to the soluble salt fraction of milk, change of acidic conditions, and fermentation of lactose to lactic acid (Mucchetti *et al.*, 1994). The electrical conductivity of milk samples was measured using conductivity meter (Seven Compact TM Conductivity S230) by placing conductivity probe in samples until constant reading of conductivity.

7.2.4. Statistical analysis

Statistical treatment of data was performed using Microsoft excel 2016, USA. Mean and standard deviation were calculated for three replicates. Single way ANOVA was applied with 95 % confidence level followed by T test using SPSS Version 25, IBM Inc., USA. The superscripts for means that are not different was obtained as described by Dallal (2015).

7.3. Results and Discussion

7.3.1. Effect of UV exposure time on protein damage

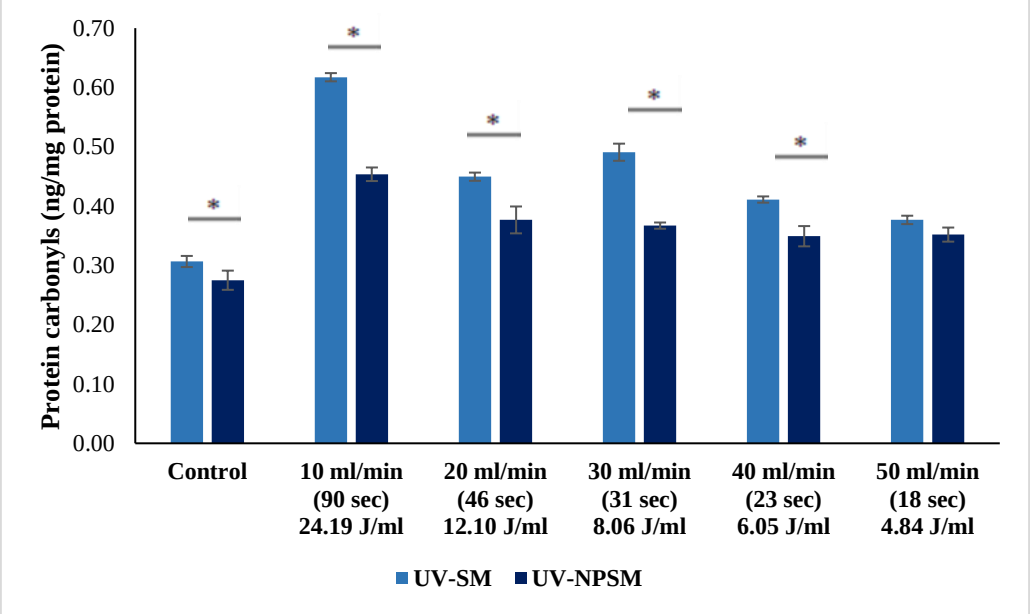


Figure 7-1: Protein carbonyls (ng/mg) of UV treated skim milk at different flow rates and residence time for N_2 purged (UV-NPSM) and non-purged milk sample (UV-SM). The error bar indicates mean ± standard deviation and * shows significantly different values ($P < 0.05$)

Amino acid derivatives are produced as a result of photooxidation in a protein rich media and hence introduce carbonyl groups (Dalsgaard *et al.*, 2007). Thereby, the most common method to assess protein oxidation are PC. PC are measured by derivatization of the carbonyls group with dinitrophenyl hydrazine (DNPH) leading to the formation of dinitro phenyl hydrazine product (Shacter, 2000). Figure 7-1 shows PC content of UV-SM and UV-NPSM samples when treated at different flow rates (10 to 50 ml/min) using the coiled tube UV reactor. Formation of PC indicates protein degradation in the presence of oxygen during UV treatment (Sattar *et al.*, 1975). As speculated, all UV treated samples show an increase in PC compared to the control. However, UV-NPSM samples resulted in lower PC levels in comparison to UV-SM ($P < 0.05$). This clearly shows that nitrogen purging is an effective pre-treatment to reduce PC content in UV treated milk. It was also shown that longer residence time (or UV exposure time) produces higher level of PC in both UV-NPSM and UV-SM samples. These observations are supported by Scheidegger *et al.* (2010) who found that irradiation time directly affected the production of PCs in a batch type UV treatment (15 W UV-C lamp). They also showed that when milk samples were exposed to different UV irradiation times of 4 h to 24 h, it resulted in PC of ~7.15 to ~18.60 nmol/mg of proteins in skim milk at a rate of 1.95 nmol/h (0.0325 nmol/min). Interestingly in the same study, Scheidegger *et al.* (2010) reported that skim milk resulted in higher levels of PC in comparison to whole milk. This may be explained by higher transmission of UV through skim milk in comparison to whole milk resulting in more UV effect and hence higher PC. Consequently, previous studies suggest that protein degradation caused by photo oxidation lead to deterioration of milk quality and production of off flavours (Skibsted, 2000). This degradation resulted as methionine reacts with singlet oxygen to yield dimethyl disulphide, methyl sulphide methionine sulfoxide, methionine sulfone and other sulphur containing compounds. By products that arises from these sulphur containing compounds produce bad aroma and flavours (Cilliers *et al.*, 2014; Dalsgaard *et al.*, 2007; Hui, 1993; Jung *et al.*, 1998; Lee *et al.*, 2009). Therefore, control of oxidative protein damage could minimize undesirable flavour in UV treated milk similar to deaeration before UHT treatment which results in improved flavour (Burton, 2012).

$A_{280\ nm}$ can also be used to determine the level of protein ring lysis among samples. The difference in $A_{280\ nm}$ between raw milk and treated milk has been used to investigate the changes in the aromatic side chains of hydrolysed casein (Dalsgaard *et al.*, 2007). Figure 7-2 shows $A_{280\ nm}$ of UV-SM and UV-NPSM with different residence time through the coiled tube UV reactor. A reduction in $A_{280\ nm}$ absorbance was observed in both UV-SM and UV-NPSM

when compared to FSM at the different flow rates considered indicting the occurrence of proteolysis in UV treated samples. Further, UV-NPSM samples showed higher absorbance values in comparison to UV-SM. These results further confirm that there has been less damage to amino acids containing aromatic rings through secondary, tertiary and quaternary structural changes in UV-NPSM samples.

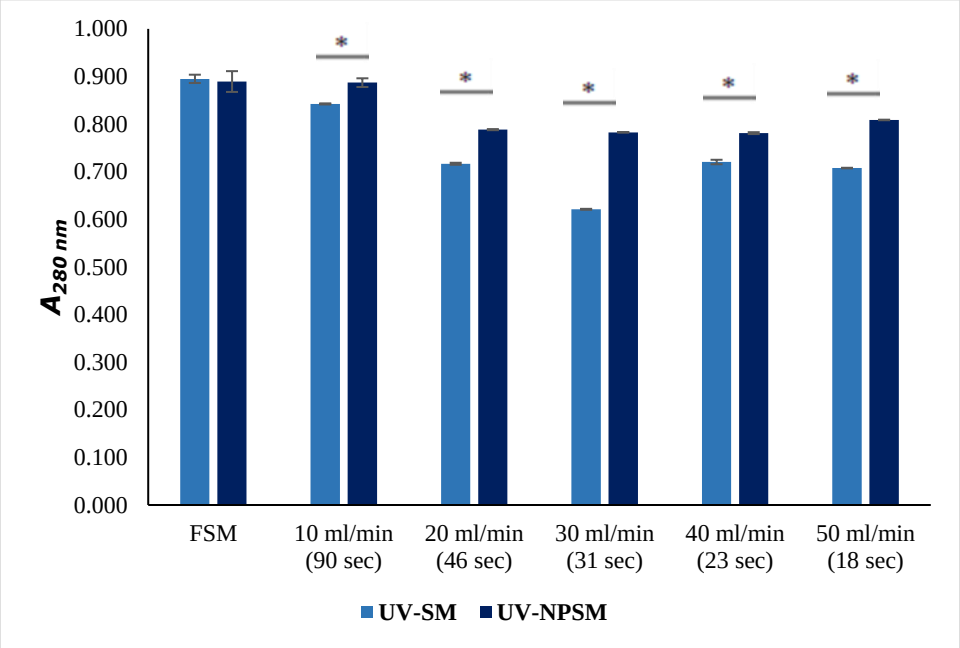


Figure 7-2: $A_{280\ nm}$ for UV treated sample at different flow rates and residence time for nitrogen purged (UV-NPSM) and non-purged milk sample (UV-SM). The error bar indicates mean ± standard deviation and * shows significantly different values ($P < 0.05$) between samples.

7.3.2. Quality study with multiple passes through the coiled tube reactor

As per preliminary studies, microbial inactivation at higher flow rates (150 mL/min) is quite considerable as a result of high Reynold's number (Chapter 4 & chapter 5). Therefore, it is needed to evaluate the effect of UV treatment on physico chemical properties of milk at 150 mL/min to justify its usage in practical conditions. Figure 7-3 shows $A_{280\ nm}$ of milk samples (FSM, UV-SM and UV-NPSM) while Figure 7-4 shows their PC content when operated at high flow rates. These values are in agreement with the results shown in Figure 7-2, where $A_{280\ nm}$ value of UV_3-SM is significantly reduced ($P < 0.05$) in comparison to nitrogen purged UV treated milk samples. Further, PCs of nitrogen purged UV samples up to 2 passes were not significantly different to FSM ($P > 0.05$). However, an increase in PCs was observed when

nitrogen purged UV treated samples were circulated 3 times (UV_3-NPSM) giving similar readings to UV_3-SM. Thereby these results showed that, the benefit of nitrogen purging diminished when UV treatment was carried out at high flow rates. These findings are also supported by Scheidegger *et al.* (2010) who showed that longer treatment time (1 h) is required for significant increase in PC (~1.81 mmol/mg of proteins) using batch UV reactor at 4 ^{o}C.

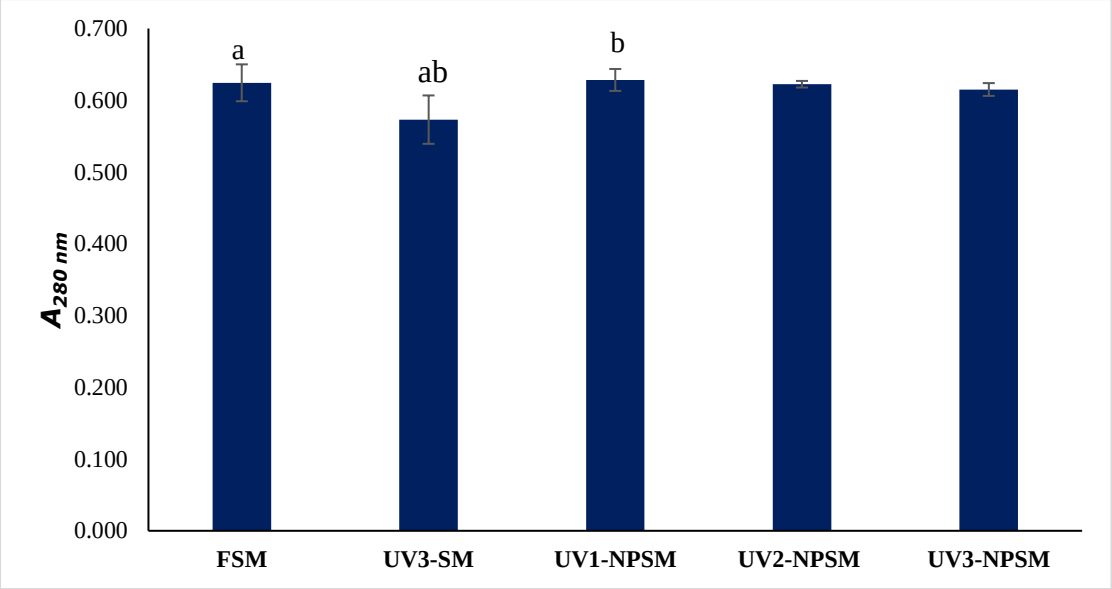


Figure 7-3: $A_{280\ nm}$ for FSM, UV_3-SM, UV_1-NPSM, UV_2-NPSM and UV_3-NPSM. The error bar indicates mean ± standard deviation and results without common letter above bars shows statistically significantly different values ($P < 0.05$) between samples.

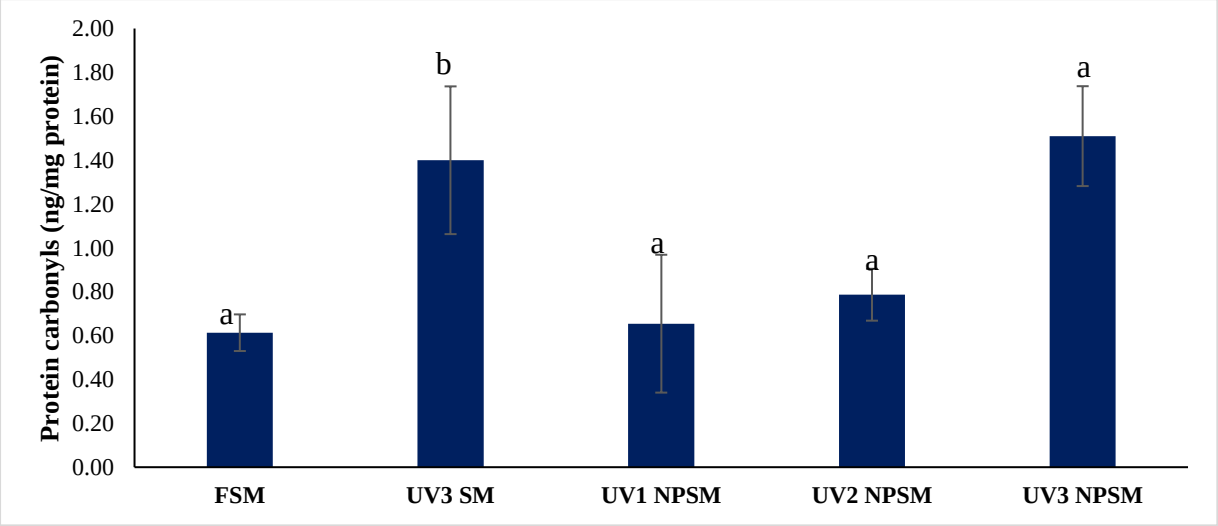


Figure 7-4: Protein carbonyls (ng/mg of proteins) for FSM, UV3-SM, UV1-NPSM, UV2-NPSM and UV3-NPSM. The error bar indicates mean ± standard deviation and results without common letter above bars shows statistically significantly different values ($P < 0.05$) between samples.

Table 7-1 shows pH, conductivity and titratable acidity of milk samples (FSM, UV_3-SM, and UV_3-NPSM). A slight change in pH was observed for UV-NPSM in comparison to FSM ($P < 0.05$) while there was no significant change in UV_3-SM ($P > 0.05$). For titratable acidity, no change was observed between samples (FSM, UV_3-SM, and UV_3-NPSM) ($P > 0.05$). These results indicate that there are only minimal change in pH, conductivity and titratable acidity in all UV treated milk samples compared to FSM. On the other hand, heating can cause multiple changes such as decrease in pH and browning reaction which can affect colour and heat sensitive proteins in milk (Cilliers *et al.*, 2014).

Table 7-1 Comparison of pH, conductivity and titratable acidity of raw skim milk, UV treated skim milk, and UV treated skim milk (N_2 purged)

Samples		pH	Conductivity	Titratable acidity (% lactic acid)
Fresh skim milk	FSM	6.76 ± 0.00	4.90 ± 0.03^{b}	0.093 ± 0.007
UV treated skim milk (4.84 J/ml)	UV_3-SM	6.77 ± 0.00	4.82 ± 0.01	0.087 ± 0.002
UV treated skim milk (N_2 purged) (1.61 J/ml)	UV_1- NPSM	6.79 ± 0.00^{a}	4.86 ± 0.01^{a}	0.092 ± 0.008
UV treated skim milk (N_2 purged) (3.23 J/ml)	UV_2- NPSM	6.79 ± 0.00^{a}	4.90 ± 0.00^{b}	0.092 ± 0.007
UV treated skim milk (N_2 purged) (4.84 J/ml)	UV_3- NPSM	6.79 ± 0.01^{a}	4.89 ± 0.02^{ab}	0.092 ± 0.007

Note: Values are shown as mean ± standard deviation of three replicates. Means without a common superscript letter (a,b) differ significantly ($P < 0.05$)

Table 7-2: Comparison of colour between raw skim milk, UV treated skim milk and UV treated skim milk (N_2 purged)

Treatment		L	A	b	ΔE
Fresh skim milk	FSM	68.47 ± 0.12[a]	-5.42 ± 0.05	-2.09 ± 0.01[a]	---
UV treated skim milk (4.84 J/ml)	UV_3-SM	67.61 ± 0.69[a]	-5.09 ± 1.25	-2.94 ± 0.54[a]	1.26
UV treated skim milk (N_2 purged) (1.61 J/ml)	UV_1- NPSM	67.94 ± 1.00[a]	-5.47 ± 0.42	-1.62 ± 0.19[ab]	0.71
UV treated skim milk (N_2 purged) (3.23 J/ml)	UV_2- NPSM	68.60 ± 0.22[a]	-4.89 ± 0.11	-2.54 ± 0.06[a]	0.71
UV treated skim milk (N_2 purged) (4.84 J/ml)	UV_3- NPSM	69.59 ± 0.57[ab]	-5.45 ± 0.54	-1.80 ± 0.51[a]	1.15

Note: Values are shown as mean ± standard deviation of three replicates. Superscripts with means (a-b) in a column without a common superscript letter differ significantly ($P < 0.05$)

Table 7-2 shows the colour values of UV treated milk samples. There was no change observed in "L" and "b" values ($P > 0.05$) for FSM, UV3-SM, UV1-NPSM, UV2-NPSM and UV3-NPSM. However, a small change was observed in "a" values ($P < 0.05$) indicating the presence of green colour components in milk (-). Literature values, on the effect of UV on colour in milk are scarce. On the other hand, there are many reports that mention about unwanted physical attributes associated with UHT milk including discolouration (Pagliarini *et al.*, 1990; Van Boekel, 1998). Further, Claeys *et al.* (2013) found that heat treatment is related to treatment times and results in an increase in total colour difference ΔE. This change in total colour difference arose due to Maillard reactions that results in brown colour products. Therefore, UV treated milk has the advantage of retaining colour when compared to high temperature processing.

Table 7-3: Particle size distribution for FSM, UV-SM and UV-NPSM

Treatment	Surface weighted mean D [3,2] µm	Volume weighted mean D [4,3] µm	D10 % µm	D50 % µm	D90 % µm	Obscuration (%)
FSM	53.52 ± 0.26[a]	87.95 ± 0.59[a]	26.75 ± 0.12[a]	72.86 ± 0.43[a]	171.13 ± 1.30[a]	3.86
UV3-SM	50.03 ± 1.58	95.69 ± 3.95	23.25 ± 1.44	76.43 ± 1.96	198.25 ± 13.41	4.09
UV1-NPSM	52.10 ± 0.82[a]	88.48 ± 1.54[a]	25.58 ± 0.84[a]	72.70 ± 0.61[a]	174.76 ± 5.62[a]	4.37
UV2-NPSM	52.97 ± 0.12[a]	86.62 ± 0.27[a]	26.51 ± 0.05[a]	71.92 ± 0.19[a]	168.15 ± 0.59[a]	4.19
UV3-NPSM	53.10 ± 0.21[a]	86.98 ± 0.48[a]	26.62 ± 0.02[a]	72.17 ± 0.34[a]	168.96 ± 1.05[a]	4.17

Note: Values are shown as mean ± standard deviation of three replicates. Superscripts with means (a-b) in a column without a common superscript letter differ significantly ($P < 0.05$)

Table 7-3 shows PSD of FSM, UV3-SM, UV1-NPSM, UV2-NPSM and UV3-NPSM. It was observed that surface weighed mean diameter of particles was similar to FSM and UV3-SM ($P > 0.05$). Similar set of results were obtained for volume weighed mean, D10 %, D 50 %, and D 90%, which shows minimal changes to milk as a result of UV treatment. However, a significant shift could be observed in PSD of UV treated milk when compared to UHT or ultra high pressure homogenization treated milk (Amador-Espejo *et al.*, 2014). This change in PSD is more likely due to homogenization treatment that reduces overall particle size. Flow behaviour of UV treated samples with and without N_2 purging also showed minimal changes when compared to FSM (Figure 7-5). The rheology profile obtained as slightly curved in comparison to Newtonian liquids. Flow behaviour during UV treatment possesses an important role in its effectiveness to inactivate microorganisms. Any increase in viscosity during treatment could affect UV transmission and thereby reduce its effectiveness in microbial reduction. However, there was no change observed in flow behaviour during UV treatment which may assist in obtaining consistent microbial inactivation for a specific UV reactor.

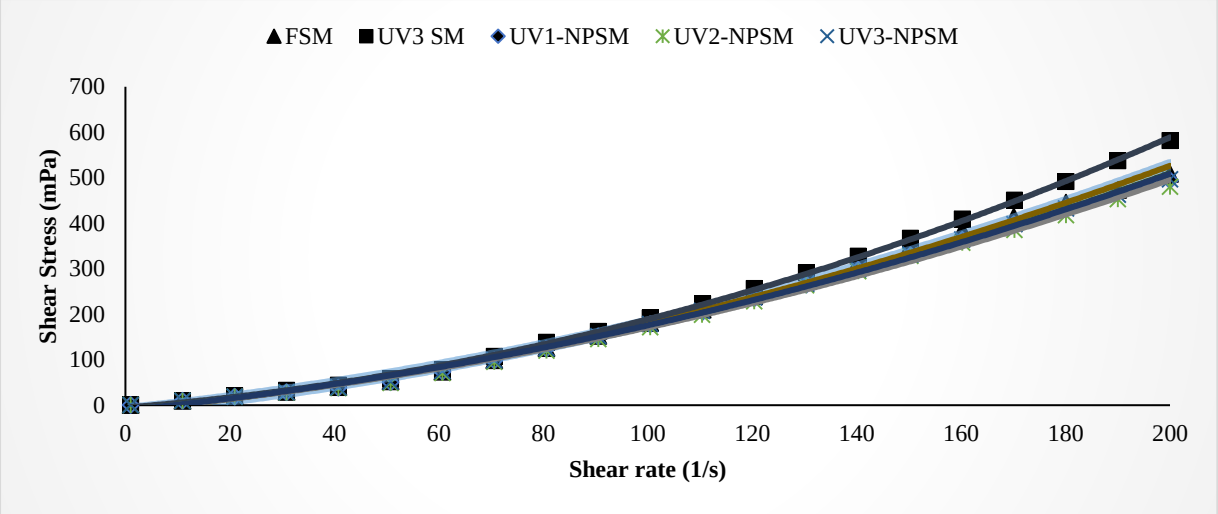


Figure 7-5: Flow curves of FSM, UV3-SM, UV1-NPSM, UV2-NPSM and UV3-NPSM

7.4. Conclusion

Ultraviolet can inactivate microorganism while the challenge remains that UV treatment can cause adverse effects from photo oxidative effects. The present study evaluated the effect of nitrogen purging prior to UV treatment of milk using continuous flow UV reactor. Nitrogen purging prior to UV treatment reveals that it can reduce the negative effects from photooxidation when exposure to air are minimized during processing. This study also shows that photo oxidative effects are directly related to UV exposure dose and nitrogen purging can reduce these effects with long UV treatment times.

CHAPTER 8

Ultraviolet treatment: A potential technology for whey preservation

This chapter is based on the draft of the paper ready for submission to an international journal.

Preface

Whey is the greenish liquid obtained as a by-product during cheese or casein manufacturing. Currently, whey is preserved through thermal pasteurization with treatment temperature that varies depending on the shelf life required. Emerging technologies can provide a better alternative to conventional thermal treatment, which tends to cause protein denaturation in the whey. Ultraviolet treatment (UV-C) is widely used because of its antimicrobial effects in water, juices and clear liquid products. One of the drawbacks of this treatment is the difficulty of UV to penetrate through opaque liquids like whey. The UV reactor used in this work consist of a simple PFA tube coiled around a UV lamp and was found very effective in lowering microbial count in whey. The effect of UVC treatment on inactivation of natural microbial load in crude whey was studied at different temperatures (4 ^{o}C, 20 ^{o}C, and 30 ^{o}C). The effect was also studied on reconstituted whey inoculated with *E. coli* ATCC 25922. With UV treatment (1.61 J/mL), approx. 6 log reduction was obtained in the natural aerobic plate count at a treatment temperature of 30 ^{o}C. With the same conditions, 3.98 ± 0.23 log reduction was obtained in *E. coli* ATCC 25922 in reconstituted whey (6 % w/v). Also, physico-chemical study reveals that UV treated crude whey and reconstituted whey maintained the same characteristics as of thermally pasteurized whey. Therefore, UV-C treatment could provide an energy efficient alternative method of pasteurization of whey

8.1. Introduction

Whey is the greenish liquid obtained as a by-product (represents 80-90 % volume of milk) during cheese or casein manufacturing that possesses more than 50 % of nutrients found in milk. It mainly composed of proteins, lactose, salts and lactic acid (Bylund, 2003; Jelen, 1992; Mahmoud *et al.*, 2004). Whey is widely used in nutrition supplements, energy drinks and other ready to drink (RTD) beverages due to its high protein content and the presence of branched-chain amino acids (Bylund, 2003). Moreover, RTD beverages are popular because it provides convenience to consumers while maintaining nutritional requirements.

Currently, whey is preserved through thermal pasteurization with treatment temperature that vary depending on the shelf life requirement (Atamer *et al.*, 2013; Bylund, 2003). Emerging technologies could provide a better alternative to conventional thermal treatment as some of the emerging technologies can process liquids at much lower temperatures for preservation and thereby, are more energy efficient. In the past few years, emerging technologies were studied

for its antimicrobial effects and consequences on physicochemical properties of whey. Table 8-1 shows an overview of literature on the effect of emerging technologies on antimicrobial and physicochemical properties of whey. Among all emerging technologies, only few technologies can inactivate microorganisms under ambient conditions. Ultraviolet (UV) possesses a strong potential to reduce the treatment temperature required during whey processing as it can inactivate bacteria under ambient conditions. Therefore, UV consumes less energy compared to other processing like ohmic heating, pulsed light, ultra high pressure homogenization (UHPH) and ultrasound. UV-C targets DNA and RNA of microbial cells and causes light induced damage through dimerization, which results in formation of cyclobutane pyrimidine dimers. This phenomena, affects replication of microorganisms leading to cell death (Dai *et al.*, 2012).

One of the drawbacks of UV-C treatment is its limited transmittance when applied to opaque liquids like whey. Multiple designs of UV reactors were studied to improve its transmittance within opaque liquids (Gayán *et al.*, 2014b). Coiled tube reactor is one of the efficient reactor that uses very low diameter tubing coiled around a UV lamp (Bandla *et al.*, 2012a). It improves microbial exposure to UV due to the induced circular flow, which is referred as Dean flow (Dean, 1927). This, result in a high microbial reduction even within opaque liquids (Bandla *et al.*, 2012a). Therefore, with a properly designed UV reactor, UV treatment of opaque liquids could be made commercially attractive since it has the additional benefit of low energy consumption with retention of quality.

As mentioned previously, whey is used as an additive in different beverages or further processed to dried powder. However, very little attempts have been made to study microbial inactivation and physicochemical properties of it using UV (Kristo *et al.*, 2012; Singh *et al.*, 2007). Kristo *et al.* (2012) studied the effect of UV treatment on structure of proteins during whey treatment using a Taylor couette type UV reactor. The authors mentioned that the extent of protein denaturation was lower than UHT sterilization and HTST pasteurization. However, the UV dosage was not clearly noted and therefore the results are not conclusive. Meanwhile Singh *et al.* (2007) studied microbial inactivation using a continuous UV reactor (total volume 0.84 L, flow rate 0.03 L /min) with 17 mm of liquid pathway and a stainless steel coil (length 448 mm, thickness 0.85 mm, pitch 20 mm) placed inside the reactor to induce circular flow. This stainless steel coil could hinder with the UV radiation. With long treatment time of 28 min (inlet temperature 24 ^{o}C, outlet temperature 32.8 ^{o}C), only 3.88 log reduction in microbial load was observed. Further, the temperature difference shows that energy consumed was quite

high (~ 36.77 J/mL). Unfortunately the power of UV lamp and the dosage were not given in the above mentioned work. On the other hand, the coiled tube UV reactor induces circular flow which allow full exposure of the fluid to UV (Bandla *et al.*, 2012a, 2012b). Thus, UV treatment using coiled tube reactor possesses immense potential to inactivate microflora considerably with lower energy consumption.

Therefore, the aim of this study is to evaluate antimicrobial efficacy of coiled tube UV reactor against native microbial load and inoculated *E. coli* ATCC 25922 in whey. The second objective is to evaluate physicochemical properties of whey using the dosage of UV treatment that gave maximum microbial reduction

Table 8-1: Literature review on the use of emerging technologies on antimicrobial and physicochemical properties of whey

Technology	Experimental parameters	Specific target	Key findings	References
Atmospheric pressure cold plasma	Two aluminium electrodes with 44 mm gap (Final temperature 14 °C to 38 °C) 70 kV applied for 1, 5, 10, 15, 30 and 60 min 2 % w/v solutions	Physicochemical properties	1. Increase in yellow colour and minor reduction in pH was observed (relates with treatment time). 2. With treatment time of 15 min, mild oxidation of protein was shown by protein carbonyls and surface hydrophobicity index 3. However, oxidation and other changes were more pronounced with treatment time increased to 30 min or 60 min.	Segat *et al.* (2015)
Ohmic heating	72-75 °C for 15 sec, (2,4,5,7, and 9 $V.cm^{-1}$) 11 % w/v whey solutions	Physicochemical properties and microbial inactivation	1. Capable to release bioactive peptides in comparison to conventional pasteurization in whey (between 4 V to 5 V) 2. Physicochemical properties (pH, microstructure, and sensory profile) did not have much difference when compared to pasteurized whey. 3. Some volatile compounds were found in Ohmic treated whey samples when compared with conventional pasteurization	Costa *et al.* (2018)

Pulsed light	Maria PUD system, France (Temperatures between 22 ^{o}C and 60 ^{o}C) 190 to 1000 nm (pulse duration 0.3 ms) 2.15 mm liquid pathway 1.1 J/cm^2	Antimicrobial effectiveness against *Listeria innocua*	1. Upon treatment with 3000 V, inlet temperature 22 ± 2 ^{o}C, 5 L/min, 11 J/cm^2 resulted in approx. 0.5 log reduction 2. With pulsed light treatment at 60 ^{o}C, using same operating conditions resulted in greater than 5 log reduction.	Artíguez *et al.* (2015)
Ultra high pressure homogenization (UHPH)	Model FPG 7400H, Stansted Fluid power, UK Homogenization valve temperatures between ~39 ^{o}C to 75 ^{o}C) 100-300 MPa Whey protein isolate (6 % or 10 % w/v)	Physicochemical characteristics (whey protein aggregation)	1. No protein aggregation was found when UHPH was applied below 225 MPa. 2. A pressure in the range 250-300 MPa resulted in protein aggregation. 3. More insoluble protein were found as a result of UHPH treatment when compared to thermal treatments.	Grácia-Juliá *et al.* (2008)
Ultrasound	400 W, 100% , 55 ^{o}C, 8 min Sweet whey (5.14 % of total solids)	Antimicrobial and sensory characteristics	1. 2.46 log reduction was observed in total viable cells and 1.34 log reduction in yeast and mould count 2. With thermosonication, brighter colour and metallic after taste were observed when compared to pasteurized and fresh whey.	Jeličič *et al.* (2012)
Ultraviolet		Reduction in native microbial load with		Singh *et al.* (2007)

	Coiled reactor with stainless steel coil placed Outlet temperature 25.5 ºC to 45.8 ºC with inlet temperature 22 ºC to 24 ºC Whey (5.68 % of total solids)	different flow characteristics	1. With Reynold number of 8.37, residence time 28 min, and Dean number 6.69 resulted in 3.88 log reduction in microbial load 2. Fouling was reduced in comparison to conventional UV reactor	
Ultraviolet	Taylor Couette reactor Temperature not given, (Flow rates 30, 40, 70, 130 and 800 ml/min), 1 % and 5 % (w/v) solutions	Structural changes to whey proteins	1. Changes in tertiary structure of protein were observed after UV exposure. 2. Total and accessible thiol groups were increased in 1 % w/v solution , but did not increase in 5 % w/v solutions 3. UV exposure resulted in aggregates and oxidative products of aromatic amino acids 4. Denaturation caused by UV was lower than UHT and HTST treatments.	Kristo *et al.* (2012)

8.2. Materials & methods

8.2.1. Media/Food matrix

Crude whey (CW) was prepared in laboratory by adding rennet enzyme (vegetarian source with activity > 60 IMCU/tablet, Mad Millie, New Zealand) into fresh raw milk as per the manufacturer's instructions. Then it was incubated at 32 ^{o}C for 12 h and filtered through a muslin cloth to collect the whey. The obtained whey was further incubated at 20 ^{o}C for 24 h to increase the initial microbial count to ~10^{8} CFU/ml. In addition, dried whey powder was purchased from Reactive supplements, New Zealand as a microbial free source to study the inactivation of surrogate organism (*E. coli* ATCC 25922). Reconstituted whey (RW) was prepared by adding the dried whey powder to sterilized water (6 % w/v) (Blaschek *et al.*, 2007).

Total solids:

Total solid content of CW was obtained by following the method AOAC 990.19. Aluminium dishes were pre-dried in an oven at 100 ^{o}C for 2 h and stored in desiccator. Whey sample (3 g) was taken in aluminium dishes and oven dried at 100 ± 1 ^{o}C for 3 h. Total solids (%) were calculated as

Equation 8-1

$$Total\ solids\ (\%) = \frac{(W_2 - W_1) - B}{Sample\ weight} \times 100$$

Where, W_2 is mass in gram of dish + dried milk, W_1 is the mass of empty dish and B is blank (difference of empty aluminium plate before and after drying)

8.2.2. Microbiological studies

8.2.2.1. Bacterial strain and culture preparation

E. coli ATCC 25922 was used as a surrogate microorganism for *E. coli* O157:H7 to assess critical safety requirement (Kim *et al.*, 2009). It was obtained in freeze dried form from Fort Richard Laboratories, New Zealand. The freeze dried culture was initially streaked on Tryptone soy agar yeast extract (0.6 %) TSAYE plates and incubated for 24 h at 35 ^{o}C. Multiple colonies from these plates were transferred to 50 ml of Tryptone soy broth yeast extract TSBYE (0.6 %) and incubated in a shaking incubator at 35 ^{o}C for 24 h. The count obtained after incubation

in broth was ~10^8 CFU/mL. This bacterial suspension was added to RW to obtain a count of ~10^6-10^7 CFU/mL

8.2.2.2. Microbial counting

For total viable count (TVC), samples were spread plated on plate count agar (DIFCO) after appropriate dilutions and incubated at 30 ^oC for 24-48 h while, microbial count of *E. coli* was found by spread plating appropriate dilutions of sample on TSAYE (0.6 %) plates and incubated at 35 ^oC for 24 h.

8.2.3. UV equipment and experimental plan

8.2.3.1. Studies on thermal inactivation of *E. coli* in RW

Thermal inactivation kinetics of *E. coli* ATCC 25922 in RW was conducted to determine decimal reduction time (D values) at different temperatures by using capillary method. The method is also used by few other researchers to determine D values in different media (Franklin *et al.*, 1958; Stern *et al.*, 1954; Van Zuijlen *et al.*, 2010). Inoculated whey was filled in capillaries and sealed from both sides by using Bunsen burner. For thermal treatment, capillaries were transferred to a water bath maintained at 51 ^oC for different time intervals. This was repeated in a water bath maintained at 57 ^oC for another set of capillaries. Treated capillaries were transferred to cooling water maintained at ~0-2 ^oC immediately after thermal treatment. Then they were washed with ethanol (70 % v/v) and sterile water. Finally, the contents were removed from capillaries using peptone water. Microbial count was determined by spread plating appropriate dilutions as described in section 8.2.2.2.

Pasteurization treatment:

Pasteurization of whey (CW and RW) was done by immersing a copper tube (length 0.29 m, ID 8 mm, OD 9.6 mm) in hot water bath by holding at 72 ^oC for 15 sec after achieving target temperature (come up time was 16 sec). A thermocouple was placed in the centre of copper tube to ensure proper treatment temperature. After thermal treatment, the copper tube was placed immediately in chilling bath (0-4 ^oC) to cool the sample to ambient conditions.

8.2.3.2. UV experiments

UV equipment

In this study, a Perfluoroalkoxy (PFA) coiled tube UV reactor was designed and constructed at the University of Auckland, similar to the one previously described by Choudhary *et al.* (2011). The UV lamp (EGPH369N/S) and quartz sleeve (EQS 450, ID 21.7 mm, OD 24.4 mm) were obtained from Davey water products, New Zealand. UV-C source power was given as 5.6 W with lamp power of 19 W and wavelength of 254 nm. The PFA tube of 7.62 m with dimensions ID 1.6 mm, OD, 3.2 mm, was obtained from Thermofischer Scientific, New Zealand. PFA tube was used because it possess high transmittance (80 %) to UV radiation, stability to high temperature and chemicals (Bandla *et al.*, 2012a; Cambié *et al.*, 2016).

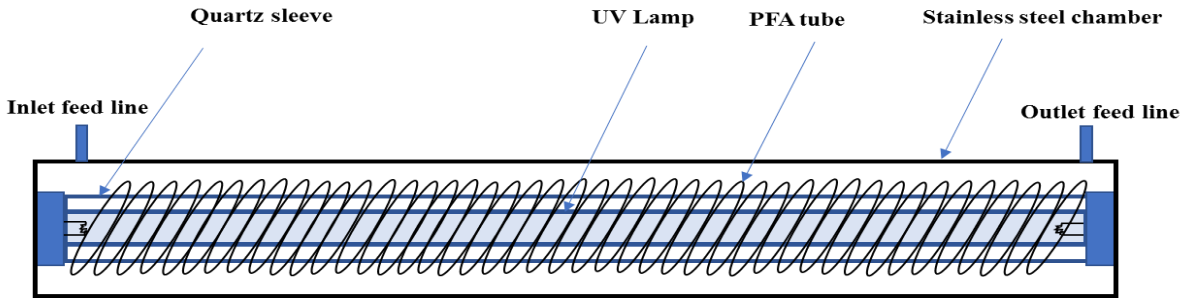


Figure 8-1: Schematic diagram of coiled tube reactor (not to scale).

Figure 8-1 represents the schematic diagram of the fabricated coiled tube reactor. UV-C lamp was placed inside the quartz sleeve. PFA tube was coiled around the quartz sleeve tightly without any spaces. The coiled reactor was placed inside a stainless steel barrel to avoid any UV exposure to the surrounding environment during UV treatment.

UV Equipment Operation and cleaning

A peristaltic pump was used for pumping whey through the UV reactor. The flow rate was set at 9 L/h and the same settings were used for microbial and physico-chemical studies. Prior to any treatment, cleaning was done by pumping ethanol (70 %) for 2-3 min followed by sterilized water for 5 min. The plate count was taken to ensure proper cleaning as described in section 8.2.2.2. Then UV lamp was switched on 5 min before any treatment, as warm up time. Whey was passed multiple times through the reactor to analyse the effect of different dosages. After the experiments, sterilant "Oxonia solution" was circulated through the reactor for 5 min followed by ethanol and sterilized water. UV-C dosage for treated sample was determined by taking the ratio of UV-C power to flow rate (Müller *et al.*, 2014). Obtained value was corrected

for T_{Quartz} (90 %) and $T_{PFA\ tube}$ (80 %)(Choudhary *et al.*, 2011). The UV dosage for one pass and second pass was calculated using the following equation.

Equation 8-2

$$\text{UV-C}\left(\frac{\text{J}}{\text{ml}}\right) = \frac{\text{UV-C Power of lamp (W)}}{\text{Flow rate}\left(\frac{\text{ml}}{\text{s}}\right)} \times T_{Quartz} \times T_{Tube}$$

Specific energy requirements during UV treatment and thermal studies:

For thermal studies, sensible heat was calculated as follows

Equation 8-3

$$Q = mC_p\Delta T$$

Where, Q is the thermal energy required (J), m is the mass of sample, C_p is the heat capacity of whey (4.082 J/g ^oC) and ΔT is the increase in temperature from only 63 ^oC to 72 ^oC, considering efficient heat recovery during pasteurization (Hammer *et al.*, 1913; Tomasula *et al.*, 2011)

During UV treatment, efficiency of lamps are quite low as some of the energy is converted into visible light and heat. Therefore, total power was calculated as follows:

Equation 8-4

$$Power\ consumption\left(\frac{J}{ml}\right) = \frac{Total\ power\ of\ UV\ Lamp}{Flow\ rate\left(\frac{ml}{s}\right)}$$

Absorption coefficient

Absorption coefficients of CW and RW were measured using UV-Vis spectrophotometer at 254 nm by diluting 1 mL of whey with 99 mL of distilled water (Bandla *et al.*, 2012b). The absorption coefficient was calculated by taking the ratio of obtained absorption values at 254 nm to the path length of quartz cuvette (1 cm).

8.2.3.3. Quality analysis

Chemical and physical parameters of untreated whey, pasteurised whey and UV treated whey with dosage of 1.61 J/mL (one pass) and 3.23 J/mL (two passes) were analysed under ambient conditions.

pH and titratable acidity

The pH was measured using pH meter (Orion star A111, Thermo scientific) calibrated with pH 4, pH 7 and pH 10 buffer solutions. The titratable acidity was measured by following AOAC method (AOAC 947.05) to analyse the effect on acidity due to subsequent treatment. Whey sample (10-15 g) was weighed in conical flask and few drops of phenolphthalein (1 % w/v in 50 % ethanol) were added. Finally, it was titrated with 0.1 N NaOH until faint pink colour persists for 30 sec. NaOH was standardized against potassium hydrogen phthalate by method AOAC 936.16. Results were expressed as % lactic acid using the following relationship:

1 mL of 0.1 N NaOH = 0.009 g lactic acid

Electrical conductivity

Conductivity measures the degree of medium to conduct electricity, indicating the ionic concentration. The conductivity of whey was measured using conductivity meter (Seven Compact TM Conductivity S230). The sample was poured into 100 mL beaker and the probe was immersed in the liquid sample until a constant reading of conductivity (mS/cm) was obtained.

Colour

Colour is an important parameter for beverages as it influence consumer perception (Imran, 1999). It was measured by using CR-400 Chroma meter as described by Makwana (2016). The colour values were obtained using CIE (*Commission Internationale d'Eclairage*) colour scale. Calibration was performed by using calibration plate (CR A43) with white background. The numerical values of L^* , a^* and b^* are used to measure the total colour difference (TCD) as given by the following equation.

Equation 8-5

$$\text{Total colour difference } (TCD) = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$$

Where, ΔL^* , Δa^* and Δb^* are the difference between values obtained from treated whey and un-treated whey.

Optical microscopy

Optical microscopy was done by transferring a small volume (20 µL) of sample on a glass slide and examining with an optical microscope (Motic microscope BA410 Series, Canada) as

described by Costa *et al.* (2018). The images were obtained using Objective 40X magnification and were captured in triplicate.

Particle size distribution (PSD)

The PSD of treated and untreated whey samples were determined using Master size 2000 (Malvern Instruments, Malvern., U.K.). Whey samples were analysed with 4-5 % obscuration value using refractive index 1.52 (whey) and 1.33 (water as dispersant). The measurements were performed under ambient conditions just after the UV treatment. d_{10} , d_{50} and d_{90} which are the particle diameter at 10 %, 50 % and 90 % of the cumulative distribution curve were noted. Further, the surface weighted mean (D [3,2]) and volume weighted mean (D [4,3]) were determined using following equations (Costa *et al.*, 2018)

Equation 8-6

$$D[3,2] = \frac{\sum n_i {d_i}^3}{\sum n_i {d_i}^2}$$

Equation 8-7

$$D[4,3] = \frac{\sum n_i {d_i}^4}{\sum n_i {d_i}^3}$$

Where d_i represents mean diameter of particle, n_i is the number of particles.

Rheology parameters

Rheology parameters were analysed to evaluate any effect of UV treatment on flow behaviour. It was measured with a rheometer (AR-G2, TA instrument, Texas, USA) by using concentric cylinder cup of 28 mm in diameter and DIN rotor as described by (Makwana, 2016). The whey sample (25 ^{o}C) was added to completely immerse the concentric cylinder and a shear rate of 300 s^{-1} was applied. The shear stress "σ" (mPa) was plotted against shear rate "γ" (s^{-1}) to evaluate the rheological differences between treated and untreated samples.

8.2.3.4. Experimental design

In the first part of experiments, thermal inactivation kinetics of *E. coli* ATCC 25922 was studied in RW at 51 ^{o}C and 57 ^{o}C as described in section 8.2.3.1 to determine D values. This temperature range was selected after a preliminary study that resulted in a measurable D value.

In the second part of experiments, CW was treated with UV-C and inactivation of native microbial count was assessed by measuring TVC at different inlet temperatures (4 ^{o}C, 20 ^{o}C

and 30 ^{o}C) as described in section 8.2.2.2. These inlet temperatures were obtained by placing whey in heated water bath. Similarly, UV-C inactivation of *E. coli* ATCC 25922 was determined in RW as it provides a microbial free source to study microbial inactivation.

In the third part of experiments, physico-chemical properties of UV treated whey (CW and RW) were studied and compared with pasteurization treatment conducted at 72 ^{o}C for 15 sec as described in section 8.2.3.3. Both types of whey were analysed for physico-chemical properties due to varied reasons. For instance RW can be an additive in beverages that can be treated with UV. On the other hand, preserving CW in liquid form by UV treatment can be beneficial after its separation from cheese or casein manufacturing. Physico-chemical properties that were assessed include colour, microstructure, flow behaviour, PSD, pH, conductivity and titratable acidity. Also, energy consumption of thermally pasteurized and UV treated whey was compared.

8.2.4. Statistical analysis

Statistical treatment of data was performed using Microsoft excel 2016. Mean and standard deviation were calculated for three replicates. Single way ANOVA was applied using $P < 0.05$ followed by T test using SPSS Version 25, IBM Inc., USA. The superscripts for means that are not different was obtained as described by Dallal (2015).

8.3. Results and discussion:

8.3.1. Thermal studies of *E. coli* ATCC 25922 in RW

Figure 8-2 shows the logarithmic reduction of *E. coli* ATCC 25922 in RW at temperatures of 51 ^{o}C and 57 ^{o}C. The linearity of graph confirms first order kinetics during thermal treatment. D values calculated from log linear kinetics are 13.67 ± 0.99 min (at 51 ^{o}C) to 0.09 ± 0.01 min (at 57 ^{o}C) ($P < 0.05$) (Table 8-2). $D_{57\,^{o}C}$ shows that 6 log reduction with *E. coli* can be obtained within 0.54 min of treatment at 57 ^{o}C. Literature D values for *E. coli* in whey are scarce. While comparing with *E. coli* ATCC 25922 in simulated milk ultra filtrate, D values of 5.2 min at 51 ^{o}C and 2.2 min at 53 ^{o}C were reported by Alkhafaji *et al.* (2008). This difference could be due to the difference in medium composition.

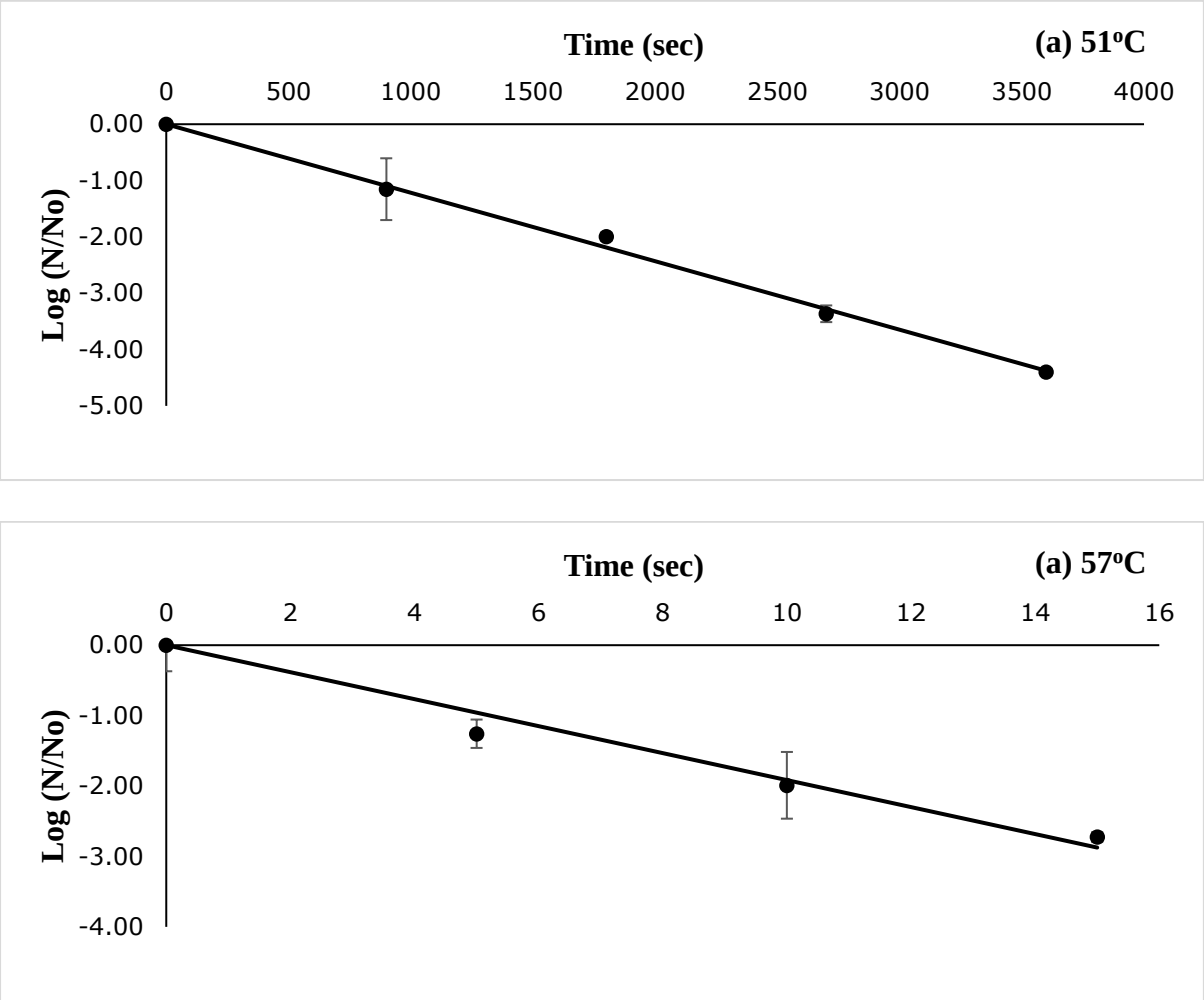


Figure 8-2: Thermal inactivation curves of *E. coli* ATCC 25922 in RW at (a) 51 ºC, and (b) 57 ºC.

Table 8-2: Thermal inactivation D values (min) of *E. coli* ATCC 25922 in RW at 51 ºC and 57 ºC.

Temperature	D values (min)
51 ºC	13.67 ± 0.99
57 ºC	0.09 ± 0.01

8.3.2. Microbial inactivation using UV treatment

Raw milk has diverse microflora which depends on hygiene during milk collection (Cilliers *et al.*, 2014). Microflora of CW thereby, arises from raw milk used for whey preparation and

contamination during cheese manufacturing. This suggests that microbial count of whey may include spores as well as vegetative cells. Vegetative cells requires much lower dosage of UV in comparison to microbial spores (Datta *et al.*, 2015).

Table 8-3 shows the inactivation in natural microbial load of CW (Total solids 8.34 ± 0.01 %, Absorption coefficient 43.53 ± 1.01 cm^{-1}) that was UV treated at different inlet temperatures. Different inlet temperatures of 4 ^{o}C, 20 ^{o}C and 30 ^{o}C were considered to resemble the temperature of whey after refrigerated, ambient and fermentation conditions respectively. The highest logarithmic inactivation was observed at 30 ^{o}C (5.8 ± 0.07) followed by 20 ^{o}C (4.92 ± 0.11) and 4 ^{o}C (3.64 ± 0.13) respectively after one pass (1.61 J/ml) through the UV reactor at 150 mL/min ($P < 0.05$). As total microbial count comprises of combination of psychrophilic, mesophilic and thermophilic organisms, log reduction obtained might include psychrotrophs in higher number. Thereby, resulted in higher inactivation with high inlet temperatures.

Also, the effect of UV treatment on inactivation of *E. coli* ATCC 25922 in RW (absorption coefficient of 156.30 ± 0.16 cm^{-1}) was assessed. Here again, the higher inactivation was observed at 30 ^{o}C (3.98 ± 0.23) in comparison to 4 ^{o}C (3.52 ± 0.14) with one pass through UV reactor (1.61 J/ml), which is similar to the trend observed for total viable count (Table 8-4). Subsequently, the second pass of UV inactivated the added *E. coli* completely (no colonies were observed during plating). This inactivation of *E. coli* with UV treatment is achieved at much lower temperature in comparison to thermal treatment (need 0.54 min at 57 ^{o}C for 6 log reduction). On the other hand, the difference in the degree of inactivation in CW and RW could be due to their difference in absorption coefficient and the difference in targeted microbial population. However, in principle, this study showed that UV is capable of reducing TVC and *E. coli* in whey in considerable quantity.

Table 8-3: UV-C inactivation of Natural microbial load in CW

<table>
<tr><th rowspan="2">Inlet temperature</th><th colspan="2">Log inactivation</th></tr>
<tr><th>UV one pass (1.61 J/ml)</th><th>UV second pass (3.23 J/ml)</th></tr>
<tr><td>4 ^{o}C</td><td>3.64 ± 0.16</td><td>5.60 ± 0.08</td></tr>
<tr><td>20 ^{o}C</td><td>4.92 ± 0.11</td><td rowspan="2">Below detection limit with initial count of 7.8</td></tr>
<tr><td>30 ^{o}C</td><td>5.8 ± 0.07</td></tr>
<tr><td>Pasteurization 72 ^{o}C for 15 sec</td><td colspan="2">Below detection limit with an initial count of 7.8</td></tr>
</table>

Note: [a-b] Means in a column without a common superscript letter differ significantly ($P < 0.05$)

Table 8-4: UV-C inactivation of *E. coli* ATCC 25922 in RW

Inlet temperature	Log inactivation	
	UV one pass (1.61 J/ml)	UV second pass (3.23 J/ml)
4 °C	3.52 ± 0.14^{a}	Below detection limit with initial count of 6.5
20 °C	3.83 ± 0.20^{ab}	
30 °C	3.98 ± 0.23^{b}	
Pasteurization 72 °C for 15 sec	Below detection limit with initial count of 6.5	

Note: [a-b] Means in a column without a common superscript letter differ significantly ($P < 0.05$)

8.3.3. Effect of UV-C treatment on physico-chemical parameters of whey

It was discussed earlier that UV-C possesses the capability to inactivate microorganisms in whey. However, it is also important to examine the effects of UV-C treatment on physico-chemical properties of it in comparison to pasteurized whey. Both types of UV treated whey (CW and RW) were considered for analysis since they can be used as additives to food products or consumed directly. Table 8-5 shows the effect of thermal and UV treatments on selected physicochemical properties of whey. Whey samples (CW) subject to UV treatment resulted in a small change in pH suggesting that UV has a minimal effect within the dosages used ($P < 0.05$), while no significant change was observed in RW ($P > 0.05$). This finding was also supported with the results of titratable acidity, as there was no significant change in titratable acidity ($P > 0.05$). Conductivity was analysed to identify if UV treatment possesses any effect on ionic changes in whey (Mucchetti *et al.*, 1994). A small change in conductivity was observed for treated whey samples for both CW and RW ($P < 0.05$)

Table 8-5: Physico-chemical properties of whey after UV treatment and pasteurization

Samples	pH	Conductivity (µS/cm)	Titratable acidity (as % Lactic acid)
		CW	
Untreated whey	6.84 ± 0.00^{ab}	1255.33 ± 2.52^{a}	0.08 ± 0.00^{a}
UV-one pass (1.61 J/ml)	6.84 ± 0.01^{a}	1249.67 ± 2.08^{ab}	0.08 ± 0.00^{abc}
UV- two passes (3.23 J/ml)	6.93 ± 0.01	1240.33 ± 3.51^{c}	0.08 ± 0.00^{abc}
Pasteurized (72 ^{o}C for 15 sec)	6.81 ± 0.02^{b}	1244.67 ± 1.53^{bcf}	0.08 ± 0.01^{ab}
		RW	
Untreated whey	7.17 ± 0.02^{c}	1281.67 ± 6.03^{d}	0.09 ± 0.00^{bc}
UV-one pass (1.61 J/ml)	7.17 ± 0.01^{c}	1275.67 ± 2.89^{de}	0.08 ± 0.00^{bc}
UV- two passes (3.23 J/ml)	7.17 ± 0.01^{c}	1270.67 ± 0.58^{7}	0.09 ± 0.00^{c}
Pasteurized (72 ^{o}C for 15 sec)	7.27 ± 0.02	1239.33 ± 1.53^{8}	0.08 ± 0.00^{abc}

Note: $^{a-f}$ Means in a column without a common superscript letter differ significantly ($P < 0.05$)

Table 8-6 shows the colour values (L^{*}, a^{*}, b^{*}) of UV-C treated and pasteurized whey. It was observed that TCD was highest for pasteurized whey in comparison to UV-C treated whey. A TCD value ~ 2.3 is considered as just noticeable difference (Sharma *et al.*, 2002). However, TCD during all treatments was lower than ~2.3 in all cases suggesting the change is not noticeable.

Further, optical microscopy was used to evaluate the microstructure of whey after UV treatment (Figure 8-3). A significant difference between CW and RW was obtained as indicated in the images (a-d & a$^{'}$-d$^{'}$). Fine particles were found in images of RW while coarse globules were shown in CW. The difference could be due to the presence of higher size fat globules in CW while whey protein concentrates that was used to prepare RW would have been processed through ultrafiltration. Optical microscopy images show no noticeable difference between fresh and treated samples for both types of whey that were UV treated.

Table 8-6: Colour parameters (L^*, a^*, b^*) of whey after UV-C treatment and pasteurization

Samples	L^*	a^*	b^*	TCD
CW				
Untreated whey	66.02 ± 0.06^{a}	-2.69 ± 0.08^{ab}	-0.66 ± 0.08^{a}	---
UV-one pass (1.61 J/ml)	66.22 ± 0.17^{ab}	-2.00 ± 0.53^{ac}	-0.31 ± 0.35^{a}	0.87 ± 0.26^{abc}
UV- two passes (3.23 J/ml)	66.11 ± 0.03^{bc}	-2.74 ± 0.13^{c}	-0.59 ± 0.05^{a}	1.02 ± 0.21^{bc}
Pasteurized (72 °C for 15 sec)	66.25 ± 0.04^{cd}	-3.02 ± 0.11^{c}	-1.62 ± 0.08^{b}	1.70 ± 0.34^{b}
RW				
Untreated whey	65.30 ± 0.21^{cd}	-2.10 ± 0.39^{ab}	-0.53 ± 0.12^{a}	---
UV-one pass (1.61 J/ml)	65.58 ± 0.08^{d}	-2.88 ± 0.16^{bc}	-0.67 ± 0.06^{a}	0.81 ± 0.55^{ac}
UV- two passes (3.23 J/ml)	65.71 ± 0.02^{d}	-2.97 ± 0.10^{abc}	-0.72 ± 0.02^{a}	0.15 ± 0.08^{a}
Pasteurized (72 °C for 15 sec)	66.00 ± 0.23^{d}	-3.17 ± 0.38^{c}	-1.55 ± 0.29^{b}	1.06 ± 0.18^{bc}

Note: $^{a\text{-}f}$ Means in a column without a common superscript letter differ significantly ($P < 0.05$)

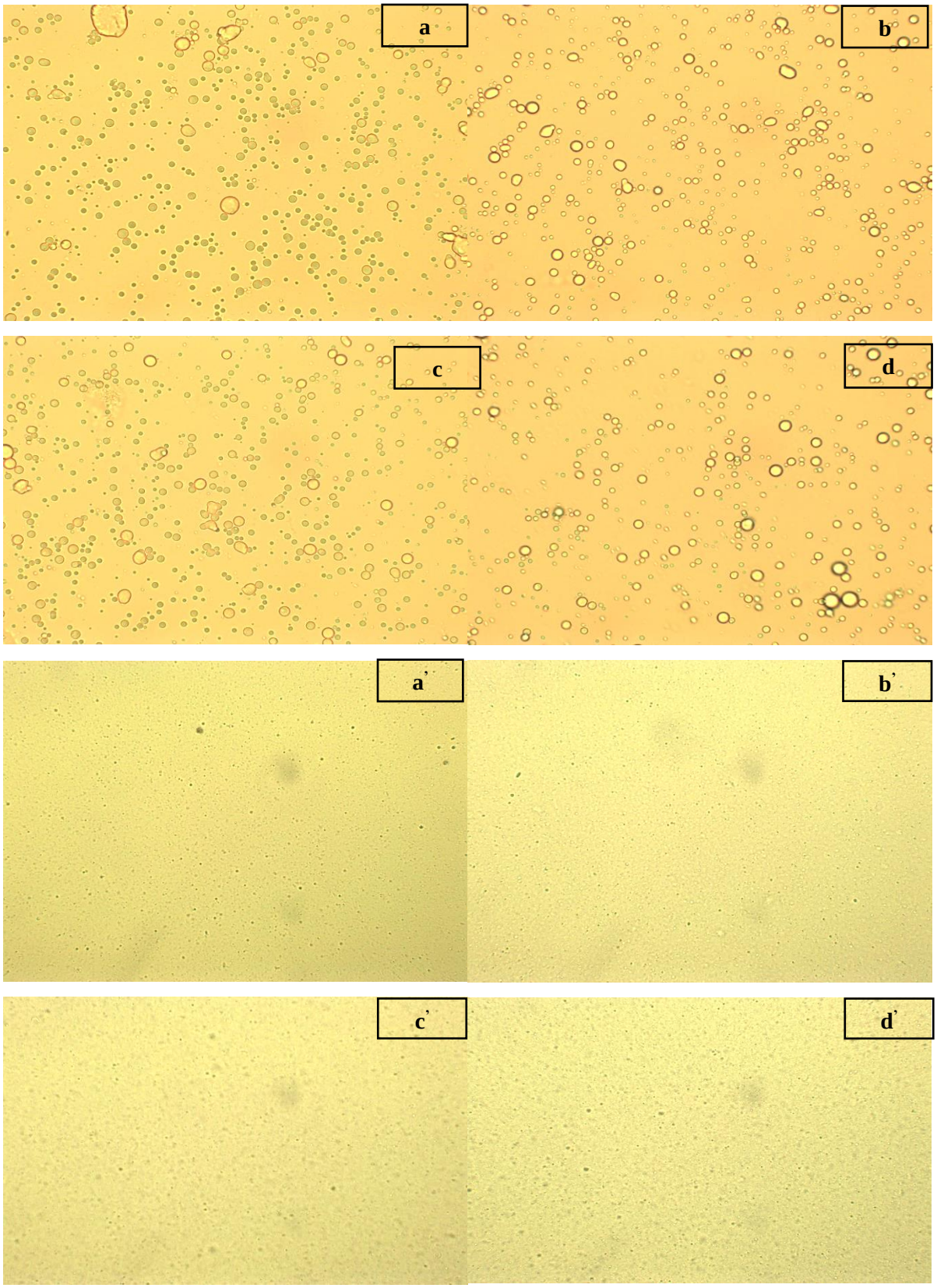


Figure 8-3: Optical microscopy (40X) images of (a) untreated whey, (b) UV treated whey (One pass, 1.61 J/ml), (c) UV treated whey (Two passes, 3.23 J/ml) and (d) Thermally treated whey (72 ^{o}C for 15 sec). (a-d represents CW while a'-d' represents RW)

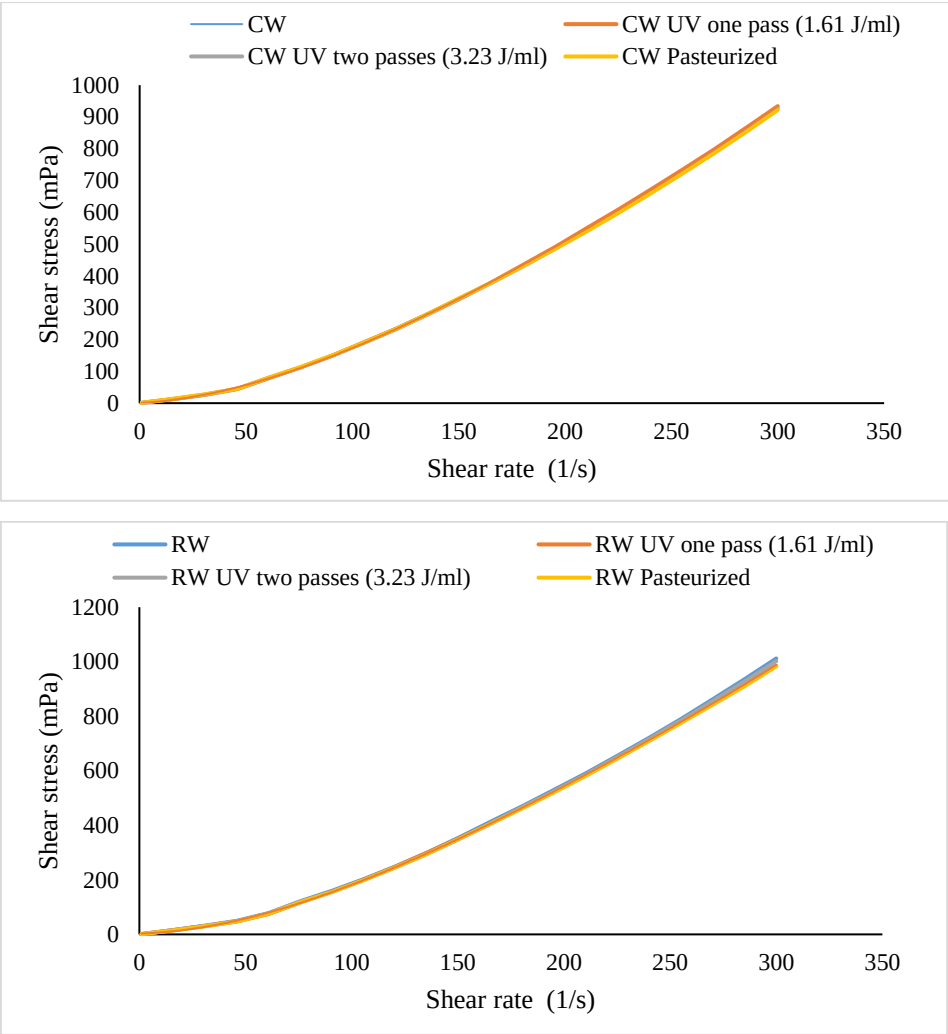


Figure 8-4: Flow curves of CW and RW after UV-C treatment and pasteurization.

Flow behaviour and particle size distribution (PSD) were also measured for UV treated, pasteurised and untreated whey for comparison (Figure 8-4 & Table 8-7). Flow rheology profile showed slightly curved behaviour in comparison to Newtonian fluids. Flow behaviour curves did not indicate any noticeable change in whey, which suggests that UV-C treatment does not affect rheological behaviour of liquid after treatment. Moreover, PSD is considered as an important parameter in defining stability and can vary with the treatment conditions indicating aggregation of components and formation of complexes in result of different treatments. No significant change was obtained in surface weighed mean D [3, 2] ($P > 0.05$). Volume mean diameter D [4, 3] is used to indicate presence of larger particles and a decrease was observed in volume weighed mean on UV treated whey samples. This could be explained as CW was forced through small diameter tubing and fat globules may had a tendency to reduce in size after pumping. Although, no significant change was observed in $D_{10\,\%}$ and $D_{50\,\%}$ ($P >$

0.05), a significant decrease was obtained in $D_{90\ \%}$ for CW ($P < 0.05$). As explained earlier, it could also be due to forced flow through small diameter tubing which resulted in this decrease.

Table 8-7: PSD of liquid whey after conventional pasteurization and UV-C treatment

Samples	Surface weighted mean D [3,2] µm	Volume weighted mean D [4,3] µm	$D_{10\ \%}$ µm	$D_{50\ \%}$ µm	$D_{90\ \%}$ µm	Obscuration (%)
CW						
Untreated whey	5.64 ± 0.06^{a}	31.31 ± 5.92	2.86 ± 0.01^{a}	6.50 ± 0.06^{a}	71.22 ± 12.14	4.63
UV-one pass (1.61 J/ml)	5.52 ± 0.06^{a}	23.38 ± 4.08	2.84 ± 0.01^{a}	6.39 ± 0.07^{a}	50.97 ± 7.68^{a}	4.54
UV- two passes (3.23 J/ml)	5.39 ± 0.05^{a}	20.87 ± 4.93	2.82 ± 0.01^{a}	6.23 ± 0.05^{a}	37.32 ± 4.95^{ab}	4.51
Pasteurized (72 ^{o}C for 15 sec)	5.40 ± 0.43^{a}	39.73 ± 0.21	2.84 ± 0.07^{a}	6.15 ± 0.44^{a}	28.40 ± 8.32^{b}	4.63
RW						
Untreated whey	0.19 ± 0.01^{b}	0.42 ± 0.06	0.09 ± 0.01^{b}	0.24 ± 0.02^{b}	0.78 ± 0.03^{c}	4.09
UV-one pass (1.61 J/ml)	0.17 ± 0.01^{b}	0.32 ± 0.01	0.09 ± 0.00^{b}	0.22 ± 0.01^{b}	0.68 ± 0.03^{c}	4.70
UV- two passes (3.23 J/ml)	0.17 ± 0.00^{b}	0.58 ± 0.03	0.08 ± 0.00^{b}	0.22 ± 0.00^{b}	0.78 ± 0.01^{c}	4.56
Pasteurized (72 ^{o}C for 15 sec)	0.17 ± 0.00^{b}	0.67 ± 0.06	0.08 ± 0.00^{b}	0.22 ± 0.00^{b}	0.82 ± 0.01^{c}	4.62

Note: ^{abcd} Means in a column without a common superscript letter differ significantly ($P < 0.05$)

8.3.4. Energy consumption

Power consumption required for UV treatment with two passes based on total lamp power was ~15.2 J/ml. This gave a microbial reduction that is equivalent to thermal pasteurization. On the other hand, thermal treatment from 63 ^{o}C to pasteurization temperature (72 ^{o}C), assuming efficient heat recovery, required ~36.738 J/ml (Figure 8-5) (Tomasula *et al.*, 2011). This study further showed that the reduction in energy is achievable while retaining the physico chemical properties of whey. Therefore, UV treatment has the capability to reduce the energy consumption needed for treatment by ~59 % in comparison to thermal pasteurization.

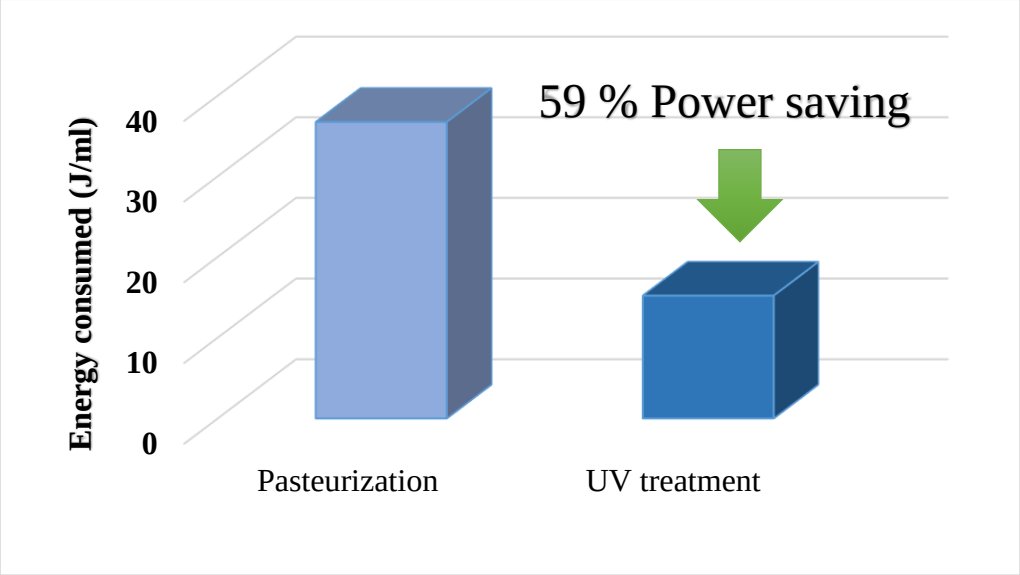


Figure 8-5: Power consumption using UV-C treatment and pasteurization.

8.4. Conclusion

The present study looked into the potential of whey treatment using UV-radiation. The results presented in this work show that UV treatment can inactivate microbial load of whey while retaining its physicochemical properties. The primary benefit of UV treatment over thermal lies in its lower energy consumption compared to thermal pasteurisation.

CHAPTER 9

Investigation of *B. subtilis* spores inactivation in different milk products using ultra high pressure homogenization

This chapter is based on the draft of the paper ready for submission to an international journal.

Preface

Ultra high temperature (UHT) thermal treatment of milk has been used for a long time because of its promising results on spore inactivation and thereby its ability to produce shelf stable product. However, this thermal treatment can adversely affect product quality as UHT treats the product at very high temperature (135-140 ^{o}C for few seconds). On the other hand, homogenization (up to 50 MPa) is a unit operation used prior to UHT treatment of milk to stabilise the product by preventing fat separation during storage. Moreover, Ultra high pressure homogenization (UHPH) is an emerging technology that applies pressures up to 420 MPa using a continuous process and possesses the capability to inactivate spores due to combined effect of high pressure and temperature at very short treatment time (less than 0.5 sec). In this study, moderate pressures (up to 250 MPa) were used in a UHPH unit to inactivate *B. subtilis* spores in different milk products that are diverse in fat and total solids contents. With high pressure homogenization at ~ 250 MPa and valve temperature of ~ 127 ^{o}C, UHPH resulted in 4.23 ± 0.50, 4.42 ± 0.100 and 3.33 ± 0.82 log reduction of *B. subtilis* spores in skimmed cow milk, whole cow milk, and sheep milk respectively. This study demonstrates that UHPH could be an alternate technique to UHT in extending shelf life of milk products.

9.1. Introduction

Spores are quite resistant to heat and thereby requires high temperature for inactivation. Currently, thermal processes like UHT is widely used to inactivate spores. However, UHT treatment can adversely affect the natural characteristics of milk (Cappozzo *et al.*, 2015; Popov-Raljić *et al.*, 2008; Renner, 1988; Van Boekel, 1998). This has led to the study of several emerging technologies, where microbial inactivation is achieved using a hurdle effect by combining more than one unit operation. One such possibility is to combine thermal treatment with homogenization that is being considered as an essential unit operation in milk processing (Bylund, 2003).

Homogenization is defined as a process of forcing liquid through a disruption valve, which results in reduction of particle size distribution (Georget *et al.*, 2014b). One of the primary objective of homogenization of milk is to attain a stabilized and a uniform emulsion of oil and water; thus preventing phase separation during storage (Perrechil *et al.*, 2010). In a typical milk processing plant, homogenization (up to 50 MPa) is carried out prior to thermal treatment. The pressure applied during conventional homogenization ranges between 30 MPa to 50 MPa;

generally, it has little or no impact on spores (Bevilacqua *et al.*, 2007; Gaulin, 1904; Georget *et al.*, 2014b). On the other hand, high pressure processing has long been studied for spore inactivation as it can be applied in multiple ways by i) application of high pressure to germinate bacterial spores followed by heating to inactivate sensitized spores (Van Opstal *et al.*, 2003), ii) the use of medium pressure processing in multiple cycles to germinate spores followed by high pressure to eliminate spores (Zhang *et al.*, 2008), iii) the simultaneous application of medium pressure and temperature as applied in pressure assisted thermal sterilization (Shibeshi *et al.*, 2011), and iv) the simultaneous use of high pressure (up to 400 MPa) and high temperature for short interval to inactivate spores through the use of high pressure homogenization (Espejo *et al.*, 2014; Georget *et al.*, 2014a; Georget *et al.*, 2014b).

High pressure homogenization (HPH) can inactivate microorganisms due to the combined effect of pressure and thermal treatment. On the basis of applied pressure, HPH can be categorized into two types. Pressure up to 200 MPa normally termed as HPH while applied pressure above 200 MPa, termed as Ultra High Pressure Homogenization (UHPH) (Georget *et al.*, 2014b). UHPH has an immense potential to inactivate different spores (Espejo *et al.*, 2014; Georget *et al.*, 2014a; Reverter-Carrión *et al.*, 2018; Roig-Sagués *et al.*, 2015). Previous literature show promising results on inactivation of heat resistant bacterial spores (*B. subtilis, G. stearothermophilus, B. cereus, B. licheniformis, B. amyloliquefaciens,* and *B. sporothermodurans*) in buffers, milk products, almond beverages (Dong *et al.*, 2015; Espejo *et al.*, 2014; Georget *et al.*, 2014a).

Milk products are quite diverse in their fat contents and total solids. Thereby, it is essential to analyse the effectiveness of a technology considering different media as inactivation of microbial spores differs with the matrix (Evelyn *et al.*, 2015b). Considering fat contents in skim cow milk (~ 0.1 wt%), whole cow milk (~ 3.2 wt%) and whole sheep milk (~ 5.24 wt%), these products provide diverse composition to analyse inactivation effects from emerging technology. Also, due to high total solids (~ 16-17 wt %), sheep milk is difficult to process with current UHT treatment as it results in protein sedimentation, affecting shelf stability, contrary to cow milk (Raynal-Ljutovac *et al.*, 2007).

B. subtilis is an aerobic organism and have the capability to spoil milk products by producing enzymes (Gopal *et al.*, 2015). It is also considered as the organism for validation of sterilization studies (Pharmacopeia., 2016). Therefore, it is the objective of the work presented in this paper to evaluate the effect of homogenization under medium pressure range (up to 250 MPa) and

heat to inactivate *B. subtilis* spores in different milk products, and especially for sheep milk which has not been studied before using UHPH.

9.2. Materials & methods

9.2.1. Media/Food matrix

Skimmed cow milk (SCM), whole cow milk (WCM) and sheep milk (SM) were used as media that contain increasing fat and total solid content. SCM and WCM were purchased in the form of UHT treated liquid milk that is aseptically packaged. SM was obtained in powdered form from Spring Sheep Milk Company, New Zealand and reconstituted to 1:6 using sterile distilled water.

9.2.2. Microbiological studies

9.2.2.1. Bacterial strains and sporulation

The freeze dried culture of *B. subtilis* ATCC 6633 was obtained from Fort Richard laboratory, New Zealand. The obtained culture was transferred to cooked meat medium (Fort Richard laboratories) and incubated for seven days at 35 ^{o}C. A wire loop of incubated culture was plated on nutrient agar (NA) (DIFCO) plates and further incubated for 24 h at 30 ^{o}C. Few colonies were transferred to nutrient broth and incubated at 37 ^{o}C for 24 h with continuous shaking. Inoculum obtained after incubation was plated (0.1 ml) to sporulation medium and incubated for 13 days at 30 ^{o}C. Sporulation medium composed of 23 g NA, 1 mg Manganese sulphate (Merck) and 0.5 g Calcium chloride (RDH) in 1 litre of water. During sporulation period, the proportion of spores was monitored by phase contrast microscopy (Motic microscope BA 410 Series, Canada) as described by Evelyn *et al.* (2015b). Spores were harvested by flooding plates with 3 ml of sterilized water and scraping plates using a spreader. The collected spore suspension was centrifuged three times at 1600 rpm (298 g), 4 ^{o}C for 15 min. After centrifugation, the supernatant was discarded, and sterilized water was used to make up the same volume. Finally, spore suspension was heat treated at 80 ^{o}C for 10 min to inactivate vegetative cells and then refrigerated at 4 ^{o}C until further use.

9.2.2.2. Enumeration of spores

Before and after treatments, spore count was determined by spread plating onto plate count agar and incubating at 30 ^{o}C for 24-48 h by making appropriate dilutions.

Spore inactivation

The spore inactivation with different treatment was measured by logarithmic ratio of spore concentration before and after treatment by using the following equation:

Equation 9-1

$$Spore\ inactivation\ = Log\ \frac{N}{N_o}$$

Where N represents initial microbial count and N_o represents microbial count after treatment

9.2.2.3. Thermal inactivation of spores

Thermal inactivation of spores was analysed by capillary tube method. The method is well known and used to study thermal inactivation kinetics by researchers due to its short come up time (Franklin *et al.*, 1958; Stern *et al.*, 1954; Van Zuijlen *et al.*, 2010). Inoculated milk samples (50 µl) were filled in capillary and sealed from both sides by using Bunsen burner with proper care to avoid heating of the liquid samples inside. For thermal treatment, these capillaries were transferred to oil bath maintained at 110 ^{o}C for different treatment time. After thermal treatment, these capillaries immediately shifted to cooling bath maintained at 0-2 ^{o}C. Later, treated capillaries were washed using 70 % ethanol solution followed by rinsing with sterilized water. Finally, the contents were flushed using sterilized water and appropriate dilutions were made before plating as per method described in section 9.2.2.2.

9.2.3. UHPH equipment and experimental plan

9.2.3.1.UHPH equipment and operation

The UHPH treatments were carried out using pilot scale unit (FPG7575:S6300, Stansted Power Fluid Ltd., UK) (Figure 9-1). The equipment comprised of two intensifiers driven by a hydraulic pump and high pressure ceramic valves where the primary valve can sustain 400 MPa. In order to achieve different inlet and outlet temperatures, the intensifiers were jacketed and could be maintained to required temperatures by circulating hot/cold water. There is a spiral type heat exchanger located between intensifiers and ceramic valves to preheat the

sample before treatment. For cooling after homogenization treatment, another spiral type heat exchanger is connected to a chiller at -4 ^{o}C. The processing parameters were indicated by pressure (P), feed temperature (T_f), inlet temperature correspond to temperature before homogenization (T_i), homogenization valve temperature (T_v) and outlet temperature that corresponds to temperature after cooling (T_o).

Prior to treatment, a batch of milk (5 litre) was inoculated with *B. subtilis* spore suspension to obtain ~10^6 cfu/ml. Then it was pre-heated to 55 ^{o}C using a waterbath. This pre-heating was necessary to achieve a high inlet and subsequent high valve temperatures. Meanwhile UHPH pressure was adjusted upto ~250 MPa to ensure achieving the required valve temperatures.Then the inoculated batch of milk was ciruclated through the UHPH unit at a flow rate of 42 L/h. Measurements were recorded after the system reached steady state.

Cleaning and sanitizing of the UHPH unit

Prior to any experiment, 70 v/v% ethanol was pumped through the unit for 2-3 min followed by rinsing with sterilized water for 15-20 min. After each experiment, 0.5 M NaOH was pumped through the system to ensure removal of any deposits followed by rinsing with water. Subsequent to any experiment, the microbial count of rinsed water was taken to ensure proper cleaning.

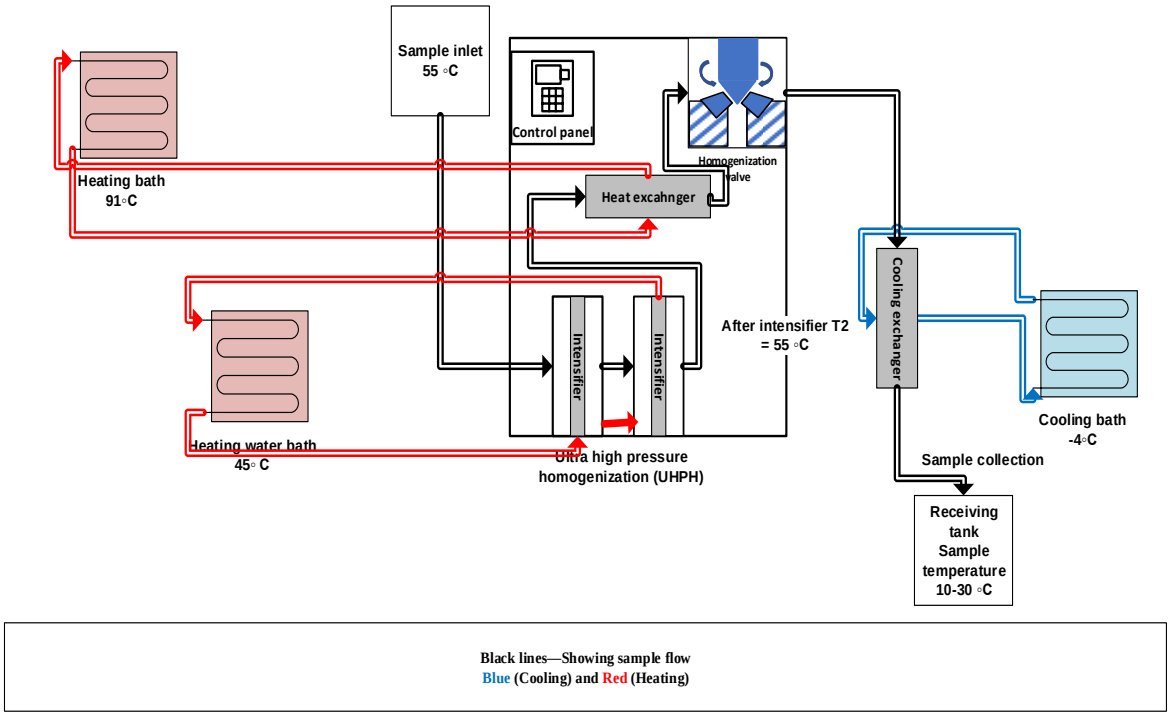


Figure 9-1: Schematic flow diagram of UHPH system

9.2.3.2. Energy consumption

The energy consumed during UHPH treatment is a combination of compression energy and that of heat generation. This could be taken as

Equation 9-2

$Q_{total} = Q_P - Q_T$

These components of energy were calculated by using following equation

Equation 9-3

$$Q_P(J) = Pressure\ (Pa) \times volume\ (m^3)$$

Q_P represents energy used during the application of constant pressure, while thermal energy (Q_T) generated was calculated by using the following equation

Equation 9-4

$$Q_T = mc\ \Delta T$$

Where Q_T represents thermal energy generation (Joules), m is the mass of sample, c is specific heat of milk samples and ΔT represents the temperature difference between T_i and T_v.

9.2.4. Statistical analysis

A single way ANOVA was used to compare means at $P < 0.05$ using SPSS followed by a Tukey test to find statistically significant differences. Data were obtained from three independent experiments carried out and results were presented as mean ± standard deviation.

9.3. Results and Discussion

9.3.1. Physical properties of milk

Table 9-1 shows the physical properties of different milk products used in this study. The total solids were highest in SM followed by WCM and SCM. These total solids arose from proteins, carbohydrates and fat content of the different milk samples. The cow's milk and SM mainly differ in protein contents. Total protein content of SM varies between 4.7 to 7.2 % which is almost twice as higher as in cow's milk (Raynal-Ljutovac *et al.*, 2008). In comparison to cow's

milk, heat sensitive proteins are higher in SM which may result in faster coagulation rate. In addition to differences in protein content, SM also contains higher fat and mineral content than cow's milk (Raynal-Ljutovac *et al.*, 2008). It has been mentioned that, milk micelles are highly mineralised in SM unlike in cow milk (Raynal-Ljutovac *et al.*, 2008). Moreover, measured viscosity values were close to the average values of WCM (0.0017 Pa s) and SM (0.00248 Pa s) as reported by Park (2007). Generally, viscosity decreases on heating but increases with coagulation. On the other hand, pH, conductivity and density of these three types of milk are slightly different. The density of SM was lower than cow's milk due to its higher fat content. Therefore, it can be noted that the density is inversely related to the fat content of milk (SCM > WCM > SM).

Table 9-1: Physical characteristics of different milk products

Characteristics	Type of milk		
	SCM	WCM	SM
Total solids (wt%)	8.81 ± 0.05	12.00 ± 0.02	16.19 ± 0.03
Moisture content (wt%)	91.19 ± 0.05	88 ± 0.05	83.81 ± 0.05
Viscosity (Pa s)	0.0017	0.0018	0.0023
pH	6.57	6.55	6.51
Conductivity (mS/cm)	5.14 ± 0.01	4.98 ± 0.01	4.52 ± 0.03
Density (g/cm^3)	1.031	1.027	1.023

9.3.2. Thermal inactivation of spores

In this study, thermal inactivation of *B. subtilis* spores was analysed in the different types of milk products. *B. subtilis* spores are mostly used for inactivation studies and also used for validation of sterilization. These spores require comparatively lower temperature for inactivation in comparison to other highly heat resistant organisms like *G. stearothermophilus* spores. Thereby, inactivation of *B. subtilis* was assessed at 110 ^{o}C to obtain a measurable log reduction. Table 9-2 gives the thermal D values of *B. subtilis* spores as 0.44 ± 0.01 min in SCM, 0.69 ± 0.04 min in WCM and 0.94 ± 0.06 min in SM at 110 ^{o}C. The increase in D values of SCM, WCM and SM could be due to increase in total solids (SCM < WCM < SM). In terms of cow's milk, Edwards *et al.* (1965) obtained a D value of 0.30 min at 112 ^{o}C in skim cow milk in *B. subtilis* spores strain A whereas, Jagannath *et al.* (2003) obtained 2.38 min at 98 ^{o}C for whole cow milk under thermal treatment for *B. subtilis* spores strain 168. These differences in D values could be due to the difference in strains, sporulation method and the composition of media.

Table 9-2: Thermal D values of *B. subtilis* spores

Media	***B. subtilis* spores D values at 110 ^{o}C (min)**
SCM	0.44 ± 0.01
WCM	0.69 ± 0.03
SM	0.94 ± 0.06

9.3.3. Pressure -Temperature variation during UHPH treatment

Table 9-3 shows the pressure-temperature variation during UHPH treatments where initial milk temperature (T_f), temperature before homogenization valve (T_i), Valve temperature (T_v), outlet temperature (T_o) and operating valve pressures were noted. The initial temperature in all treatments was set to 55 ^{o}C and UHPH was operated at three different pressures of 175 MPa, 200 MPa and 250 MPa. A temperature increase of 16 ^{o}C, 17.5 ^{o}C, 17.2 ^{o}C/100 MPa was obtained with pressure 175 MPa, 200 MPa, and 250 MPa respectively. These results are closer to the laboratory scale homogenization unit used in the study by Espejo *et al.* (2014), that found 17.83 ^{o}C / 100 MPa with an inlet temperature of 85 ^{o}C with valve pressure of 320 MPa. Also,

Hayes *et al.* (2003) obtained 17.6 ºC /100 MPa with a pressure treatment between 50-200 MPa. The temperature increase during UHPH treatment is due to the combined effect of shear stress, cavitation, and change of kinetic energy to pressure (Espejo *et al.*, 2014).

Subsequently, the batch of milk that was treated was rapidly cooled down to 20-30 ºC to maintain high temperature short time treatment conditions. Thereby, the residence time in UHPH treatment was quite low (less than one second) in comparison to UHT treatment. It is hypothesized that the < 1 sec residence time that was achieved in UHPH treatment could retain quality factors in milk.

Table 9-3: Pressure -Temperature variation in UHPH treatment (Water)

Initial milk temperature T_{feed} (º C)	**Pressure (MPa)**	**T_I (º C)**	**T_V (º C)**	**T_O (º C)**
55	174.67 ± 6.03	84.33 ± 1.53	112.67 ± 1.53	27.8 ± 1.82
55	205 ± 7	84.67 ± 1.52	117 ± 2.65	26.57 ± 2.25
55	254 ± 10.07	84 ± 1	127.33 ± 2.52	25.4 ± 3.80

9.3.4. UHPH Inactivation of spores

The inactivation of the *B. subtilis* spores in SCM, WCM, and SM when treated at the three different UHPH pressure settings are given in Table 9-4. When the valve temperature of UHPH treatment was at around 112 ºC at 174 MPa, no considerable inactivation of *B. subtilis* spores was obtained. This minimum inactivation is comparable to thermal only inactivation at 110 ºC for few seconds, suggesting that inactivation in UHPH treatment at these settings may have driven by temperature for all three types of milk. On the other hand, UHPH treatment at 250 MPa, with a valve temperature of 127.33 ± 2.52 ºC resulted in inactivation greater than 4 log in SCM and WCM while 3.33 log reduction in SM. The inactivation in SM is significantly

lower than SCM or WCM ($P < 0.05$) and inversely related to their viscosity. These differences in viscosity of milk samples may have contributed towards to a lesser extent of turbulence and cavitation in the media and thereby affected the degree of inactivation in UHPH treatment.

Table 9-4: Inactivation of *B. subtilis* spores in UHPH treatments

Pressure (MPa)	T Valve (º C)	*B. subtilis* inactivation (log reduction)		
		SCM	WCM	SM
174.67 ± 6.03	112.67 ± 1.53	0.16 ± 0.17	0.07 ± 0.12	0.341 ± 0.36
205 ± 7	117 ± 2.65	0.22 ± 0.10	0.57 ± 0.20	0.778 ± 0.41
254 ± 10.07	127.33 ± 2.52	4.23 ± 0.50	4.42 ± 0.10	3.33 ± 0.82

Literature found on HPH/UHPH inactivation can be divided into two categories as, application of HPH/UHPH with low valve temperature (Tv < 100 ºC) and high valve temperatures (T_v > 100 ºC). The use of HPH/UHPH at low temperatures was not promising since it gives low reduction in spore count. For e.g. Feijoo *et al.* (1997) studied the effect of inlet temperatures from 33ºC to 50 ºC on *B. licheniformis* spores reduction at 200 MPa, and noticed only an increase from 0.53 log to 0.75 log reduction. On the other hand, Espejo *et al.* (2014) studied the effects of inlet temperatures of 55 to 85 ºC, which results into valve temperatures from 113 ºC to 139 ºC when the pressure applied was 300 MPa in WCM. They showed that increase in inlet temperature resulted in increase in spore inactivation. With T_i of 65 ºC, T_v of 121 ºC, log reduction of 4.41 ± 0.29, 2.64 ± 0.35, 2.19 ± 0.63, 4.81 ± 0.47, 2.27 ± 1.10, and 0.74 ± 0.13 was obtained for *B. cereus*, *B. licheniformis*, *B. sporothermodurans*, *B. coagulans*, *G. stearothermophilus* and *B. subtilis* respectively. Therefore, these studies together with the present study showed that operating UHPH at higher temperatures has given promising results. Similar trend of high inactivation with high inlet temperature was obtained by Georget *et al.* (2014a) on spores of *Bacillus subtilis* and *Geobacillus stearothermophilus* in PBS buffer. At pressures of 300 MPa and 350 MPa, it was found that there was no inactivation in spores at inlet temperatures of 37 ºC (*B. subtilis*) and 55 ºC (*G. stearothermopilus*). Consequently, increase in inlet temperature to 80 ºC, resulted up to five log reduction in *B. subtilis* and two log reduction in *G. stearothermophilus* spores. In another study, Roig-Sagués *et al.* (2015) showed that the effect of different inlet temperatures on *A. acidoterrestris* and *A. hesperidum*. On increasing inlet temperature from 20 ºC to 80 ºC, inactivation of spores increased from 0.5

log to 5.5 log approximately for *A. acidoterrestris* spores and 0.2 to 5 log approximately for *A. hesperidum*. On the other hand, Dong *et al.* (2015) reported only 3.5 log reduction in spores of *A.acidoterrestris* at 350 MPa with valve temperature higher than 150 ^{o}C.

It is important to consider the mechanism of inactivation during high pressure homogenization. Previous work has suggested that high pressure (up to 150 MPa) can induce spore germination by releasing calcium dipicolinic acid in the core of the spores and thereby sensitize spores to thermal treatment (Chaves-López *et al.*, 2009). However, high pressure (dynamic pressure) applied in UHPH may not germinate spores with very short time (less than 1 sec) contrary to high pressure processing (static pressure). Hence, the benefits of inactivation may be driven by thermal treatment during UHPH treatment. Georget *et al.* (2014a) also observed that inactivation during UHPH were closer to modelled thermal inactivation. One of the primary benefit of UHPH over UHT is its short treatment time, which can minimize adverse effect on milk quality. Poliseli-Scopel *et al.* (2014) findings supported quality effects when they showed lower furan concentration in UHPH treated milk in comparison to UHT milk. In another study, Cruz *et al.* (2007) found that UHPH treated soymilk was more stable than UHT treated product.

9.3.5. Energy consumption during UHPH

The energy consumed (Q_p) during UHPH when operated at a pressure of 250 MPa was calculated using Equation 9-3 as 250 J/ml. Also, this pressure application resulted in temperature increase of approx. 43 ^{o}C suggesting the possibility of energy recovery after homogenization process. Based on this study, it showed that UHPH process contributed 73 % of its energy as heat (Q_T) (Equation 9-4). Moreover, the use of UHPH has the added benefit of eliminating conventional homogenization which may result in saving capital and operating costs. Based on the results, total energy required (Q_t) during UHPH was 67.5 J/ml.

9.4. Conclusion

This study analysed the inactivation of *B. subtilis* spores in different milk products (SCM, WCM and SM) using UHPH treatment. The obtained results show that UHPH possesses the capability to inactivate spores in milk products even with high total solid content. The energy consumption with UHPH shows the possibility to lower operating cost as it can combine homogenization and UHT treatments.

Conclusions

Low acid liquid foods have limited shelf life as they provide favourable conditions for microorganisms survival. Moreover, microorganisms have the possibility to convert themselves to spores which are quite resistant to thermal treatment. Food shelf life is directly related to the extent of microbial or spore inactivation, which could be achieved through processing. This thesis mainly evaluated the use of ultrasound, ultraviolet and ultra high pressure homogenization in combination with heat with an objective to reduce temperature during conventional thermal treatment.

At the outset of this work, ultrasonication followed by heat was assessed to analyse any potential sensitization effect of ultrasonication on spores. The microbiological studies conducted using *B. subtilis* spores showed only a slight advantage in spore inactivation in water, whole milk and rice porridge and may not be justifiable to apply this combination of technology in industry. Therefore it was relevant to continue to explore combination of other emerging technologies with heat to assess inactivation of spores at lower temperatures while retaining quality factors.

Subsequently, the study on ultraviolet in combination with heat was more extensive covering different media and types of microorganisms. The initial studies investigated the potential of using UV in combination with heat in inoculated media (*B. subtilis* and *G. stearothermophilus* spores) as an alternative method for thermal sterilization. The results show that ultraviolet treatment in combination with heat is more efficient in spore inactivation, especially for skimmed cow milk. This benefit was less for whole cow milk and sheep milk as their transmittance to ultraviolet is lower than skimmed cow milk due to their high total solid content. In addition, whole cow milk and sheep milk have high viscosity that minimizes the exposure under similar flow conditions. The results showed that ultraviolet pre-treatment could lower the adverse effects during thermal sterilization. Therefore, this combined technology could be an economical alternative to conventional sterilization for skimmed cow milk and probably for other types of milk if ultraviolet exposure could be improved further.

The ultraviolet treatment was applied on pasteurized skimmed cow milk to analyze its effect on shelf life extension. Microbiological and physicochemical studies revealed that the shelf life of milk increased with the increase in ultraviolet dosage. It was also found that this extension was mainly due to the sporicidal effect of ultraviolet treatment and thereby assisted in obtaining an extension of shelf life of milk approximately by 50 days. The outcome of this study could help to meet the logistics and transportation challenges in the global trade of ESL milk.

Even though our study showed that ultraviolet is capable to inactivate microorganisms, the challenge remains in the adverse effects of ultraviolet treatment due to photo oxidation. The present study evaluated the effect of nitrogen purging prior to ultraviolet treatment of milk using a continuous flow reactor. It was revealed that the negative effects of photo oxidation can be reduced when there is no exposure to atmosphere during processing. However, protein oxidation studies showed that the advantage of nitrogen purging is considerable only during prolong exposure to ultraviolet. The outcome of this study provides possibility of nitrogen purging prior to milk processing to minimize oxidative changes.

Based on the results of microbial inactivation with skimmed cow milk, the present study also looked into the potential of whey treatment using ultraviolet radiation due to better transmission to ultraviolet. The results presented in this work showed that ultraviolet treatment can inactivate microbial load of whey while retaining its physicochemical properties. It was proved that the primary advantage of ultraviolet treatment over thermal lies in its lower energy consumption compared to thermal pasteurization and thereby, it might be used for ready to drink whey beverages.

The benefits of ultraviolet remains limited to low total solids product like skimmed cow milk and whey. For high total solid content, we have investigated the use of ultra high pressure homogenization in which the application of combined pressure, temperature and homogenization was studied for inactivation of *B. subtilis* spores in different milk products (Skimmed cow milk, whole cow milk and sheep milk) that vary in their total solids. The results obtained in this study showed that UHPH could inactivate spores in different milk products (with different levels) while providing lower holding time in comparison to thermal processes. The results also showed that the extent of inactivation of spores depends on the valve temperature and pressure. Consequently, preheating of the milk was found to improve inactivation.

Thus, this study established that UV followed by heat can inactivate spores at lower temperature. However, extent of spore inactivation obtained from UV is directly related to the total solids content and properties of milk. Hence, such a combination could be successfully employed for skim milk and whey having considerable transmission properties. Based on this study, sterilization for skim milk can be achieved at 120 ^{o}C for 10 sec in combination with UV (D_{ACT} 2.37 J/ml), which resulted in 5.38 log reduction of *G. stearothermophilus* spores while longer treatment time is required for whole milk. Ultrasonication followed by heat gave only

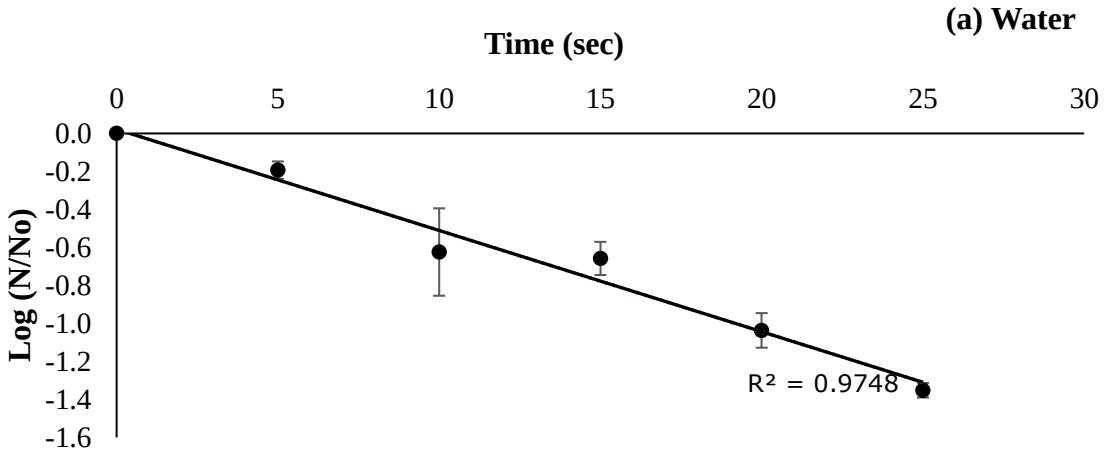
minor advantage during spore inactivation and thereby is not feasible to replace conventional sterilization. In comparison, UHPH inactivation of spores is mainly driven by thermal effects while providing short treatment time.

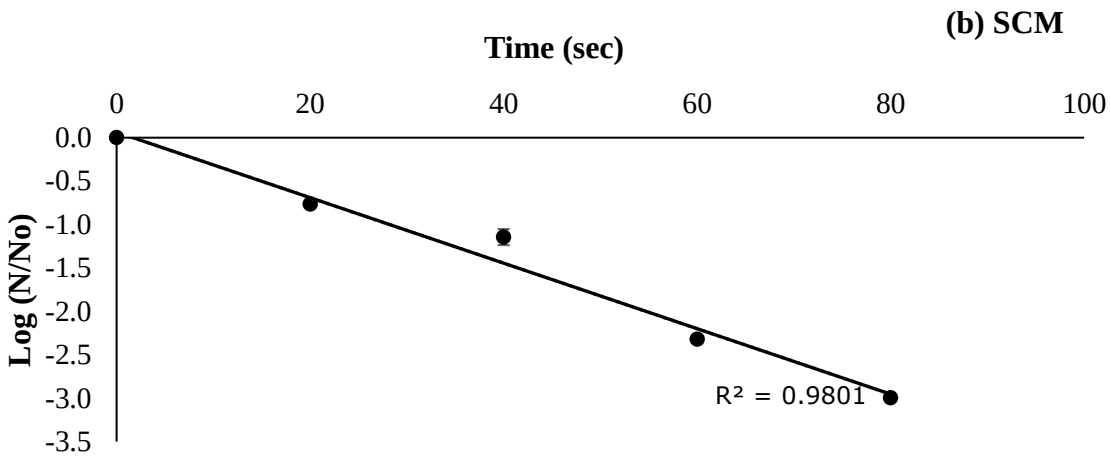
Future Recommendations

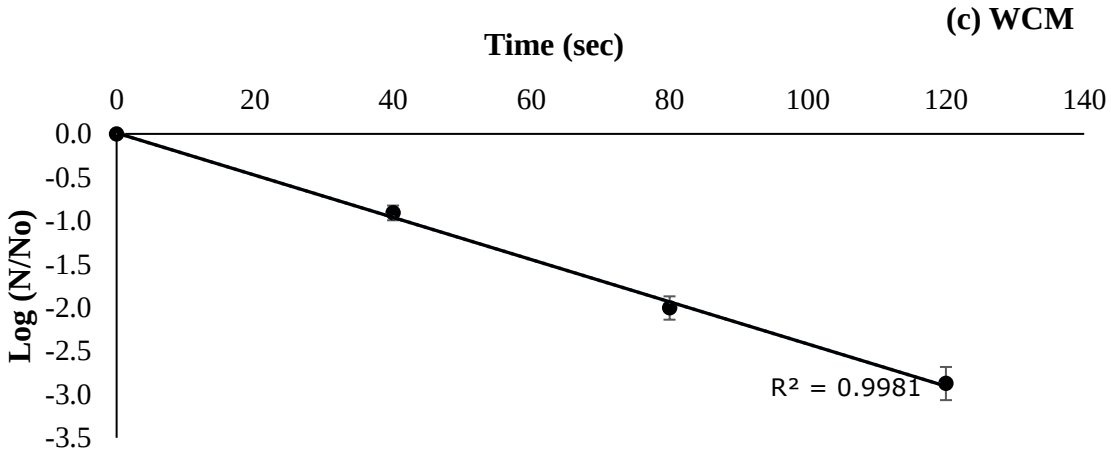
The use of emerging technologies for minimizing the use of heat during processing of milk were investigated. For future works, the recommendations are given as below:

- To identify the targeted microorganism and to investigate the mechanism of microbial inactivation with different emerging technologies.
- To study the use of UV-C LEDs to further reduce energy consumption of a UV treatment process.
- To compare the effects on food components like α-lactalbumin and β-lactoglobulin by emerging technologies with the operating conditions that cause sufficient microbial inactivation.
- To make a holistic comparison of emerging technologies with conventional treatment to assess commercial benefits.
- To address legislative requirements with the use of emerging technologies as necessary for commercialization.

Supplementary material:







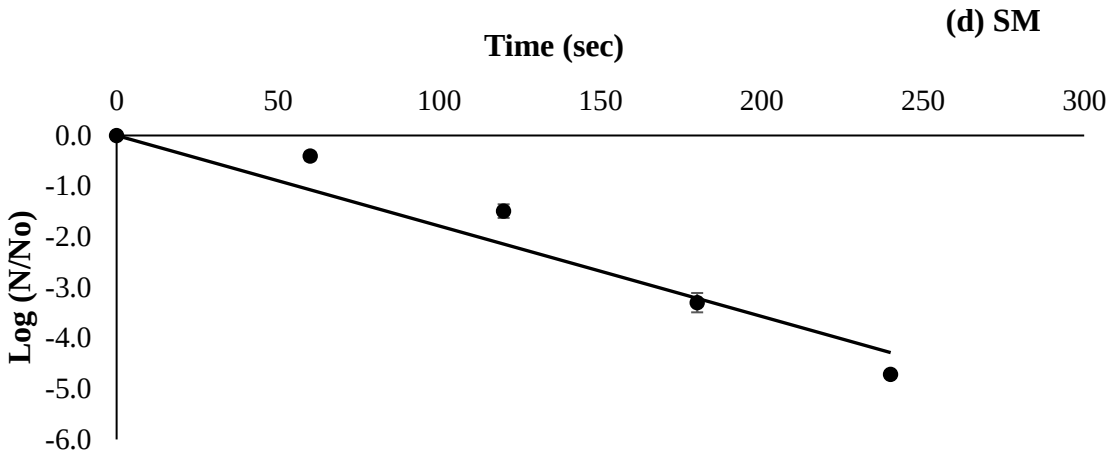


Figure: Thermal inactivation curves for *B. subtilis* ATCC 6633 spores at 110 ^{o}C (a) Water, (b) SCM (c) WCM (d) SM

References

Al-Attabi, Z., D'Arcy, B. R., & Deeth, H. C. (2009). Volatile sulphur compounds in UHT milk. *Critical Reviews in Food Science and Nutrition, 49*(1), 28-47. 10.1080/10408390701764187

Alberini, F., Simmons, M. J. H., Parker, D. J., & Koutchma, T. (2015). Validation of hydrodynamic and microbial inactivation models for UV-C treatment of milk in a swirl-tube 'SurePure Turbulator™'. *Journal of Food Engineering, 162*, 63-69. http://dx.doi.org/10.1016/j.jfoodeng.2015.04.009

Alkhafaji, S., & Farid, M. (2008). Modelling the inactivation of *Escherichia coli* ATCC 25922 using pulsed electric field. *Innovative Food Science & Emerging Technologies, 9*(4), 448-454. https://doi.org/10.1016/j.ifset.2008.02.003

Alkhafaji, S., & Farid, M. M. (2012). PEF Assisted Thermal Sterilization (PEF-ATS) Process-Inactivation of *Geobacillus sterothermophilus* Spores. *Journal of Food Science and Engineering*, *2*, 403-410.

Allen, C., Loo, J. F., Yu, S., Kong, S., & Chan, T.-F. (2014). Monitoring bacterial growth using tunable resistive pulse sensing with a pore-based technique. *Applied Microbiology and Biotechnology, 98*(2), 855-862.

Amador-Espejo, G. G., Suàrez-Berencia, A., Juan, B., Bárcenas, M. E., & Trujillo, A. J. (2014). Effect of moderate inlet temperatures in ultra-high-pressure homogenization treatments on physicochemical and sensory characteristics of milk. *Journal of Dairy Science, 97*(2), 659-671. https://doi.org/10.3168/jds.2013-7245

Ansari, J. A., Ismail, M., & Farid, M. (2017). Investigation of the use of ultrasonication followed by heat for spore inactivation. *Food and Bioproducts Processing, 104*, 32-39. https://doi.org/10.1016/j.fbp.2017.04.005

Artíguez, M. L., & Martínez de Marañón, I. (2015). Improved process for decontamination of whey by a continuous flow-through pulsed light system. *Food Control, 47*, 599-605. https://doi.org/10.1016/j.foodcont.2014.08.006

Atamer, Z., Samtlebe, M., Neve, H., Heller, K. J., & Hinrichs, J. (2013). Review: elimination of bacteriophages in whey and whey products. *Frontiers in Microbiology, 4*, 191.

Augustyniak, E., Adam, A., Wojdyla, K., Rogowska-Wrzesinska, A., Willetts, R., Korkmaz, A., Atalay, M., Weber, D., Grune, T., Borsa, C., Gradinaru, D., Chand Bollineni, R., Fedorova, M., & Griffiths, H. R. (2015). Validation of protein carbonyl measurement: a multi-centre study. *Redox Biology, 4*, 149-157. 10.1016/j.redox.2014.12.014

Bakshi, A. S., & Smith, D. E. (1984). Effect of Fat Content and Temperature on Viscosity in Relation to Pumping Requirements of Fluid Milk Products1. *Journal of Dairy Science, 67*(6), 1157-1160. 10.3168/jds.S0022-0302(84)81417-4

Ballingall, J., & Pambudi, D. (2017). Dairy trade's economic contribution to New Zealand.

Bandla, S., Choudhary, R., Watson, D. G., & Haddock, J. (2012a). Impact of UV-C processing of raw cow milk treated in a continuous flow coiled tube ultraviolet reactor. *Agricultural Engineering International: CIGR Journal, 14*(2), 86-93.

Bandla, S., Choudhary, R., Watson, D. G., & Haddock, J. (2012b). UV-C treatment of soymilk in coiled tube UV reactors for inactivation of *Escherichia coli* W1485 and *Bacillus cereus* endospores. *LWT-Food Science and Technology, 46*(1), 71-76.

Baysal, A. H., Molva, C., & Unluturk, S. (2013). UV-C light inactivation and modeling kinetics of *Alicyclobacillus acidoterrestris* spores in white grape and apple juices. *International Journal of Food Microbiology, 166*(3), 494-498. 10.1016/j.ijfoodmicro.2013.08.015

Berk, Z. (2009). Chapter 17 - Thermal processing *Food Process Engineering and Technology* (pp. 355-373). San Diego: Academic Press.

Bevilacqua, A., Campaniello, D., Sinigaglia, M., & Corbo, M. R. (2015). Combination of ultrasound and antimicrobial compounds towards *Pichia spp*. and *Wickerhamomyces anomalus* in pineapple juice. *LWT - Food Science and Technology, 64*(2), 616-622. http://dx.doi.org/10.1016/j.lwt.2015.06.038

Bevilacqua, A., Cibelli, F., Corbo, M. R., & Sinigaglia, M. (2007). Effects of high-pressure homogenization on the survival of *Alicyclobacillus acidoterrestris* in a laboratory medium. *Letters in Applied Microbiology, 45*(4), 382-386. 10.1111/j.1472-765X.2007.02219.x

Bevilacqua, A., Corbo, M. R., & Sinigaglia, M. (2012). High-pressure homogenisation and benzoate to control *Alicyclobacillus acidoterrestris*: a possible way? *International Journal of Food Science & Technology, 47*(4), 879-883. 10.1111/j.1365-2621.2011.02913.x

Black, E. P., Setlow, P., Hocking, A. D., Stewart, C. M., Kelly, A. L., & Hoover, D. G. (2007). Response of Spores to High-Pressure Processing. *Comprehensive Reviews in Food Science and Food Safety, 6*(4), 103-119. 10.1111/j.1541-4337.2007.00021.x

Blaschek, K. M., Wendorff, W. L., & Rankin, S. A. (2007). Survey of Salty and Sweet Whey Composition from Various Cheese Plants in Wisconsin. *Journal of Dairy Science, 90*(4), 2029-2034. https://doi.org/10.3168/jds.2006-770

Block, S. S. (1977). Disinfection, sterilization, and preservation. *Soil Science, 124*(6), 378.

Bottone, E. J. (2010). *Bacillus cereus*, a volatile human pathogen. *Clinical Microbiology Reviews, 23*(2), 382-398. 10.1128/CMR.00073-09

Briviba, K., Gräf, V., Walz, E., Guamis, B., & Butz, P. (2016). Ultra high pressure homogenization of almond milk: Physico-chemical and physiological effects. *Food Chemistry, 192*, 82-89. http://dx.doi.org/10.1016/j.foodchem.2015.06.063

Bull, M., Olivier, S., Van Diepenbeek, R., Kormelink, F., & Chapman, B. (2009). Synergistic inactivation of spores of proteolytic *Clostridium botulinum* strains by high pressure and heat is strain and product dependent. *Applied and Environmental Microbiology, 75*(2), 434-445.

Burgos, J., Ordonez, J. A., & Sala, F. (1972). Effect of Ultrasonic Waves on the Heat Resistance of *Bacillus cereus* and *Bacillus licheniformis* Spores. *Applied Microbiology, 24*(3), 497-498.

Burton, H. (2012). *Ultra-high-temperature processing of milk and milk products*: Springer Science & Business Media.

Butler, I. B., Schoonen, M. A. A., & Rickard, D. T. (1994). Removal of dissolved oxygen from water: A comparison of four common techniques. *Talanta, 41*(2), 211-215. http://dx.doi.org/10.1016/0039-9140(94)80110-X

Bylund, G. (2003). *Dairy processing handbook*: Tetra Pak Processing Systems AB.

Cambié, D., Bottecchia, C., Straathof, N. J., Hessel, V., & Noël, T. (2016). Applications of continuous-flow photochemistry in organic synthesis, material science, and water treatment. *Chemical Reviews, 116*(17), 10276-10341.

Cano, R. J., & Borucki, M. K. (1995). Revival and identification of bacterial spores in 25-to 40-million-year-old Dominican amber. *Science, 268*(5213), 1060.

Cappozzo, J. C., Koutchma, T., & Barnes, G. (2015). Chemical characterization of milk after treatment with thermal (HTST and UHT) and nonthermal (turbulent flow ultraviolet) processing technologies. *Journal of Dairy Science, 98*(8), 5068-5079. http://dx.doi.org/10.3168/jds.2014-9190

Casillas-Buenrostro, R. M., Heredia, N. L., Benesh, D. L., & García, S. (2012). Efficacy of 3M™ Petrifilm™ aerobic count plates for enumerating *Bacillus sporothermodurans* and *Geobacillus stearothermophilus* in UHT milk. *International Dairy Journal, 25*(2), 147-149. https://doi.org/10.1016/j.idairyj.2012.01.004

Chavan, Y., & Singhal, R. S. (2013). Ultrasound-assisted extraction (UAE) of bioactives from arecanut (*Areca catechu* L.) and optimization study using response surface

methodology. *Innovative Food Science & Emerging Technologies, 17*, 106-113. http://dx.doi.org/10.1016/j.ifset.2012.10.001

Chaves-López, C., Lanciotti, R., Serio, A., Paparella, A., Guerzoni, E., & Suzzi, G. (2009). Effect of high pressure homogenization applied individually or in combination with other mild physical or chemical stresses on *Bacillus cereus* and *Bacillus subtilis* spore viability. *Food Control, 20*(8), 691-695. http://dx.doi.org/10.1016/j.foodcont.2008.09.001

Chemat, F., Grondin, I., Costes, P., Moutoussamy, L., Sing, A. S. C., & Smadja, J. (2004). High power ultrasound effects on lipid oxidation of refined sunflower oil. *Ultrasonics Sonochemistry, 11*(5), 281-285. http://dx.doi.org/10.1016/j.ultsonch.2003.07.004

Chen, W., Harte, F. M., Davidson, P. M., & Golden, D. A. (2013). Inactivation of *Alicyclobacillus acidoterrestris* using high pressure homogenization and dimethyl dicarbonate. *Journal of Food Protection, 76*(6), 1041-1045. 10.4315/0362-028x.jfp-12-443

Choudhary, R., Bandla, S., Watson, D. G., Haddock, J., Abughazaleh, A., & Bhattacharya, B. (2011). Performance of coiled tube ultraviolet reactors to inactivate *Escherichia coli* W1485 and *Bacillus cereus* endospores in raw cow milk and commercially processed skimmed cow milk. *Journal of Food Engineering, 107*(1), 14-20. http://dx.doi.org/10.1016/j.jfoodeng.2011.06.009

Christiansson, A., Naidu, A. S., Nilsson, I., Wadström, T., & Pettersson, H. (1989). Toxin production by *Bacillus cereus* dairy isolates in milk at low temperatures. *Applied and Environmental Microbiology, 55*(10), 2595-2600.

Cilliers, F. P., Gouws, P. A., Koutchma, T., Engelbrecht, Y., Adriaanse, C., & Swart, P. (2014). A microbiological, biochemical and sensory characterisation of bovine milk treated by heat and ultraviolet (UV) light for manufacturing Cheddar cheese. *Innovative Food Science & Emerging Technologies, 23*, 94-106.

Claeys, W. L., Cardoen, S., Daube, G., De Block, J., Dewettinck, K., Dierick, K., De Zutter, L., Huyghebaert, A., Imberechts, H., Thiange, P., Vandenplas, Y., & Herman, L. (2013). Raw or heated cow milk consumption: Review of risks and benefits. *Food Control, 31*(1), 251-262. http://dx.doi.org/10.1016/j.foodcont.2012.09.035

Conesa, R., Periago, P. M., Esnoz, A., López, A., & Palop, A. (2003). Prediction of *Bacillus subtilis* spore survival after a combined non-isothermal-isothermal heat treatment. *European Food Research and Technology, 217*(4), 319-324.

Coohill, T. P., & Sagripanti, J. L. (2008). Overview of the inactivation by 254 nm ultraviolet radiation of bacteria with particular relevance to biodefense. *Photochemistry and Photobiology, 84*(5), 1084-1090. 10.1111/j.1751-1097.2008.00387.x

Costa, N. R., Cappato, L. P., Ferreira, M. V. S., Pires, R. P. S., Moraes, J., Esmerino, E. A., Silva, R., Neto, R. P. C., Tavares, M. I. B., Freitas, M. Q., Silveira Júnior, R. N., Rodrigues, F. N., Bisaggio, R. C., Cavalcanti, R. N., Raices, R. S. L., Silva, M. C., & Cruz, A. G. (2018). Ohmic Heating: A potential technology for sweet whey processing. *Food Research International, 106*, 771-779. https://doi.org/10.1016/j.foodres.2018.01.046

Cregenzán-Alberti, O., Arroyo, C., Dorozko, A., Whyte, P., & Lyng, J. G. (2017). Thermal characterization of *Bacillus subtilis* endospores and a comparative study of their resistance to high temperature pulsed electric fields (HTPEF) and thermal-only treatments. *Food Control, 73*, 1490-1498. http://dx.doi.org/10.1016/j.foodcont.2016.11.012

Crook, J. A., Rossitto, P. V., Parko, J., Koutchma, T., & Cullor, J. S. (2015). Efficacy of Ultraviolet (UV-C) Light in a Thin-Film Turbulent Flow for the Reduction of Milkborne Pathogens. *Foodborne Pathogens and Disease, 12*(6), 506-513. 10.1089/fpd.2014.1843

Cruz, N., Capellas, M., Hernández, M., Trujillo, A. J., Guamis, B., & Ferragut, V. (2007). Ultra high pressure homogenization of soymilk: Microbiological, physicochemical and microstructural characteristics. *Food Research International, 40*(6), 725-732. 10.1016/j.foodres.2007.01.003

Dai, T., Vrahas, M. S., Murray, C. K., & Hamblin, M. R. (2012). Ultraviolet C irradiation: an alternative antimicrobial approach to localized infections? *Expert Review of Anti-Infective Therapy, 10*(2), 185-195. 10.1586/eri.11.166

Dallal, G. (2015). Obtaining superscripts to affix to means that are not significantly different from each other.

Dalsgaard, T. K., Otzen, D., Nielsen, J. H., & Larsen, L. B. (2007). Changes in structures of milk proteins upon photo-oxidation. *Journal of Agricultural and Food Chemistry, 55*(26), 10968-10976.

Datta, N., & Tomasula, P. M. (2015). *Emerging dairy processing technologies: Opportunities for the dairy industry*: John Wiley & Sons.

Davies, M. J., & Truscott, R. J. W. (2001). Photo-oxidation of proteins and its role in cataractogenesis. *Journal of Photochemistry and Photobiology B: Biology, 63*(1–3), 114-125. https://doi.org/10.1016/S1011-1344(01)00208-1

De Buyser, M.-L., Dufour, B., Maire, M., & Lafarge, V. (2001). Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *International Journal of Food Microbiology, 67*(1), 1-17. https://doi.org/10.1016/S0168-1605(01)00443-3

De Pree, C. G. (2010). *Physics Made Simple: A Complete Introduction to the Basic Principles of This Fundamental Science*: Three Rivers Press.

Dean, W. R. (1927). XVI. Note on the motion of fluid in a curved pipe. *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science, 4*(20), 208-223.

Deeth, H. (2017). Optimum Thermal Processing for Extended Shelf-Life (ESL) Milk. *Foods, 6*(11), 102. 10.3390/foods6110102

Deeth, H. C., & Lewis, M. J. (2017). *High Temperature Processing of Milk and Milk Products*: John Wiley & Sons.

Delmas, H., & Barthe, L. (2015). Ultrasonic mixing, homogenization, and emulsification in food processing and other applications. In J. A. Gallego-Juárez & K. F. Graff (Eds.), *Power Ultrasonics* (pp. 757-791). Oxford: Woodhead Publishing.

Dobrynin, D., Fridman, G., Mukhin, Y. V., Wynosky-Dolfi, M. A., Rieger, J., Rest, R. F., Gutsol, A. F., & Fridman, A. (2010). Cold plasma inactivation of *Bacillus cereus* and *Bacillus anthracis* (anthrax) spores. *IEEE Transactions on Plasma Science, 38*(8), 1878-1884.

Doll, E. V., Scherer, S., & Wenning, M. (2017). Spoilage of Microfiltered and Pasteurized Extended Shelf Life Milk Is Mainly Induced by Psychrotolerant Spore-Forming Bacteria that often Originate from Recontamination. *Frontiers in Microbiology, 8*, 135. 10.3389/fmicb.2017.00135

Dong, P., Georget, E. S., Aganovic, K., Heinz, V., & Mathys, A. (2015). Ultra high pressure homogenization (UHPH) inactivation of *Bacillus amyloliquefaciens* spores in phosphate buffered saline (PBS) and milk. *Frontiers in Microbiology, 6*, 712. 10.3389/fmicb.2015.00712

Donk, P. (1920). A highly resistant thermophilic organism. *Journal of Bacteriology, 5*(4), 373-374.

Donnelly, C. W. (2014). *Cheese and Microbes*: ASM Press.

Douki, T., Setlow, B., & Setlow, P. (2005). Photosensitization of DNA by dipicolinic acid, a major component of spores of *Bacillus* species. *Photochemical & Photobiological Sciences, 4*(8), 591-597. 10.1039/b503771a

Duba, K. S., & Fiori, L. (2015). Extraction of bioactives from food processing residues using techniques performed at high pressures. *Current Opinion in Food Science, 5*, 14-22. http://dx.doi.org/10.1016/j.cofs.2015.06.009

Earnshaw, R. G., Appleyard, J., & Hurst, R. M. (1995). Understanding physical inactivation processes: combined preservation opportunities using heat, ultrasound and pressure. *International Journal of Food Microbiology, 28*(2), 197-219.

Edwards, J., Busta, F., & Speck, M. (1965). Thermal inactivation characteristics of *Bacillus subtilis* spores at ultrahigh temperatures. *Applied Microbiology, 13*(6), 851-857.

Efigênia, M., Povoa, B., & Moraes-Santos, T. (1997). Effect of heat treatment on the nutritional quality of milk proteins. *International Dairy Journal, 7*(8–9), 609-612. http://dx.doi.org/10.1016/S0958-6946(97)00049-6

Ercan, S. Ş., & Soysal, Ç. (2013). Use of ultrasound in food preservation. *Natural Science, 5*(08), 5.

Espejo, G. G. A., Hernández-Herrero, M. M., Juan, B., & Trujillo, A. J. (2014). Inactivation of *Bacillus* spores inoculated in milk by Ultra High Pressure Homogenization. *Food Microbiology, 44*, 204-210. 10.1016/j.fm.2014.06.010

Evelyn, E., & Silva, F. V. M. (2015a). Inactivation of *Byssochlamys nivea* ascospores in strawberry puree by high pressure, power ultrasound and thermal processing. *International Journal of Food Microbiology, 214*, 129-136. http://dx.doi.org/10.1016/j.ijfoodmicro.2015.07.031

Evelyn, E., & Silva, F. V. M. (2015b). Thermosonication versus thermal processing of skim milk and beef slurry: Modeling the inactivation kinetics of psychrotrophic *Bacillus cereus* spores. *Food Research International, 67*, 67-74. http://dx.doi.org/10.1016/j.foodres.2014.10.028

Evelyn, E., & Silva, F. V. M. (2015c). Use of power ultrasound to enhance the thermal inactivation of *Clostridium perfringens* spores in beef slurry. *International Journal of Food Microbiology, 206*, 17-23. http://dx.doi.org/10.1016/j.ijfoodmicro.2015.04.013

Feijoo, S. C., Hayes, W. W., Watson, C. E., & Martin, J. H. (1997). Effects of Microfluidizer® Technology on *Bacillus licheniformis* Spores in Ice Cream Mix. *Journal of Dairy Science, 80*(9), 2184-2187.

Fenaille, F., Parisod, V., Visani, P., Populaire, S., Tabet, J.-C., & Guy, P. A. (2006). Modifications of milk constituents during processing: A preliminary benchmarking study. *International Dairy Journal, 16*(7), 728-739. http://dx.doi.org/10.1016/j.idairyj.2005.08.003

Feng, H., Barbosa-Cánovas, G. V., & Weiss, J. (2011). *Ultrasound technologies for food and bioprocessing* (Vol. 1). New York: Springer.

Feng, H., & Yang, W. (2010). Ultrasonic Processing *Nonthermal Processing Technologies for Food* (pp. 135-154): Wiley-Blackwell.

Ferragut, V., Hernández-Herrero, M., Poliseli, F., Valencia, D., & Guamis, B. (2011). Ultra high pressure homogenization (UHPH) treatment of vegetable milks: improving hygienic and colloidal stability. Paper presented at the 11th International Congress on Engineering and Food (ICEF11)–Food Process Engineering in a Changing World, Athens.

Flores-Cervantes, D., Palou, E., & López-Malo, A. (2013). Efficacy of individual and combined UVC light and food antimicrobial treatments to inactivate Aspergillus flavus or A. niger spores in peach nectar. *Innovative Food Science & Emerging Technologies, 20*, 244-252.

Franklin, J. G., Williams, D. J., & Clegg, L. F. L. (1958). Methods of assessing the sporicidal efficiency of an ultra-high-temperature milk sterilizing plant. Iii. Laboratory determinations of the heat resistance of spores of *bacillus subtilis* in water and in milk. *Journal of Applied Bacteriology, 21*(1), 51-57. 10.1111/j.1365-2672.1958.tb00114.x

Friedberg, E. C., Walker, G. C., Siede, W., & Wood, R. D. (2005). *DNA repair and mutagenesis*: American Society for Microbiology Press.

Fung, F., Wang, H.-S., & Menon, S. (2018). Food safety in the 21st century. *Biomedical Journal, 41*(2), 88-95. https://doi.org/10.1016/j.bj.2018.03.003

Garcia, M. L., Burgos, J., Sanz, B., & Ordonez, J. A. (1989). Effect of heat and ultrasonic waves on the survival of two strains of *Bacillus subtilis*. *The Journal of Applied Bacteriology, 67*(6), 619-628.

Gaulin, A. (1904). System for intimately mixing milk: Google Patents.

Gayán, E., Álvarez, I., & Condón, S. (2013). Inactivation of bacterial spores by UV-C light. *Innovative Food Science & Emerging Technologies, 19*, 140-145. 10.1016/j.ifset.2013.04.007

Gayán, E., Condón, S., & Álvarez, I. (2014a). Biological aspects in food preservation by ultraviolet light: A review. *Food and Bioprocess Technology, 7*(1), 1-20.

Gayán, E., Condón, S., & Álvarez, I. (2014b). Continuous-Flow UV Liquid Food Pasteurization: Engineering Aspects. *Food and Bioprocess Technology, 7*(10), 2813-2827. 10.1007/s11947-014-1267-0

Georget, E., Miller, B., Aganovic, K., Callanan, M., Heinz, V., & Mathys, A. (2014a). Bacterial spore inactivation by ultra-high pressure homogenization. *Innovative Food Science & Emerging Technologies, 26*, 116-123. http://dx.doi.org/10.1016/j.ifset.2014.08.004

Georget, E., Miller, B., Callanan, M., Heinz, V., & Mathys, A. (2014b). (Ultra) High Pressure Homogenization for Continuous High Pressure Sterilization of Pumpable Foods – A Review. *Frontiers in Nutrition, 1*, 15. 10.3389/fnut.2014.00015

Geveke, D. J. (2005). UV inactivation of bacteria in apple cider. *Journal of Food Protection, 68*(8), 1739-1742.

Gopal, N., Hill, C., Ross, P. R., Beresford, T. P., Fenelon, M. A., & Cotter, P. D. (2015). The Prevalence and Control of *Bacillus* and Related Spore-Forming Bacteria in the Dairy Industry. *Frontiers in Microbiology, 6*, 1418. 10.3389/fmicb.2015.01418

Grácia-Juliá, A., René, M., Cortés-Muñoz, M., Picart, L., López-Pedemonte, T., Chevalier, D., & Dumay, E. (2008). Effect of dynamic high pressure on whey protein aggregation: A comparison with the effect of continuous short-time thermal treatments. *Food Hydrocolloids, 22*(6), 1014-1032. https://doi.org/10.1016/j.foodhyd.2007.05.017

Groenewald, W., Gouws, P., Cilliers, F., & Witthuhn, R. (2013). The use of ultraviolet radiation as a non-thermal treatment for the inactivation of *Alicyclobacillus acidoterrestris* spores in water, wash water from a fruit processing plant and grape juice concentrate. *Journal for New Generation Sciences, 11*(2), 19-32.

Guerrero-Beltrán, J., & Barbosa-C, G. (2004). Advantages and limitations on processing foods by UV light. *Food Science and Technology International, 10*(3), 137-147.

Guerrouj, K., Sánchez-Rubio, M., Taboada-Rodríguez, A., Cava-Roda, R. M., & Marín-Iniesta, F. (2016). Sonication at mild temperatures enhances bioactive compounds and microbiological quality of orange juice. *Food and Bioproducts Processing, 99*, 20-28. http://dx.doi.org/10.1016/j.fbp.2016.03.007

Guneser, O., & Karagul Yuceer, Y. (2012). Effect of ultraviolet light on water- and fat-soluble vitamins in cow and goat milk. *Journal of Dairy Science, 95*(11), 6230-6241. 10.3168/jds.2011-5300

Halpin, R. M., Cregenzán-Alberti, O., Whyte, P., Lyng, J. G., & Noci, F. (2013). Combined treatment with mild heat, manothermosonication and pulsed electric fields reduces

microbial growth in milk. *Food Control, 34*(2), 364-371. http://dx.doi.org/10.1016/j.foodcont.2013.05.008

Hammer, B. W., & Johnson, A. (1913). The specific heat of milk and milk derivatives. *Research Bulletin (Iowa Agriculture and Home Economics Experiment Station), 1*(14), 1.

Haque, F., Dutta, A., Thimmanagari, M., & Chiang, Y. W. (2016). Intensified green production of astaxanthin from *Haematococcus pluvialis*. *Food and Bioproducts Processing, 99*, 1-11. http://dx.doi.org/10.1016/j.fbp.2016.03.002

Harvey, E. N., & Loomis, A. L. (1929). The destruction of luminous bacteria by high frequency sound waves. *Journal of Bacteriology, 17*(5), 373-376.

Hayes, M. G., & Kelly, A. L. (2003). High pressure homogenisation of raw whole bovine milk (a) effects on fat globule size and other properties. *Journal of Dairy Research, 70*(3), 297-305. 10.1017/S0022029903006320

Hewedy, M. M., Kiesner, C., Meissner, K., Hartkopf, J., & Erbersdobler, H. F. (1994). Effects of UHT heating of milk in an experimental plant on several indicators of heat treatment. *Journal of Dairy Research, 61*(02), 305-309.

Hielscher, U. G. (n.d.). Ultrasonication Saves Energy and Costs. Retrieved December 25, 2015, from http://www.hielscher.com/energy_efficiency_01.htm

Hucker, G. J., & Conn, H. J. (1923). Methods of Gram staining.

Hui, Y. H. (1993). *Dairy science and technology handbook.*

Imran, N. (1999). The role of visual cues in consumer perception and acceptance of a food product. *Nutrition & Food Science, 99*(5), 224-230. doi:10.1108/00346659910277650

Jacobo, Á. S., Saldo, J., & Gervilla, R. (2014). Influence of High-Pressure and Ultra-High-Pressure Homogenization on Antioxidants in Fruit Juice. *In Processing and Impact on Antioxidants in Beverages* (pp. 185-193). Academic Press.

Jagannath, A., & Tsuchido, T. (2003). Validation of a polynomial regression model: the thermal inactivation of *Bacillus subtilis* spores in milk. *Letters in Applied Microbiology, 37*(5), 399-404. doi:10.1046/j.1472-765X.2003.01416.x

Jelen, P. (1992). Whey: composition, properties, processing and uses. *Encyclopedia of Food Science and Technology. New York: Wiley*, 2835-2845.

Jeličič, I., Božanić, R., Brnčic, M., & Tripalo, B. (2012). Influence and comparison of thermal, ultrasonic and thermo-sonic treatments on microbiological quality and sensory properties of rennet cheese whey. *Mljekarstvo/Dairy, 62*(3)

Jenness, R. (1988). Composition of Milk. In N. P. Wong, R. Jenness, M. Keeney & E. H. Marth (Eds.), *Fundamentals of Dairy Chemistry* (pp. 1-38). Boston, MA: Springer US.

Jung, M. Y., Yoon, S. H., Lee, H. O., & Min, D. B. (1998). Singlet Oxygen and Ascorbic Acid Effects on Dimethyl Disulfide and Off-Flavor in Skim Milk Exposed to Light. *Journal of Food Science, 63*(3), 408-412. doi:10.1111/j.1365-2621.1998.tb15753.x

Juodeikiene, G., Bartkiene, E., Viskelis, P., Urbonaviciene, D., Eidukonyte, D., & Bobinas, C. (2012). Fermentation processes using lactic acid bacteria producing bacteriocins for preservation and improving functional properties of food products *Advances in Applied Biotechnology*: InTech.

Kadam, S. U., Tiwari, B. K., Álvarez, C., & O'Donnell, C. P. (2015). Ultrasound applications for the extraction, identification and delivery of food proteins and bioactive peptides. *Trends in Food Science & Technology, 46*(1), 60-67. http://dx.doi.org/10.1016/j.tifs.2015.07.012

Kakagianni, M., Gougouli, M., & Koutsoumanis, K. P. (2016). Development and application of *Geobacillus stearothermophilus* growth model for predicting spoilage of evaporated milk. *Food Microbiology, 57*, 28-35.

Kessler, H.-G., & Beyer, H.-J. (1991). Thermal denaturation of whey proteins and its effect in dairy technology. *International Journal of Biological Macromolecules, 13*(3), 165-173. http://dx.doi.org/10.1016/0141-8130(91)90043-T

Keyser, M., Műller, I. A., Cilliers, F. P., Nel, W., & Gouws, P. A. (2008). Ultraviolet radiation as a non-thermal treatment for the inactivation of microorganisms in fruit juice. *Innovative Food Science & Emerging Technologies, 9*(3), 348-354. http://dx.doi.org/10.1016/j.ifset.2007.09.002

Khan, M. N., & Panigrahi, S. (2017). *Principles of Engineering Physics 1*: Cambridge University Press.

Khanal, S. N., Anand, S., & Muthukumarappan, K. (2014). Evaluation of high-intensity ultrasonication for the inactivation of endospores of 3 *bacillus* species in nonfat milk. *Journal of Dairy Science, 97*(10), 5952-5963. http://dx.doi.org/10.3168/jds.2014-7950

Kilshaw, P. J., Heppell, L. M., & Ford, J. E. (1982). Effects of heat treatment of cow's milk and whey on the nutritional quality and antigenic properties. *Archives of Disease in Childhood, 57*(11), 842-847. 10.1136/adc.57.11.842

Kim, J. K., & Harrison, M. A. (2009). Surrogate selection for *Escherichia coli* O157:H7 based on cryotolerance and attachment to romaine lettuce. *Journal of Food Protection, 72*(7), 1385-1391.

Koutchma, T. (2009). Advances in ultraviolet light technology for non-thermal processing of liquid foods. *Food and Bioprocess Technology, 2*(2), 138-155.

Koutchma, T., & Barnes., G. (2013). Shelf life enhancement of milk products. *Food Technology, 10*, 68-69.

Koutchma, T., Forney, L. J., & Moraru, C. I. (2009). *Ultraviolet light in food technology: principles and applications*: CRC press, New York.

Kristo, E., Hazizaj, A., & Corredig, M. (2012). Structural changes imposed on whey proteins by UV irradiation in a continuous UV light reactor. *Journal of Agricultral and Food Chemistry, 60*(24), 6204-6209. 10.1021/jf300278k

Kwan, K., Nakai, S., & Skura, B. (1983). Comparison of four methods for determining protease activity in milk. *Journal of Food Science, 48*(5), 1418-1421.

Lanzanova, M., Mucchetti, G., & Neviani, E. (1993). Analysis of conductance changes as a growth index of lactic acid bacteria in milk. *Journal of Dairy Science, 76*(1), 20-28.

Ledenbach, L. H., & Marshall, R. T. (2009). Microbiological Spoilage of Dairy Products. In W. H. Sperber & M. P. Doyle (Eds.), *Compendium of the Microbiological Spoilage of Foods and Beverages* (pp. 41-67). New York, NY: Springer New York.

Lee, J. H., & Min, D. B. (2009). Changes of Headspace Volatiles in Milk with Riboflavin Photosensitization. *Journal of Food Science, 74*(7), C563-C568. doi:10.1111/j.1750-3841.2009.01295.x

Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., Ahn, B. W., Shaltiel, S., & Stadtman, E. R. (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology, 186*, 464-478.

Li, X., & Farid, M. (2016). A review on recent development in non-conventional food sterilization technologies. *Journal of Food Engineering, 182*, 33-45. http://dx.doi.org/10.1016/j.jfoodeng.2016.02.026

Liem, D. G., Bolhuis, D. P., Hu, X., & Keast, R. S. J. (2016). Short communication: Influence of labeling on Australian and Chinese consumers' liking of milk with short (pasteurized) and long (UHT) shelf life. *Journal of Dairy Science, 99*(3), 1747-1754. 10.3168/jds.2015-10516

Lopez, M., Mazas, M., Gonzalez, I., Gonzalez, J., & Bernardo, A. (1996). Thermal resistance of *Bacillus stearothermophilus* spores in different heating systems containing some approved food additives. *Letters in Applied Microbiology, 23*(3), 187-191.

Lu, G., Li, C., Liu, P., Cui, H., Xia, Y., & Wang, J. (2010). Inactivation of microorganisms in apple juice using an ultraviolet silica-fiber optical device. *Journal of Photochemistry and Photobiology B: Biology, 100*(3), 167-172.

Lu, M., & Wang, N. S. (2017). Spoilage of Milk and Dairy Products *The Microbiological Quality of Food* (pp. 151-178): Elsevier.

Mahmoud, N. S., & Ghaly, A. E. (2004). On-line sterilization of cheese whey using ultraviolet radiation. *Biotechnology Progress, 20*(2), 550-560. 10.1021/bp030050i

Makwana, B. (2016). Investigation of safety and quality of raw milk during PEF and HPP treatments (Master's thesis) *University of Auckland, New Zealand*

Månsson, L. H. (2008). Fatty acids in bovine milk fat. *Food & Nutrition Research, 52*(1), 1821.

Marquis, R. E., & Bender, G. R. (1985). Mineralization and heat resistance of bacterial spores. *Journal of Bacteriology, 161*(2), 789-791.

Matak, K., Sumner, S., Duncan, S., Hovingh, E., Worobo, R., Hackney, C., & PIERSON, M. (2004). Effects of UV light on chemical indicators of goat's milk. *Department Food Science and Technology*, 49-84.

Matak, K., Sumner, S., Hovingh, E., Worobo, R., Hackney, C., & Pierson, M. (2007). Inactivation by UV Irradiation of *Escherichia coli* in Milk of different temperatures and milk fat percentages. *Página da internet acessada julho*

Matser, A. M., Krebbers, B., van den Berg, R. W., & Bartels, P. V. (2004). Advantages of high pressure sterilisation on quality of food products. *Trends in Food Science & Technology, 15*(2), 79-85. 10.1016/j.tifs.2003.08.005

Mazas, M., Gonzalez, I., Lopez, M., GONZÁLEZ, J., & Sarmiento, R. M. (1995). Effects of sporulation media and strain on thermal resistance of Bacillus cereus spores. *International Journal of Food Science & Technology, 30*(1), 71-78.

Mazri, C., Sánchez, L., Ramos, S. J., Calvo, M., & Pérez, M. D. (2012). Effect of high-pressure treatment on denaturation of bovine lactoferrin and lactoperoxidase. *Journal of Dairy Science, 95*(2), 549-557. https://doi.org/10.3168/jds.2011-4665

MBIE, C. a. (2017). The investor's guide to the New Zealand dairy industry 2017. Retrieved 13/11/2018, from http://www.mbie.govt.nz/

McDonald, K. F., Curry, R. D., Clevenger, T. E., Unklesbay, K., Eisenstark, A., Golden, J., & Morgan, R. D. (2000). A comparison of pulsed and continuous ultraviolet light sources for the decontamination of surfaces. *Plasma Science, IEEE Transactions on, 28*(5), 1581-1587.

McDonough, J., Murta, S., Law, R., & Harvey, A. (2019). Oscillatory fluid motion unlocks plug flow operation in helical tube reactors at lower Reynolds numbers (Re≤ 10). *Chemical Engineering Journal, 358*, 643-657.

Meurant, G. (1995). *Handbook of milk composition*: Elsevier.

Milani, E. A., Ramsey, J. G., & Silva, F. V. M. (2016). High pressure processing and thermosonication of beer: comparing the energy requirements and *Saccharomyces cerevisiae* ascospores inactivation with thermal processing and modeling. *Journal of Food Engineering* http://dx.doi.org/10.1016/j.jfoodeng.2016.02.023

Minolta. (1994). *Precise color communication: color control from feeling to instrumentation*: Minolta.

Moeller, R., Douki, T., Cadet, J., Stackebrandt, E., Nicholson, W. L., Rettberg, P., Reitz, G., & Horneck, G. (2007). UV-radiation-induced formation of DNA bipyrimidine photoproducts in *Bacillus subtilis* endospores and their repair during germination. *International Microbiology, 10*(1), 39-46.

Mosley, G. A. (2008). *Sterility Assurance Level (SAL): the term and its definition continues to cause confusion in the industry*. Paper presented at the Pharmaceutical Microbiology Forum Newsletter.

Mucchetti, G., Gatti, M., & Neviani, E. (1994). Electrical Conductivity Changes in Milk Caused by Acidification: Determining Factors. *Journal of Dairy Science, 77*(4), 940-944. 10.3168/jds.S0022-0302(94)77029-6

Müller, A., Orlowska, M., Knörr, M., Stahl, M. R., Greiner, R., & Koutchma, T. (2017). Actinometric and biodosimetric evaluation of UV-C dose delivery in annular, Taylor–Coutte and coiled tube continuous systems. *Food Science and Technology International, 23*(3), 222-234. 10.1177/1082013216679010

Müller, A., Stahl, M. R., Greiner, R., & Posten, C. (2014). Performance and dose validation of a coiled tube UV-C reactor for inactivation of microorganisms in absorbing liquids. *Journal of Food Engineering, 138*(Supplement C), 45-52. https://doi.org/10.1016/j.jfoodeng.2014.04.013

Nangpal, A., & Reuter, H. (1990). Formation of lactulose during UHT treatment of milk-comparison between direct and indirect heating. *Kieler Milchwirtschaftliche Forschungsberichte, 42*(1), 31-41.

Navarro, J. L., Izquierdo, L., Carbonell, J. V., & Sentandreu, E. (2014). Effect of pH, temperature and maturity on pectinmethylesterase inactivation of citrus juices treated

by high-pressure homogenization. *LWT - Food Science and Technology*, *57*(2), 785-788. http://dx.doi.org/10.1016/j.lwt.2014.01.033

Ordoñez, J. A., & Burgos, J. (1976). Effect of ultrasonic waves on the heat resistance of *Bacillus* spores. *Applied and Environmental Microbiology*, *32*(1), 183-184.

Pagán, R., Mañas, P., Raso, J., & Condón, S. (1999). Bacterial Resistance to Ultrasonic Waves under Pressure at Nonlethal (Manosonication) and Lethal (Manothermosonication) Temperatures. *Applied and Environmental Microbiology, 65*(1), 297-300.

Pagliarini, E., Vernile, M., & Peri, C. (1990). Kinetic study on color changes in milk due to heat. *Journal of Food Science, 55*(6), 1766-1767.

Palacios, P., Burgos, J., Hoz, L., Sanz, B., & Ordonez, J. A. (1991). Study of substances released by ultrasonic treatment from *Bacillus stearothermophilus* spores. *Journal of Applied Bacteriology, 71*(5), 445-451.

Palgan, I., Muñoz, A., Noci, F., Whyte, P., Morgan, D. J., Cronin, D. A., & Lyng, J. G. (2012). Effectiveness of combined Pulsed Electric Field (PEF) and Manothermosonication (MTS) for the control of Listeria innocua in a smoothie type beverage. *Food Control, 25*(2), 621-625. http://dx.doi.org/10.1016/j.foodcont.2011.11.009

Park, Y. W. (2007). Rheological characteristics of goat and sheep milk. *Small Ruminant Research, 68*(1), 73-87. https://doi.org/10.1016/j.smallrumres.2006.09.015

Patton, S. (1955). Browning and associated changes in milk and its products: A review. *Journal of Dairy Science, 38*(5), 457-478.

Pennell, K. G., Naunovic, Z., & Blatchley III, E. R. (2008). Sequential inactivation of *bacillus subtilis* spores with ultraviolet radiation and iodine. *Journal of Environmental Engineering, 134*(7), 513-520.

Pereda, J., Ferragut, V., Quevedo, J. M., Guamis, B., & Trujillo, A. J. (2007). Effects of ultra-high pressure homogenization on microbial and physicochemical shelf life of milk. *Journal of Dairy Science, 90*(3), 1081-1093. 10.3168/jds.S0022-0302(07)71595-3

Perrechil, F. A., & Cunha, R. L. (2010). Oil-in-water emulsions stabilized by sodium caseinate: Influence of pH, high-pressure homogenization and locust bean gum addition. *Journal of Food Engineering, 97*(4), 441-448. http://dx.doi.org/10.1016/j.jfoodeng.2009.10.041

Pharmacopeia. (2016). Biological indicators for sterilization. Retrieved 10th March 2016, 2016, from http://www.pharmacopeia.cn/v29240/usp29nf24s0_c1035.html

Pinho, C. R. G., Franchi, M. A., Tribst, A. A. L., & Cristianinia, M. (2011). Effect of High Pressure Homogenization Process on *Bacillus Stearothermophilus* and *Clostridium*

Sporogenes Spores in Skim Milk. *Procedia Food Science, 1*, 869-873. http://dx.doi.org/10.1016/j.profoo.2011.09.131

Piyasena, P., Mohareb, E., & McKellar, R. C. (2003). Inactivation of microbes using ultrasound: a review. *International Journal of Food Microbiology, 87*(3), 207-216. http://dx.doi.org/10.1016/S0168-1605(03)00075-8

Poliseli-Scopel, F. H., Hernández-Herrero, M., Guamis, B., & Ferragut, V. (2012). Comparison of ultra high pressure homogenization and conventional thermal treatments on the microbiological, physical and chemical quality of soymilk. *LWT - Food Science and Technology, 46*(1), 42-48. http://dx.doi.org/10.1016/j.lwt.2011.11.004

Poliseli-Scopel, F. H., Hernández-Herrero, M., Guamis, B., & Ferragut, V. (2014). Sterilization and aseptic packaging of soymilk treated by ultra high pressure homogenization. *Innovative Food Science & Emerging Technologies, 22*, 81-88. http://dx.doi.org/10.1016/j.ifset.2014.01.001

Ponraj, S. B., Sharp, J. A., Kanwar, J. R., Sinclair, A. J., Kviz, L., Nicholas, K. R., & Dai, X. J. (2017). Argon gas plasma to decontaminate and extend shelf life of milk. *Plasma processes and polymers, 14*(11)

Popov-Raljić, J. V., Lakić, N. S., Laličić-Petronijević, J. G., Barać, M. B., & Sikimić, V. M. (2008). Color Changes of UHT Milk During Storage. *Sensors, 8*(9), 5961-5974. 10.3390/s8095961

Poulsen, M. (2016). Safety of UV-treated milk as a novel food pursuant to Regulation (EC) No 258/97.

Rahn, R. O., Stefan, M. I., Bolton, J. R., Goren, E., Shaw, P. S., & Lykke, K. R. (2003). Quantum yield of the iodide-iodate chemical actinometer: dependence on wavelength and concentrations. *Photochemistry and Photobiology, 78*(2), 146-152.

Raso, J., & Barbosa-Canovas, G. V. (2003). Nonthermal preservation of foods using combined processing techniques. *Critical Reviews in Food Science and Nutrition, 43*(3), 265-285. 10.1080/10408690390826527

Raso, J., Pagán, R., Condón, S., & Sala, F. J. (1998a). Influence of Temperature and Pressure on the Lethality of Ultrasound. *Applied and Environmental Microbiology, 64*(2), 465-471.

Raso, J., Palop, A., Pagán, R., & Condón, S. (1998b). Inactivation of *Bacillus subtilis* spores by combining ultrasonic waves under pressure and mild heat treatment. *Journal of Applied Microbiology, 85*(5), 849-854. 10.1046/j.1365-2672.1998.00593.x

Raynal-Ljutovac, K., Lagriffoul, G., Paccard, P., Guillet, I., & Chilliard, Y. (2008). Composition of goat and sheep milk products: An update. *Small Ruminant Research, 79*(1), 57-72. http://dx.doi.org/10.1016/j.smallrumres.2008.07.009

Raynal-Ljutovac, K., Park, Y. W., Gaucheron, F., & Bouhallab, S. (2007). Heat stability and enzymatic modifications of goat and sheep milk. *Small Ruminant Research, 68*(1), 207-220. https://doi.org/10.1016/j.smallrumres.2006.09.006

Renner, E. (1988). Storage stability and some nutritional aspects of milk powders and ultra high temperature products at high ambient temperatures. *Journal of Dairy Research, 55*(01), 125-142.

Reverter-Carrión, L., Sauceda-Gálvez, J. N., Codina-Torrella, I., Hernández-Herrero, M. M., Gervilla, R., & Roig-Sagués, A. X. (2018). Inactivation study of *Bacillus subtilis*, *Geobacillus stearothermophilus*, *Alicyclobacillus acidoterrestris* and *Aspergillus niger* spores under Ultra-High Pressure Homogenization, UV-C light and their combination. *Innovative Food Science & Emerging Technologies, 48,* 258-264. https://doi.org/10.1016/j.ifset.2018.06.011

Richardson, P. (2001). *Thermal technologies in food processing*: Taylor & Francis.

Rodriguez, A. C., Larkin, J. W., Dunn, J., Patazca, E., Reddy, N. R., Alvarez-Medina, M., Tetzloff, R., & Fleischman, G. J. (2004). Model of the Inactivation of Bacterial Spores by Moist Heat and High Pressure. *Journal of Food Science, 69*(8), E367-E373. 10.1111/j.1365-2621.2004.tb09897.x

Roig-Sagués, A. X., Asto, E., Engers, I., & Hernández-Herrero, M. M. (2015). Improving the efficiency of ultra-high pressure homogenization treatments to inactivate spores of *Alicyclobacillus spp*. in orange juice controlling the inlet temperature. *LWT - Food Science and Technology, 63*(2), 866-871. 10.1016/j.lwt.2015.04.056

Roig-Sagués, A. X., Gervilla, R., Pixner, S., Terán-Peñafiel, T., & Hernández-Herrero, M. M. (2018). Bactericidal effect of ultraviolet-C treatments applied to honey. *LWT - Food Science and Technology, 89*, 566-571. https://doi.org/10.1016/j.lwt.2017.11.010

Rossitto, P. V., Cullor, J. S., Crook, J., Parko, J., Sechi, P., & Cenci-Goga, B. T. (2012). Effects of UV irradiation in a continuous turbulent flow UV reactor on microbiological and sensory characteristics of cow's milk. *Journal of Food Protection, 75*(12), 2197-2207. 10.4315/0362-028x.jfp-12-036

Rug-Lenartowicz, R. (1954). *The influence of acidity on the electrical conductivity of milk.* Paper presented at the Dairy Sci. Abstr.

Rysstad, G., & Kolstad, J. (2006). Extended shelf life milk—advances in technology. *International Journal of Dairy Technology, 59*(2), 85-96. 10.1111/j.1471-0307.2006.00247.x

Salaün, F., Mietton, B., & Gaucheron, F. (2005). Buffering capacity of dairy products. *International Dairy Journal, 15*(2), 95-109. https://doi.org/10.1016/j.idairyj.2004.06.007

Sale, A. J. H., Gould, G. W., & Hamilton, W. A. (1970). Inactivation of Bacterial Spores by Hydrostatic Pressure. *Microbiology, 60*(3), 323-334. doi:10.1099/00221287-60-3-323

Samaržija, D., Zamberlin, Š., & Pogačić, T. (2012). Psychrotrophic bacteria and milk and dairy products quality. *Mljekarstvo, 62*(2), 77-95.

Samuelsson, E.-G., & Harper, J. W. (1961). *Degradation of methionine by light and its dependence on pH and presence of oxygen*: Carl.

Sattar, A., deMan, J. M., & Furia, T. E. (1975). Photooxidation of milk and milk products: A review. *Critical Reviews in Food Science & Nutrition, 7*(1), 13-37.

Saunal, H., Laget, J. P., Alvarado, D. E., & Delonca, E. H. (1982). Influence of homogenization on emulsion stability. *International Journal of Cosmetic Science, 4*(5), 207-218. 10.1111/j.1467-2494.1982.tb00315.x

Scheidegger, D., Pecora, R., Radici, P., & Kivatinitz, S. (2010). Protein oxidative changes in whole and skim milk after ultraviolet or fluorescent light exposure. *Journal of Dairy Science, 93*(11), 5101-5109.

Scherba, G., Weigel, R. M., & O'Brien, W. D. (1991). Quantitative assessment of the germicidal efficacy of ultrasonic energy. *Applied and Environmental Microbiology, 57*(7), 2079-2084.

Scurrah, K. J., Robertson, R. E., Craven, H. M., Pearce, L. E., & Szabo, E. A. (2006). Inactivation of *Bacillus* spores in reconstituted skim milk by combined high pressure and heat treatment. *Journal of Applied Microbiology, 101*(1), 172-180. 10.1111/j.1365-2672.2006.02897.x

Segat, A., Misra, N. N., Cullen, P. J., & Innocente, N. (2015). Atmospheric pressure cold plasma (ACP) treatment of whey protein isolate model solution. *Innovative Food Science & Emerging Technologies, 29*, 247-254. https://doi.org/10.1016/j.ifset.2015.03.014

Senhaji, A. (1977). The protective effect of fat on the heat resistance of bacteria (II). *International Journal of Food Science & Technology, 12*(3), 217-230.

Sepulveda, D. R., Góngora-Nieto, M. M., Guerrero, J. A., & Barbosa-Cánovas, G. V. (2005). Production of extended-shelf life milk by processing pasteurized milk with pulsed electric fields. *Journal of Food Engineering, 67*(1), 81-86. https://doi.org/10.1016/j.jfoodeng.2004.05.056

Setlow, B., Atluri, S., Kitchel, R., Koziol-Dube, K., & Setlow, P. (2006). Role of Dipicolinic Acid in Resistance and Stability of Spores of *Bacillus subtilis* with or without DNA-Protective α/β-Type Small Acid-Soluble Proteins. *Journal of Bacteriology, 188*(11), 3740-3747. 10.1128/JB.00212-06

Setlow, P. (2003). Spore germination. *Current opinion in microbiology, 6*(6), 550-556.

Setlow, P. (2006a). Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology, 101*(3), 514-525. 10.1111/j.1365-2672.2005.02736.x

Setlow, P. (2006b). Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology, 101*(3), 514-525.

Setlow, P. (2014). Germination of Spores of *Bacillus* Species: What We Know and Do Not Know. *Journal of Bacteriology, 196*(7), 1297-1305. 10.1128/JB.01455-13

Shacter, E. (2000). Quantification and significance of protein oxidation in biological samples*. *Drug Metabolism Reviews, 32*(3-4), 307-326. 10.1081/DMR-100102336

Sharma, G., & Bala, R. (2002). *Digital color imaging handbook*: CRC press.

Shibeshi, K., & Farid, M. M. (2011). Scale-up unit of a unique moderately high pressure unit to enhance microbial inactivation. *Journal of Food Engineering, 105*(3), 522-529. http://dx.doi.org/10.1016/j.jfoodeng.2011.03.016

Shimamura, T., & Ukeda, H. (2012). Maillard reaction in milk—effect of heat treatment. *Milk protein. InTechOpen Science. p*, 147-158.

Siemer, C., Toepfl, S., & Heinz, V. (2014). Inactivation of *Bacillus subtilis* spores by pulsed electric fields (PEF) in combination with thermal energy II. Modeling thermal inactivation of *B. subtilis* spores during PEF processing in combination with thermal energy. *Food Control, 39*, 244-250. 10.1016/j.foodcont.2013.09.067

Sierra, G., & Boucher, R. M. (1971). Ultrasonic synergistic effects in liquid-phase chemical sterilization. *Applied Microbiology, 22*(2), 160-164.

Silcock, P., Alothman, M., Zardin, E., Heenan, S., Siefarth, C., Bremer, P. J., & Beauchamp, J. (2014). Microbially induced changes in the volatile constituents of fresh chilled pasteurised milk during storage. *Food Packaging and Shelf Life, 2*(2), 81-90. https://doi.org/10.1016/j.fpsl.2014.08.002

Simmons, M. J., Alberini, F., Tsoligkas, A. N., Gargiuli, J., Parker, D. J., Fryer, P. J., & Robinson, S. (2012). Development of a hydrodynamic model for the UV-C treatment of turbid food fluids in a novel 'SurePure turbulator™'swirl-tube reactor. *Innovative Food Science & Emerging Technologies, 14*, 122-134.

Singh, J., & Ghaly, A. (2007). Effect of flow characteristics on online sterilization of cheese whey in UV reactors. *Applied Biochemistry and Biotechnology, 142*(1), 1.

Singh, R. P., & Heldman, D. R. (2001). *Introduction to food engineering*: Gulf Professional Publishing.

Singleton, J., Aurand, L., & Lancaster, F. (1963). Sunlight Flavor in Milk. I. A Study of Components Involved in the Flavor Development1. *Journal of Dairy Science, 46*(10), 1050-1053.

Skibsted, L. (2000). Light-induced changes in dairy products. *Bulletin of the International Dairy Federation* (346), 4-9.

Spilimbergo, S., Bertucco, A., Lauro, F. M., & Bertoloni, G. (2003). Inactivation of *Bacillus subtilis* spores by supercritical CO2 treatment. *Innovative Food Science & Emerging Technologies, 4*(2), 161-165. http://dx.doi.org/10.1016/S1466-8564(02)00089-9

Stern, J. A., & Proctor, B. E. (1954). A micro-method and apparatus for the multiple determination of rates of destruction of bacteria and bacterial spores subjected to heat. *Food Technology, 8*, 139-143.

Stoscheck, C. M. (1990). [6] Quantitation of protein. In M. P. Deutscher (Ed.), *Methods in Enzymology* (Vol. 182, pp. 50-68): Academic Press.

Sun, D.-W. (2011). *Handbook of food safety engineering*: John Wiley & Sons.

Suzuki, Y. J., Carini, M., & Butterfield, D. A. (2010). Protein carbonylation. *Antioxidants & Redox Signaling, 12*(3), 323-325.

Swindells, J., Coe Jr, J., & Godfrey, T. (1952). Absolute viscosity of water at 20 C. *Journal of Research of the National Bureau of Standard, 48*, 1.

Tewari, G., & Juneja, V. (2008). *Advances in thermal and non-thermal food preservation*: John Wiley & Sons.

Tiwari, B. K., O' Donnell, C. P., Muthukumarappan, K., & Cullen, P. J. (2009). Ascorbic acid degradation kinetics of sonicated orange juice during storage and comparison with thermally pasteurised juice. *LWT - Food Science and Technology, 42*(3), 700-704. http://dx.doi.org/10.1016/j.lwt.2008.10.009

Tomasula, P. M., & Nutter, D. W. (2011). Chapter 2 - Mitigation of Greenhouse Gas Emissions in the Production of Fluid Milk. In S. L. Taylor (Ed.), *Advances in Food and Nutrition Research* (Vol. 62, pp. 41-88): Academic Press.

Tran, M. T. T., & Farid, M. (2004). Ultraviolet treatment of orange juice. *Innovative Food Science & Emerging Technologies, 5*(4), 495-502. http://dx.doi.org/10.1016/j.ifset.2004.08.002

Tucker, G. S., & Featherstone, S. (2011). *Essentials of thermal processing*: John Wiley & Sons.

USFDA. (2009). Grade "A" pasteurized milk ordinance. *US Food and Drug Administration, Washington, DC*, 52.

Valencia-Flores, D. C., Hernandez-Herrero, M., Guamis, B., & Ferragut, V. (2013). Comparing the effects of ultra-high-pressure homogenization and conventional thermal treatments on the microbiological, physical, and chemical quality of almond beverages. *Journal of Food Science, 78*(2), E199-205. 10.1111/1750-3841.12029

Van Boekel, M. A. J. S. (1998). Effect of heating on Maillard reactions in milk. *Food Chemistry, 62*(4), 403-414. http://dx.doi.org/10.1016/S0308-8146(98)00075-2

Van Opstal, I., Vanmuysen, S. C., Wuytack, E. Y., & Michiels, C. W. (2003). Inactivation of *Bacillus cereus* spores in milk by mild pressure and heat treatments. *Communications in Agricultural and Applied Biological Sciences, 68*(3), 7-10.

Van Zuijlen, A., Periago, P. M., Amézquita, A., Palop, A., Brul, S., & Fernández, P. S. (2010). Characterization of *Bacillus sporothermodurans* IC4 spores; putative indicator microorganism for optimisation of thermal processes in food sterilisation. *Food Research International, 43*(7), 1895-1901. http://dx.doi.org/10.1016/j.foodres.2009.11.011

Vercet, A., Burgos, J., & López-Buesa, P. (2001). Manothermosonication of Foods and Food-Resembling Systems: Effect on Nutrient Content and Nonenzymatic Browning. *Journal of Agricultural and Food Chemistry, 49*(1), 483-489. 10.1021/jf000438i

Vercet, A., Lopez, P., & Burgos, J. (1999). Inactivation of Heat-Resistant Pectinmethylesterase from Orange by Manothermosonication. *Journal of Agricultural and Food Chemistry, 47*(2), 432-437. 10.1021/jf980566v

Vinatoru, M. (2001). An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrasonics Sonochemistry, 8*(3), 303-313. 10.1016/S1350-4177(01)00071-2

Vreeland, R. H., Rosenzweig, W. D., & Powers, D. W. (2000). Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. *Nature, 407*(6806), 897-900.

Wang, D., Oppenländer, T., El-Din, M. G., & Bolton, J. R. (2010). Comparison of the disinfection effects of vacuum-UV (VUV) and UV light on *Bacillus subtilis* spores in aqueous suspensions at 172, 222 and 254 nm. *Photochemistry and photobiology, 86*(1), 176-181.

Warth, A., Ohye, D., & Murrell, W. (1963). The composition and structure of bacterial spores. *The Journal of Cell Biology, 16*(3), 579-592.

Warth, A. D. (1980). Heat stability of *Bacillus cereus* enzymes within spores and in extracts. *Journal of Bacteriology, 143*(1), 27-34.

Wimalaratne, S. K., & Farid, M. M. (2008). Pressure assisted thermal sterilization. *Food and Bioproducts Processing, 86*(4), 312-316. 10.1016/j.fbp.2007.08.001

Wu, H., Hulbert, G. J., & Mount, J. R. (2000). Effects of ultrasound on milk homogenization and fermentation with yogurt starter. *Innovative Food Science & Emerging Technologies, 1*(3), 211-218. http://dx.doi.org/10.1016/S1466-8564(00)00020-5

Ye, Z., Forney, L. J., Koutchma, T., Giorges, A. T., & Pierson, J. A. (2008). Optimum UV Disinfection between Concentric Cylinders. *Industrial & Engineering Chemistry Research, 47*(10), 3444-3452. 10.1021/ie0703641

Yu, H., Chen, S., & Cao, P. (2012). Synergistic bactericidal effects and mechanisms of low intensity ultrasound and antibiotics against bacteria: a review. *Ultrasonics Sonochemistry, 19*(3), 377-382. 10.1016/j.ultsonch.2011.11.010

Zhang, H., & Mittal, G. S. (2008). Effects of high-pressure processing (HPP) on bacterial spores: an overview. *Food Reviews International, 24*(3), 330-351.

Zink, D. L. (1997). The impact of consumer demands and trends on food processing. *Emerging Infectious Diseases, 3*(4), 467.